

DOCTORAMENTO EM CIÊNCIAS FARMACÊUTICAS
FITOQUÍMICA E FARMACOGNOSIA

Guinea-Bissau's plants for pest management: A research to discover novel multi-target fungicides and insecticides

Rute Moreira

D
2024



Rute Carina Pinto Martins Moreira

**Guinea-Bissau's plants for pest management: A research
to discover novel multi-target fungicides and insecticides**

Thesis for Doctor Degree in Pharmaceutical Sciences

Phytochemistry and Pharmacognosy Specialty

Work performed under the supervision of

Prof. Doctor Paula Cristina Branquinho de Andrade

and co-supervision of

Prof. Doctor Romeu António Videira

and

Prof. Doctor Patrícia Carla Ribeiro Valentão

(until September 13th, 2022)

March 2024

***The more I learn,
the more I realize how much I don't know.***

- Albert Einstein

The following funds and institutions supported this work:

Rute Moreira thanks FCT (Fundação para a Ciência e Tecnologia) and ESF (European Social Fund) through POCH (Programa Operacional Capital Humano) for her PhD grant (SFRH/BD/148029/2019).

PT national funds (FCT/MCTES, Fundação para a Ciência e Tecnologia and Ministério da Ciência, Tecnologia e Ensino Superior) through the project UIDB/50006/2020 and UIDP/560006/2020.



IT IS AUTHORIZED THE REPRODUCTION OF THIS THESIS ONLY FOR RESEARCH PURPOSES, UNDER THE WRITTEN STATEMENT OF THE INTERESTED PART, COMMITTING ITSELF TO DO IT.

Publications

The data contained in the following works make up part of this thesis.

Publication in international peer-reviewed journals indexed at the Journal Citation Reports (JCR) of the ISI Web of Knowledge:

1. **Moreira R**, Ferreres F, Gil-Izquierdo Á, Gomes NGM, Araújo L, Pinto E, Andrade PB, Videira RA. Antifungal activity of *Guiera senegalensis*: From the chemical composition to the mitochondrial toxic effects and tyrosinase inhibition. *Antibiotics*. 2023;12(5):869.

Manuscripts under evaluation in international peer-reviewed journals indexed at the Journal Citation Reports (JCR) of the ISI Web of Knowledge:

1. **Moreira R**, Ferreres F, Gil-Izquierdo Á, Gomes NGM, Araújo L, Pinto E, Andrade PB, Videira RA. *Ficus polita* leaves: from the chemical characterization to the pesticide potential.
2. **Moreira R**, Andrade PB, Videira RV. Medicinal plants from Sub-Saharan Africa and fungal diseases: An ethnopharmacological knowledge-guided research strategy to address the pressing issue of antifungal resistance.

Oral communications:

1. **Moreira R**, Ferreres F, Gil-Izquierdo Á, Gomes NGM, Araújo L, Pinto E, Andrade PB, Videira RA. *Guiera senegalensis*: On the track of biofungicides development. XXVI Galician Portuguese Chemistry Meeting. November 16-18, 2022. Santiago de Compostela, Spain.
2. **Moreira R**, Ferreres F, Gil-Izquierdo Á, Gomes NGM, Araújo L, Pinto E, Andrade PB, Videira RA. Experimental approaches to find plant extracts with multi target fungicide activity. LAQV Webinar – Natural Products – Chemistry and Bioactivity. July 19, 2023. Porto, Portugal.
3. **Moreira R**, Ferreres F, Gil-Izquierdo Á, Gomes NGM, Araújo L, Pinto E, Andrade PB, Videira RA. *Ficus polita* leaves methanol extract: from the chemical

characterization to the biopesticide potential. XXVII Luso-Galician Chemistry Meeting. November 22-24, 2023. Porto, Portugal.

Poster communications:

1. **Moreira R**, Ferreres F, Videira R, Valentão P, Gil-Izquierdo A, Gomes NGM, Araújo L, Andrade PB. Delivering a chemical fingerprint of *Guiera senegalensis* leaves by HPLC-DAD-ESI/MS. IBERPHENOL International Conference - Advances in the role of phenols in health effects and other uses. November 5-6, 2020. Coimbra, Portugal.
2. **Moreira R**, Ferreres F, Videira R, Valentão P, Gil-Izquierdo A, Gomes NGM, Araújo L, Andrade PB. Botanical biopesticides research and development: the potential of *Guiera senegalensis*. Science 2021 – Meeting with Science and Technology in Portugal. June 28-30, 2021. Lisboa, Portugal.
3. **Moreira R**, Ferreres F, Gil-Izquierdo A, Gomes NGM, Araújo L, Valentão P, Videira, RA, Andrade PB. *Guiera senegalensis* J. F. Gmel. leaves methanol extract: chemical characterization and bioinsecticide potential. 14th National Organic Chemistry Meeting & 7th National Medicinal Chemistry Meeting. April 20-22, 2022. Lisboa, Portugal.
4. **Moreira R**, Ferreres F, Gil-Izquierdo Á, Gomes NGM, Araújo L, Pinto E, Andrade PB, Videira RA. R. *Guiera senegalensis*: a renewable resource to develop fungicides with multi-target activity. Science 2023 – Meeting with Science and Technology in Portugal. July 5-7, 2023. Aveiro, Portugal.

Acknowledgments

Throughout this journey, several people and institutions have crossed my path, each playing a key role in the development of this work. I am sincerely grateful for your contribution to the accomplishment of this PhD thesis.

To “Fundação para a Ciência e a Tecnologia” (FCT) and European Social Fund (ESF) through “Programa Operacional Capital Humano” (POCH) for the PhD fellowship (SFRH/BD/148029/2019) that supported this work.

To my supervisor, Prof. Doctor Paula Andrade, for, firstly, accepting me in the Laboratory of Pharmacognosy of the Faculty of Pharmacy of the University of Porto as a Master student and, later, for the opportunity to join this doctoral program and accomplish this PhD thesis. Thank you for the supervision, encouragement, availability, and the knowledge transmitted.

To my co-supervisor, Prof. Doctor Romeu Videira, I would like to express my sincere gratitude for all the guidance, support, dedication and for the knowledge he shared with me throughout this long journey. Thank you for sharing your enthusiasm for science and for inspiring me to explore new horizons and broaden my perspectives.

To Prof. Doctor Eugénia Pinto I would like to express my thankfulness for welcoming me into the Laboratory of Microbiology of the Faculty of Pharmacy of the University of Porto and allowing me to carry out part of my work there, which was crucial for the development of this project. Thank you for all the availability.

To Prof. Doctor Federico Ferreres from Molecular Recognition and Encapsulation (REM) Group of Catholic University of Murcia, and to Prof. Doctor Ángel Gil-Izquierdo from the Research Group on Quality, Safety and Bioactivity of Plant Foods (CEBAS), Campus University Espinardo, I would like to thank the important contribution to the identification of phenolic compounds.

To Doctor Luísa Araújo I would like to thank for providing Guinea-Bissau plant species to REQUIMTE-LAQV.

To Doctor Nelson Gomes I would like to express my gratitude for the availability and for the knowledge in chromatography that he taught me at the beginning of my PhD journey.

I would like to also thank my PhD colleagues and friends, for all the companionship and support. Thank you for all the good times and laughs you gave me. Among them, I extend a heartfelt appreciation to my friend Tiago Macedo, who followed me closely and

Acknowledgments

always encouraged me throughout this journey. To my friend Lúdia Rocha, who was present at the beginning of my research journey and who continues to be an unwavering presence in my everyday life. And to my friend Dina Neves for consistently providing me encouragement during my most challenging moments.

To my boyfriend and my parents, I am eternally grateful for always believing in me. Thank you for the unvaluable support, uplifting words, endless patience, and profound understanding.

Abstract

Abstract

The increase in prevalence of drug-resistant insects and pathogenic fungi stands as a global public health concern, intensified by the selective pressure exerted by human activities and climate change on fungal and insect species. The emergence of drug-resistant species mainly arises from the lack of selectivity and persistence of the drugs in the environment. Specifically, in the case of antifungal agents, limited drug options contribute to cross-usage between agricultural and clinical practice, further exacerbating the issue. Over 1.5 million people die every year from fungal infections, making this one of the top global causes of death, ahead of vector-borne diseases, such as malaria. Vulnerable populations in developing countries persist as the hardest hit by this health issue. The highest global incidence is particularly focused in regions grappling with a history of human immunodeficiency virus (HIV) infections and malnutrition, such as Sub-Saharan Africa. However, it is important to highlight that the magnitude of the problem is underestimated due to poor diagnosis and measurement of disease morbidity.

In Sub-Saharan African region, medicinal plants stand as the primary resource supporting the healthcare system, often serving as the sole remedy for treating fungal diseases. World Health Organization (WHO) recognizes the need to develop an integrative approach that combines ethnopharmacological knowledge and conventional medicine, which underscores the importance of integrating the assessment of antifungal and insecticidal activities into medicinal plant research programs. Thus, the control of fungal and vector-borne diseases hindering resistance progress is the main challenge of the antifungal and insecticidal drug development programs. To address this challenge, the canonical paradigm of drug discovery, focused on synthetic molecules with a single-site mode of action, is shifting towards natural products-based approaches, aiming the development of novel multi-target antifungal and insecticidal formulations. In the first chapter of this thesis, information of the plants used in the Sub-Saharan African region against fungal diseases is gathered. Considering the unmet research and development (R&D) requirements along with the fungal priority list and concerns related to vector-borne diseases outlined by the WHO, a research strategy is proposed to pinpoint the plant compounds responsible for the antifungal and insecticidal activities.

To address the pressing issue of drug-resistance, two plants of Guinea-Bissau with ethnopharmacological significance were selected, namely *Guiera senegalensis* J.F.Gmel from Combretaceae family and *Ficus polita* L. from Moraceae family.

The chemical characterization of *G. senegalensis* crude methanol extract (CME) shows that it is a complex mixture of compounds, with the presence of sugars, phospholipids, guieranone A, porphyrin-containing compounds, steroids and/or triterpenes, and phenolic compounds. Aiming the establishment of a correlation between chemical composition and bioactivity, solid-phase extraction was used to discard water-soluble compounds with low affinity for the octadecyl phase (C18) column and to obtain an ethyl acetate fraction (EAF) that concentrates guieranone A and chlorophylls, and a methanol fraction (MF) with remaining compounds, that include phenolic compounds and phospholipids. While the CME and MF exhibited poor antifungal activity against *Aspergillus fumigatus*, *Fusarium oxysporum* and *Colletotrichum gloeosporioides*, EAF demonstrates antifungal activity against these filamentous fungi, particularly against *C. gloeosporioides*. Assays with yeasts revealed that EAF has strong effectiveness against the pathogenic *Cryptococcus neoformans* and *Candida krusei* with MICs of 8 and 16 µg/mL, respectively, as well as for non-pathogenic *Saccharomyces cerevisiae* (MIC = 8 µg/mL). Using *S. cerevisiae* as yeast model and combining *in vivo/ex vivo* assays with *in vitro* enzymatic assays, it was revealed the mechanism of action of *G. senegalensis* EAF. It works as a mitochondrial toxin, compromising complexes I and II activities, and as a strong inhibitor of fungal tyrosinase.

Neuronal *Drosophila melanogaster* (ML-DmBG2-c2) cell line was used to reveal that *G. senegalensis* CME has competence to promote insect cells toxicity, a bioactivity enhanced by its lipophilic fraction (EAF). The lactate dehydrogenase (LDH) leakage assay data indicate that this toxicity is not associated with the rupture of plasma membrane, suggesting that it does not involve cell death by necrosis. Cellular assays showed that the insect cells toxicity is associated with a significant enhancement of reactive oxygen species (ROS) generation and with mitochondrial dysfunction. It is shown that *G. senegalensis* CME has compounds capable of overcoming cellular barriers and targeting insect cell's mitochondrion, promoting inhibition of mitochondrial complex II and the depolarization of mitochondrial membrane potential.

The selectivity degree for fungal organisms and insect cells was achieved by assessing the toxicological profile of *G. senegalensis* CME and its fractions on cellular models of human neuronal (SH-SY5Y) and skin (HaCaT) cells. The obtained findings suggest that *G. senegalensis* CME and its fractions display selective toxicity towards neuronal insect cells and an improved degree of selectivity for pathogenic yeasts. Furthermore, *G. senegalensis* CME revealed capacity to protect HaCaT cells from the harmful effects of UVA radiation, by decreasing ROS generation.

The chemical characterization of *F. polita* CME leaves also revealed the presence of sugars, phospholipids, steroids and/or triterpenes, phenolic compounds, and porphyrin-containing compounds. A fractionation plan in a reversed-phase C18 SPE cartridge allows to obtain two fractions with a well-defined and distinctive chemical composition. Data show that porphyrin-containing compounds, steroids and/or triterpenes, as well as herniarin are largely retained in EAF, while the other phenolic compounds are mainly obtained in the MF. Whereas CME and MF display poor antifungal activity against filamentous fungi *A. fumigatus*, *F. oxysporum* and *C. gloeosporioides*, EAF demonstrates activity with MICs ranging from 1000 to 1500 µg/mL. In the same range of concentrations, EAF also exhibits fungistatic activity against yeasts (*S. cerevisiae*, *C. albicans*, *C. krusei* and *C. neoformans*). By assessing the oxygen consumption rate of *S. cerevisiae*, it is revealed that *F. polita* EAF exhibits yeast toxicity for concentrations equal to or higher than 150 µg/mL, highlighting the higher sensitivity of the metabolic assays. Investigation on the mechanisms of action revealed that *F. polita* EAF works as mitochondrial toxin for yeasts, by inhibiting complex II.

Assays focused on insecticidal potential of *F. polita* extract and fractions, assessed in neuronal *D. melanogaster* cells, reveal that *F. polita* CME affects the cell viability of ML-DmBG2-c2 cells at concentrations equal to or higher than 100 µg/mL. This insecticidal activity is improved by *F. polita* EAF. Thus, the lipophilic compounds enriched in EAF must be primarily responsible for the activity.

The toxicological profile of *F. polita* CME and its fractions in human HaCaT and SH-SY5Y cells depends on the cell type. Thus, the viability of HaCaT cells remained unaffected by *F. polita* CME or MF up to 200 µg/mL, while neuronal cells demonstrated sensitivity at the highest tested concentration of MF. Conversely, the EAF has negative impact on the viability of both human neuronal and skin cells at 200 µg/mL. Therefore, the degree of selectivity of the *F. polita* CME and its fractions for insect cells and yeasts is very limited.

Despite the *F. polita* CME and its fractions do not show insecticidal potential, *F. polita* EAF mitochondrial toxicity for yeasts endow it with some potential to be considered for the development of antifungal formulations. Additionally, *F. polita* leaves are an excellent renewable resource to obtain herniarin, a coumarin with multiple applications. On the other hand, *G. senegalensis* leaves are a renewable resource to obtain mixtures of bioactive compounds with high potential to support the development of innovative multi-target formulations with antifungal and insecticidal activities. Thus, the fraction of ethyl acetate obtained from the CME of *G. senegalensis* leaves emerges as a new tool to tackle the global challenge of resistance to antifungal drugs and insecticides. Additionally, this tool is

also an accessible and promising alternative for Sub-Saharan African countries to address public health issues related to both mycoses and insect-borne infectious diseases.

Keywords: *Guiera senegalensis*; *Ficus polita*; antifungal and insecticidal activities; mitochondria; tyrosinase.

Resumo

Resumo

O aumento da prevalência de espécies de insetos e fungos patogénicos resistentes a pesticidas e fármacos antifúngicos é uma preocupação global de saúde pública, que tem sido agravada pela pressão seletiva das atividades humanas e das alterações climáticas. A falta de seletividade e a persistência no ambiente destes compostos são a principal causa do aparecimento de novas espécies resistentes. No caso específico dos agentes antifúngicos, o número reduzido de compostos disponíveis resulta no seu uso cruzado entre a agricultura e a clínica, agravando o problema. Mais de 1,5 milhões de pessoas morrem todos os anos devido a infeções fúngicas, sendo esta uma das principais causas de morte a nível mundial à frente das doenças infecciosas transmitidas por insetos, como a malária. Esta problemática de saúde pública atinge de forma mais severa as populações vulneráveis dos países em vias de desenvolvimento, especialmente em regiões da África Subsaariana, onde o histórico de infeções pelo vírus da imunodeficiência humana e a desnutrição tornam essas comunidades ainda mais suscetíveis. No entanto, é importante sublinhar que a magnitude do problema é subestimada, devido à falta de diagnóstico e de avaliação da morbilidade das doenças provocadas por fungos e das doenças infecciosas transmitidas por insetos.

Na África Subsaariana, as plantas medicinais constituem o principal recurso que suporta o sistema de saúde, servindo frequentemente como o único tratamento para doenças fúngicas. A Organização Mundial de Saúde reconhece a necessidade de desenvolver uma abordagem integrativa que combine conhecimento etnofarmacológico e medicina convencional, destacando a importância de integrar a avaliação das atividades inseticida e antifúngica em programas de investigação de plantas medicinais. Desta forma, controlar as doenças fúngicas e as doenças transmitidas por vetores, travando o desenvolvimento de resistência a medicamentos e pesticidas, é o principal desafio dos programas de desenvolvimento de fármacos antifúngicos e de inseticidas. Para enfrentar este desafio, o paradigma canónico da descoberta de medicamentos, focado em moléculas sintéticas com um único mecanismo de ação, está a ser conduzido para abordagens baseadas em produtos naturais, visando o desenvolvimento de novas formulações antifúngicas e inseticidas multialvo.

No primeiro capítulo desta tese, apresenta-se uma compilação detalhada da informação disponível sobre as plantas medicinais utilizadas no tratamento de doenças fúngicas na África Subsaariana. Para além disso, é também racionalizada uma estratégia de investigação para identificar os compostos bioativos responsáveis pelas atividades

antifúngicas e inseticidas dessas plantas medicinais. Esta estratégia foi desenhada para colmatar as necessidades não atendidas pelos programas convencionais de investigação e desenvolvimento baseados em plantas medicinais, considerando a lista de prioridade de fungos patogénicos e as preocupações relacionadas com as doenças infecciosas transmitidas por insetos identificadas pela Organização Mundial de Saúde.

Para abordar a questão premente da resistência de pragas aos compostos farmacológicos e pesticidas disponíveis, foram selecionadas duas plantas da Guiné-Bissau com importância etnofarmacológica, nomeadamente, *Guiera senegalensis* J.F.Gmel pertencente à família das Combretáceas e *Ficus polita* L. da família das Moráceas.

A caracterização química do extrato metanólico bruto de *G. senegalensis* mostrou que este é uma mistura complexa de compostos, constituída por açúcares, fosfolípidios, guieranona A, compostos contendo porfirinas, esteróides e/ou triterpenos, bem como compostos fenólicos. Com o objetivo de estabelecer uma relação entre composição química e bioatividade, o extrato metanólico bruto foi submetido a um procedimento de extração em fase sólida para descartar compostos solúveis em água com baixa afinidade para a coluna octadecil (C18) e obter uma fração de acetato de etilo, que concentra guieranona A e clorofilas, bem como uma fração metanólica com os compostos remanescentes na coluna, que inclui compostos fenólicos e fosfolípidos. Enquanto o extrato metanólico bruto e a fração de metanol exibem fraca atividade antifúngica contra *Aspergillus fumigatus*, *Fusarium oxysporum* e *Colletotrichum gloeosporioides*, a fração de acetato de etilo demonstra atividade antifúngica relevante contra estes fungos filamentosos, particularmente contra *C. gloeosporioides*. Os ensaios com leveduras revelaram que a fração de acetato de etilo tem uma grande eficácia contra as leveduras patogénicas *Cryptococcus neoformans* e *Candida krusei* com concentrações inibitórias mínimas de 8 e 16 µg/mL, respetivamente, bem como para a levedura não patogénica *Saccharomyces cerevisiae* (MIC = 8 µg/mL). Usando *S. cerevisiae* como organismo-modelo e combinando ensaios *in vivo/ex vivo* com ensaios enzimáticos *in vitro*, foi possível revelar o mecanismo de ação da fração de acetato de etilo de *G. senegalensis*. Esta fração atua como uma toxina mitocondrial, comprometendo as atividades dos complexos I e II, e como um forte inibidor da enzima tirosinase fúngica.

Uma linha celular neuronal de *Drosophila melanogaster* (ML-DmBG2-c2) foi utilizada para revelar que o extrato metanólico bruto de *G. senegalensis* promove toxicidade em células de inseto. Uma bioatividade que é melhorada pela fração lipofílica. Os dados do ensaio com a enzima lactato desidrogenase indicam que a toxicidade celular não está associada à rutura da membrana plasmática, sugerindo que a morte celular não ocorre por

necrose. Para além disso, os ensaios celulares mostraram que a toxicidade exibida em células de inseto está relacionada com aumento da produção de espécies reativas de oxigénio e com disfunção mitocondrial. Os estudos mostraram que o extrato metanólico bruto de *G. senegalensis* possui compostos capazes de atravessar as barreiras das células de inseto e atingir a mitocôndria, diminuindo a atividade do complexo II da cadeia respiratória e promovendo a despolarizando da membrana interna da mitocôndria, i.e., redução do potencial elétrico transmembranar.

A determinação do grau de seletividade para fungos e células de insetos foi obtida através da avaliação do perfil toxicológico do extrato metanólico bruto de *G. senegalensis* e das suas frações em modelos de células neuronais (SH-SY5Y) e de células da pele (HaCaT) humanas. Os resultados obtidos sugerem que o extrato metanólico bruto e as suas frações apresentam toxicidade seletiva para as células neuronais de inseto e um maior grau de seletividade para leveduras patogénicas. Para além disso, o extrato metanólico bruto de *G. senegalensis* revelou capacidade para proteger os queratinócitos dos efeitos nocivos da radiação UVA, através do decréscimo da produção de espécies reativas do oxigénio.

A caracterização química do extrato metanólico bruto das folhas de *F. polita* também demonstrou a presença de açúcares, fosfolípidos, compostos fenólicos, esteroides e/ou triterpenos, bem como compostos contendo porfirinas. Um protocolo de fracionamento usando uma coluna de fase reversa para extração em fase sólida permitiu obter duas frações com composição química distinta e bem definida. Os dados mostram que os compostos contendo porfirinas, os esteroides e/ou triterpenos, bem como a herniarina são amplamente retidos na fração de acetato de etilo, enquanto os demais compostos fenólicos são obtidos principalmente na fração de metanol. Enquanto o extrato metanólico bruto e a fração de metanol apresentam baixa atividade antifúngica contra fungos filamentosos *A. fumigatus*, *F. oxysporum* e *C. gloeosporioides*, a fração de acetato de etilo apresenta concentrações inibitórias mínimas que variam entre os 1000 e os 1500 µg/mL. Através da avaliação da taxa de consumo de oxigénio de leveduras *S. cerevisiae*, foi demonstrado que a fração de acetato de etilo de *F. polita* exibe toxicidade em leveduras para concentrações iguais ou superiores a 150 µg/mL, destacando a maior sensibilidade dos ensaios metabólicos. A investigação dos mecanismos de ação mostrou que, em leveduras, a fração de acetato de etilo de *F. polita* funciona como uma toxina mitocondrial, promovendo a inibição do complexo II da cadeia respiratória.

O potencial inseticida do extrato metanólico bruto de *F. polita* e das suas frações foi avaliado em células neuronais de *D. melanogaster*. Os resultados obtidos mostram que o

extrato metanólico bruto de *F. polita* afeta a viabilidade celular das células ML-DmBG2-c2 em concentrações iguais ou superiores a 100 µg/mL. Este potencial inseticida é melhorado pela fração de acetato de etilo de *F. polita*. Assim, os compostos lipofílicos enriquecidos na fração de acetato de etilo parecem ser os principais responsáveis pela atividade.

O perfil toxicológico do extrato metanólico bruto de *F. polita* e das suas frações em células HaCaT e SH-SY5Y é dependente do tipo de célula. Os ensaios de viabilidade com HaCaT mostraram que a viabilidade destas células não é afetada pelo extrato metanólico bruto ou pela fração metanólica de *F. polita* até à concentração de 200 µg/mL. Contudo, as células neuronais demonstram maior sensibilidade aos efeitos da fração de metanol. Por outro lado, a fração de acetato de etilo à concentração de 200 µg/mL tem um impacto negativo sobre a viabilidade celular de ambas as linhas celulares humanas. Portanto, o grau de seletividade do extrato metanólico bruto de *F. polita* e das suas frações para células de inseto e para leveduras é limitado.

Apesar do extrato metanólico bruto de *F. polita* e das suas frações não apresentarem potencial inseticida, a toxicidade mitocondrial da fração de acetato de etilo revelada pelos ensaios metabólicos confere-lhe potencial para ser considerada no desenvolvimento de formulações antifúngicas. Adicionalmente, as folhas de *F. polita* são também uma excelente fonte renovável de herniarina, uma cumarina conhecida pelas suas múltiplas aplicações. Por outro lado, as folhas de *G. senegalensis* são um recurso renovável para obter misturas de compostos bioativos com um elevado potencial para suportar o desenvolvimento de inovadoras formulações com atividade antifúngica e inseticida baseadas em múltiplos modos de ação. Assim, a fração de acetato de etilo obtida do extrato metanólico bruto das folhas de *G. senegalensis* emerge como uma nova ferramenta para enfrentar o desafio global da resistência a fármacos antifúngicos e pesticidas. Além disso, esta ferramenta também é uma alternativa acessível e promissora para os países da África Subsaariana abordarem os problemas de saúde pública, tanto relacionados a micoses quanto a doenças infecciosas transmitidas por insetos.

Palavras-chave: *Guiera senegalensis*; *Ficus polita*; atividades antifúngica e inseticida; mitocôndria; tirosinase.

Table of Contents

Table of Contents

Publications	vii
Acknowledgments	ix
Abstract	xii
Resumo	xvii
List of Figures	xxviii
List of Tables	xxxv
List of Abbreviations	xxxviii
Thesis Outline	2
CHAPTER I: General Introduction and Objectives	3
1. Emerging fungal infections - an urgent public health problem	4
2. Fungal infections and traditional medicine in Sub-Saharan Africa – Challenges and opportunities	5
3. Research strategy to reveal the chemical compounds that ensure antifungal activity to the medicinal plants	20
3.1. Plant extract preparation and fractionation	21
3.2. Chemical analysis of the plant crude extract and fractions	22
3.3. Selection of the relevant pathogenic fungal species and assessment of the antifungal activity	24
3.4. The challenge of the relationship between chemical profile and bioactivity	27
4. The growing problem of the insecticide resistance	27
5. Research strategy to reveal the chemical compounds that ensure insecticidal activity to the medicinal plants	28
5.1. Selection of the relevant insect species and assessment of the insecticidal activity	28
6. Selection of the plant species	30
6.1. <i>Guiera senegalensis</i>	30
6.2. <i>Ficus polita</i>	32
	xxii

7. Objectives	34
CHAPTER II: Experimental Section.....	36
1. Standards and reagents	37
2. Sampling, preparation of extracts and fractionation	38
2.1. Sampling	38
2.2. Preparation of leaves crude methanol extracts.....	38
2.3. Fractionation of leaves crude methanol extracts.....	38
2.4. Alkaloid-targeted extraction	39
3. Chemical characterization	39
3.1. Total phenolic compounds.....	39
3.2. Sugars	40
3.3. Phospholipids.....	40
3.4. Liebermann-Burchard reactive compounds.....	41
3.5. Chlorophylls	41
3.6. Alkaloids	41
3.7. Compounds identification by HPLC-DAD-ESI(Ion Trap)-MS ⁿ and UPLC-ESI-QTOF-MS ² analyses.....	42
3.7.1. Phenolic compounds	42
3.7.2. Chlorophylls.....	42
3.7.3. Alkaloids.....	43
3.8. Quantification of compounds by HPLC-DAD	43
3.8.1. Phenolic compounds	43
3.8.2. Chlorophylls.....	46
4. Antioxidant activity	46
4.1. Nitric oxide radical scavenging activity	46
4.2. Superoxide anion radical scavenging activity	47
5. Antifungal activity assessment	48
5.1. Selection of fungal species.....	48
5.2. <i>In vivo</i> and <i>ex vivo</i> assays.....	49
5.2.1. Effects on growth.....	49
5.2.2. Effects on the mitochondrial respiratory function.....	50

5.2.3. Preparation of mitochondria-enriched fractions from <i>S. cerevisiae</i> yeasts.....	50
5.2.4. Effects on mitochondrial respiratory chain complexes	51
5.3. <i>In vitro</i> assays.....	52
5.3.1. Tyrosinase activity.....	52
5.3.2. Xanthine oxidase activity.....	53
6. Insecticidal activity assessment	53
6.1. Assays with ML-DmBG2-c2 <i>Drosophila melanogaster</i> cell line	53
6.1.1. Effects on cell viability	54
6.1.2. Effects on membrane integrity.....	54
6.1.3. Effects on the generation of intracellular reactive oxygen species.....	55
6.1.4. Effects on mitochondrial membrane potential.....	56
6.1.5. Preparation of mitochondria-enriched fractions from ML-DmBG2-c2 cells.....	57
6.1.6. Effects on mitochondrial respiratory chain complex activities	57
6.2. Effects on acetylcholinesterase activity.....	57
7. Toxicological profile.....	58
7.1. Cell conditions	58
7.2. Effects on cell viability.....	59
7.3. Effects on membrane integrity	59
7.4. Effects on the generation of intracellular reactive oxygen species.....	59
7.5. Effects on mitochondrial membrane potential.....	60
7.6. Effects on cell viability and intracellular reactive oxygen species after UVA irradiation	60
CHAPTER III: Results and Discussion	61
1. <i>G. senegalensis</i> leaves: from the chemical characterization to the antifungal and insecticide potential.....	62
1.1. Chemical characterization.....	62
1.1.1. Chemical composition of crude methanol extract and fractions	62
1.1.2. Characterization of the phenolic profile of <i>G. senegalensis</i> CME	65
1.1.3. Characterization of the phenolic and chlorophyll profiles of <i>G. senegalensis</i> EAF	73

1.2.	Antifungal activity	77
1.2.1.	Fungal susceptibility to <i>G. senegalensis</i> CME, MF and EAF	77
1.2.2.	<i>G. senegalensis</i> EAF exhibits antifungal activity related with mitochondrial dysfunction	80
1.2.3.	<i>G. senegalensis</i> EAF inhibits the activity of tyrosinase	83
1.3.	Insecticidal activity	86
1.3.1.	<i>G. senegalensis</i> CME and its fractions, MF and EAF, exhibit toxicity for insect cells.....	86
1.3.2.	<i>G. senegalensis</i> extract triggers toxicity for neuronal insect cells related with mitochondrial dysfunction.....	88
1.3.3.	The insecticidal potential of <i>G. senegalensis</i> CME is not related with the inhibition of acetylcholinesterase activity	91
1.4.	Toxicological profile in human neuronal and skin cells.....	93
1.4.1.	Effects on cell viability and membrane integrity	93
1.4.2.	Positive impact of <i>G. senegalensis</i> CME on ROS generation by human cell lines and protection against the harmful effects of UV radiation	95
1.5.	Main conclusions.....	97
2.	<i>Ficus polita</i> leaves: from the chemical characterization to the antifungal and insecticide potential	99
2.1.	Chemical characterization	99
2.1.1.	Chemical composition of <i>F. polita</i> CME, MF and EAF	99
2.1.2.	Characterization of the phenolic profile of <i>F. polita</i> CME and its fractions, MF and EAF.....	100
2.1.3.	Characterization of the chlorophyll profile of <i>F. polita</i> EAF	105
2.1.4.	Antioxidant activity of CME, MF and EAF	107
2.2.	Antifungal activity	108
2.2.1.	Fungal susceptibility to <i>F. polita</i> CME, MF and EAF	108
2.2.2.	<i>F. polita</i> EAF exhibits antifungal activity related with mitochondrial dysfunction.....	112
2.2.3.	Antifungal activity of <i>F. polita</i> is not related to tyrosinase inhibition ...	114
2.3.	Insecticidal activity	115
2.3.1.	Effects of <i>F. polita</i> CME and its fractions, MF and EAF, on ML-DmBG2- c2.....	115
2.4.	Toxicological profile on human neuronal and skin cells.....	117
2.5.	Main conclusions.....	119

CHAPTER IV: Integrated Discussion, Conclusion Remarks and Future Perspectives	
.....	120
CHAPTER V: References	
.....	127

List of Figures

List of Figures

Figure 1. <i>Guiera senegalensis</i> J. F. Gmel in Eticoga, Guinea-Bissau (photographs by Nelson Gomes).	31
Figure 2. <i>Ficus polita</i> Vahl. in Varela, Guinea-Bissau (photographs by Nelson Gomes).	33
Figure 3. Hydrolyzation of sugars that will react with phenol forming orange-yellow compounds.	40
Figure 4. Determination of nitrite by Griess reaction.	47
Figure 5. $O_2^{\cdot -}$ is formed by the NADH/PMS system and reacts with NBT to form a diformazan product.	48
Figure 6. Metabolism of L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosinase and formation of dopachrome.	52
Figure 7. Reduction of xanthine to uric acid by xanthine oxidase.	53
Figure 8. Reduction of MTT to formazan by mitochondrial dehydrogenases of metabolically active cells.	54
Figure 9. Reduction of pyruvate to lactate by LDH.	55
Figure 10. DCFH-DA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation yields the fluorescent adduct DCF....	56
Figure 11. Reactions underlying Ellman's method. Thiocoline results from the hydrolysis of acetylthiocholine iodide by AChE. Then, thiocoline reacts with DTNB to generate the anion TNB with yellow color.	58
Figure 12. HPLC-UV profile of the alkaloid-targeted <i>G. senegalensis</i> leaves extract, using a reverse phase Luna C18 (Phenomenex, California, USA) with spectra acquisition in continuous scan mode from 200 to 600 nm, and the chromatogram recorded at 280 nm.	64
Figure 13. HPLC-UV profile of CME obtained from the leaves of <i>G. senegalensis</i> . Polyphenolic compounds were analyzed by reverse-phase HPLC-DAD-ESI-MS ⁿ using a C18 column with spectra acquisition in continuous scan mode from 200 to 600 nm. The chromatograms were recorded at 280 and 340 nm. Compound identifications are indicated in Table 7	65

- Figure 14.** HPLC-UV profile of *G. senegalensis* EAF obtained by SPE (Hypersep C18) from the *G. senegalensis* leaves CME. Polyphenolic compounds were analyzed by reverse-phase HPLC-DAD using a C18 column with spectra acquisition in continuous scan mode from 200 to 600 nm. The chromatogram was recorded at 280 nm. 72
- Figure 15.** HPLC-UV profile of *G. senegalensis* EAF obtained by solid-phase extraction (Hypersep C18) from the *G. senegalensis* leaves CME, using a C30 column with spectra acquisition in continuous scan mode from 200 to 600 nm and the chromatogram recorded at 420 nm. Chlorophylls were also analyzed by reverse-phase HPLC-ESI-MSⁿ. The compound identification is indicated in **Table 8** and text. 74
- Figure 16.** Effects of *G. senegalensis* leaves CME and of its MF and EAF on the growth of *S. cerevisiae* (**A,C**), and *C. neoformans* (**B,D**), at 30 °C, in the respective standard media. Time-dependent growth of *S. cerevisiae* (**A**) and *C. neoformans* (**B**) cultures in the absence and presence of 50 µg/mL of *G. senegalensis* leaves CME or in the presence of its fractions, MF and EAF, at 50 µg/mL. Effects of increasing concentrations of EAF on *S. cerevisiae* (**C**) and *C. neoformans* (**D**) growth. The cell number was measured by the optical density at 610 nm. The results shown are representative of three independent experiments using three started yeast cultures. 80
- Figure 17.** Effects of the exposure of *S. cerevisiae* cultures to 50 µg/mL of *G. senegalensis* EAF, for 8 h, on the activity of the mitochondrial redox complexes I, II, and IV and citrate synthase (**A–D**). The activity of mitochondrial redox chain complexes was normalized by citrate synthase activity. *In vitro* effects of *G. senegalensis* EAF on the activity of complexes I and II of mitochondria isolated from *S. cerevisiae* cultures grown in control condition, i.e., in the absence of EAF (**E, F**). Results are presented as mean ± SD of three independent assays. *, **, *** Significantly different from control condition, with $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Black bars—control; gray bars— *G. senegalensis* EAF treatment. 82
- Figure 18.** Effects of increasing concentrations of *G. senegalensis* EAF on the activity of pure mushroom tyrosinase assessed in the presence of low (90 µM – white circles) and high (900 µM – black circles) L-DOPA (enzyme substrate) concentration (**A**). Kinetics of tyrosinase activity in the absence (black circles) and presence of 10 (squares) or 25 (triangles) µg/mL of *G. senegalensis* EAF using seven concentrations of substrate (**B**). Data were fitted into a Michaelis-Menten kinetic equation and used to construct the double-reciprocal plot analysis (**C**). Table with the apparent Michaelis–Menten kinetic parameters and K_i values obtained with a noncompetitive inhibition model using GraphPad Prism software (**D**). *, ** Significantly different from control condition, with $p \leq 0.05$, $p \leq 0.01$, respectively. 84

- Figure 19.** Proliferation curves of ML-DmBG2-c2 cells built with MTT reduction assay data obtained at different time-points, over 120 h, using cultures seeded in 96-well plates with 3.5×10^4 (white symbols) or with 7.5×10^4 (black symbols) cells/well..... 86
- Figure 20.** Effect of increasing concentrations of *G. senegalensis* leaves CME on the viability (A) and membrane integrity (B) of ML-DmBG2-c2, evaluated, respectively, by the MTT reduction and the LDH leakage assays, after a 48 h incubation period. The decrease in cell viability as function of extract concentration is shown in the insert A. Results are expressed as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of five independent assays, each one performed in triplicate. *, *** Significantly different from the control cells, with $p \leq 0.05$ and $p \leq 0.001$, respectively. 87
- Figure 21.** Effect of increasing concentrations of *G. senegalensis* MF (A) and EAF (B) on ML-DmBG2-c2 cells, evaluated by the MTT reduction, after a 48 h incubation period. Results are expressed as percentage of control (cells treated with vehicle) and presented as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of three independent assays, each one performed in triplicate. *, **, *** Significantly different from the control cells, with $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. 87
- Figure 22.** Effect of increasing concentrations of *G. senegalensis* leaves CME on the cellular ROS generation of ML-DmBG2-c2 assessed by using DCFH-DA probe. Results are expressed as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of four independent assays, each one performed in triplicate. ** Significantly different from the control cells, with $p \leq 0.01$ 88
- Figure 23.** Effect of 25 $\mu\text{g/mL}$ of *G. senegalensis* leaves CME on the activity of the mitochondrial complexes I (A), II (B), and IV (C). The activity of mitochondrial redox chain complexes was assessed in mitochondria-enriched fraction obtained from untreated or treated ML-DmBG2-c2 cells and normalized by citrate synthase activity (D). Results are presented as mean $\pm$ SD of four independent assays. * Significantly different from untreated cells, with $p \leq 0.05$ 89
- Figure 24.** (A) Direct effects of *G. senegalensis* leaves CME on the activity of the complex II of mitochondria isolated from ML-DmBG2-c2 cells. (B) $\Delta\Psi_m$ of the ML-DmBG2-c2 cells exposed for 30 minutes to increasing concentrations of *G. senegalensis* leaves CME, expressed by ratio of red (aggregate) to green (monomer) fluorescence JC-1. FCCP (12 μM) were used as positive control. For both parameters, results are presented as mean $\pm$ SD of at least three independent experiments, each one performed in triplicate. **, ***

Significantly different from the matched control cells, with $p \leq 0.01$ and $p \leq 0.001$, respectively.	90
Figure 25. Effects of increasing concentrations of <i>G. senegalensis</i> CME on the activity of acetylcholinesterase assessed in the presence of low (A - 0.625 mM) and high (B - 20 mM) acetylthiocholine concentrations, respectively. Results are expressed as percentage of control and presented as mean $\pm$ SD of three independent assays, each one performed in triplicate.....	92
Figure 26. Effect of increasing concentrations of <i>G. senegalensis</i> leaves CME on the viability (A, C) and membrane integrity (B, D) of HaCaT (A, B) and SH-SY5Y (C, D), evaluated respectively by the MTT reduction and the LDH leakage assays, after a 48 h incubation period. Results are expressed as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of three independent assays, each one performed in triplicate. *, **, *** Significantly different from the control cells, with $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.....	93
Figure 27. Effect of increasing concentrations of <i>G. senegalensis</i> MF (A, C) and EAF (B, D) on HaCaT (A, B) and SH-SY5Y (C, D) cells, evaluated by the MTT reduction, after a 48 h incubation period. Results are expressed as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of, at least, three independent assays, each one performed in triplicate. *** Significantly different from the control cells, with $p \leq 0.001$	94
Figure 28. Effect of increasing concentrations of <i>G. senegalensis</i> leaves CME on the cellular ROS generation of HaCaT (A) and SH-SY5Y (B) cells assessed by using DCFH-DA probe. Results are expressed as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of four independent assays, each one performed in triplicate. *, **, *** Significantly different from the control cells, with $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.	95
Figure 29. (A) Effects of the pre-incubation of HaCaT cells with <i>G. senegalensis</i> CME for 48 h on the harmful effects of exposure to UV radiation (365 nm) for 15 min, assessed by cell viability (MTT reduction assay). (B) Effects of the pre-treatment of 50 μ g/mL of <i>G. senegalensis</i> CME for 0.5 h before the exposure to UV radiation (365 nm) for 15 min, assessed by intracellular ROS generation (DCFH-DA fluorescence assay). *, **, *** Significantly different from control (untreated cells) with $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. #, ## Significantly different from the cells exposed to UV radiation, with $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. n.s., without significant difference.	96

- Figure 30.** HPLC-UV profile of *F. polita* leaves CME (A), MF (B) and EAF (C). Compounds were analyzed by reverse phase HPLC-DAD-ESI-MSⁿ using a C18 column with spectra acquisition in continuous scan mode from 200 to 600 nm, and the chromatograms recorded at 335, 280 and 320 nm. Compounds identification as indicated in the insert of chromatogram B, in Table 12 and text. N.Q. Not quantified. 101
- Figure 31.** HPLC-UV profile of *F. polita* leaves EAF. Compounds were analyzed by reverse phase HPLC-DAD-ESI-MSⁿ using a C30 column with spectra acquisition in continuous scan mode from 200 to 600 nm, and the chromatograms recorded at 320 and 420 nm. Compounds identification as indicated in Table 13 and text. 105
- Figure 32.** Effect of increasing concentrations of *F. polita* leaves CME and of its fractions, MF and EAF, on the scavenging of the •NO (A) and of O₂•⁻ (B) and on the activity of xanthine oxidase (C) assessed in the presence of xanthine (enzyme substrate). Results are expressed as percentage of control and presented as mean ± SD of, at least, three independent assays, each one performed in triplicate. 108
- Figure 33.** Time-dependent growth of *S. cerevisiae* (A) and *C. neoformans* (B) cultures in SAB medium, at 30 °C, in the absence and presence of 100 µg/mL of *F. polita* leaves CME, MF or EAF. Effects of increasing concentrations of EAF (0 - 400 µg/mL) on *S. cerevisiae* (C) and *C. neoformans* (D) growth. The cell number was measured by the optical density at 610 nm. The results shown are representative of three independent experiments using three started yeast cultures. 111
- Figure 34.** Effect of increasing concentrations of *F. polita* EAF and herniarin (100 µg/mL) on the mitochondrial respiration of *S. cerevisiae* yeasts, at 30 °C, in the respective media (A). Typical assay of the oxygen consumption promoted by 2.2 million yeasts exposed to *F. polita* EAF recorded by a Clark-type electrode in 1 mL of culture medium (B). *In vitro* effects of *F. polita* EAF on the activity of complexes I, II and IV of mitochondria isolated from *S. cerevisiae* cultures grown in control condition, i.e., in the absence of EAF (C, D, E). Results are expressed as percentage of control (cells treated with vehicle) and presented as mean ± SD of three independent assays. *, **, *** Significantly different from control condition, with $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. 113
- Figure 35.** Effect of increasing concentrations of *F. polita* CME on the activity of pure mushroom tyrosinase assessed in the presence of 90 µM of L-DOPA (enzyme substrate). Results are expressed as percentage of control and presented as mean ± SD of three independent assays, each one performed in triplicate. 114

- Figure 36.** Effect of increasing concentrations of *F. polita* leaves CME and its fractions, MF and EAF, on the viability of ML-DmBG2-c2 (**A, B, C**), evaluated by the MTT reduction, after a 48 h incubation period. Results are expressed as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of three independent assays, each one performed in triplicate. *, **, *** Significantly different from the control cells, with $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively..... 115
- Figure 37.** Effects of increasing concentrations of *F. polita* CME on the activity of AChE assessed in the presence of 0.625 (**A**) and 20 mM (**B**) acetylthiocholine, respectively. Results are expressed as percentage of control and presented as mean $\pm$ SD of three independent assays, each one performed in triplicate. 116
- Figure 38.** Effect of increasing concentrations of *F. polita* leaves CME and of its fractions, MF and EAF, on the viability of HaCaT (**A, B, C**) and SH-SY-5Y (**D, E, F**) cells evaluated by the MTT reduction assay after a 48 h incubation period. Results are expressed as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of at least three independent assays, respectively, each one performed in triplicate. * $p \leq 0.05$ ** $p \leq 0.01$, *** $p \leq 0.001$ 117

List of Tables

List of Tables

Table 1. Main plants used in the treatment of mycoses in Sub-Saharan Africa.....	9
Table 2. Panel of pathogenic fungal species to assess the antifungal activity.....	24
Table 3. Linear regression equation analysis, limit of detection (LOD) and limit of quantification (LOQ) for external standards used in the quantification of <i>G. senegalensis</i> phenolic compounds.	44
Table 4. Linear regression equation analysis, LOD and LOQ for external standards used in the quantification of <i>F. polita</i> phenolic compounds.....	45
Table 5. Linear regression equation analysis, LOD and LOQ for chlorophylls <i>a</i> and <i>b</i>	46
Table 6. Extraction yields of <i>G. senegalensis</i> leaves with methanol, CME, and of its solid phase fractionation obtained with ethyl acetate, EAF, and methanol, MF, and their general characterization in terms of total levels of Folin-Ciocalteu reactive phenolic compounds, sugars, phospholipids, Liebermann-Burchard reactive compounds, chlorophylls and alkaloids indicating the EF for each one.	63
Table 7. Identification and quantification (dry weight basis) of each phenolic compound detected in <i>G. senegalensis</i> leaves CME by HPLC-DAD-ESI-MS ⁿ : Rt (retention time), UV-Vis absorption features, molecular formula, MS features in negative or positive (for guieranone A) ion modes with indication of main MS ² detected fragments.	66
Table 8. Identification and quantification (dry weight basis) of each chlorophyll detected in <i>G. senegalensis</i> leaves EAF by HPLC-ESI-MS ⁿ : Rt, UV absorption features, molecular formula, MS features in positive ion mode with indication of main MS ² detected fragments.	76
Table 9. Antifungal activity of <i>G. senegalensis</i> CME and its fractions, EAF and MF, against three species of filamentous fungi, assessed by MIC and MFC.....	77
Table 10. Antifungal activity of <i>G. senegalensis</i> EAF against four yeast strains, assessed by MIC and MFC. The fungicide penconazole and the antifungal fluconazole were used as positive controls.	78
Table 11. Extraction yields of <i>F. polita</i> leaves with methanol (CME) and of its solid-phase fractionation obtained with ethyl acetate (EAF) and methanol (MF). There is also a general characterization in terms of total levels of Folin–Ciocalteu reactive phenolic compounds,	

sugars, phospholipids, Liebermann–Burchard reactive compounds, and chlorophylls with the EF indicated for each compound..... 99

Table 12. Identification and quantification (dry weight basis) of each phenolic compound detected in the *F. polita* leaves CME by HPLC-DAD-ESI-MSⁿ: Rt, UV absorption features, molecular formula, MS features in negative or positive (for herniarin) ion modes with indication of main MS² detected fragments..... 102

Table 13. Identification and quantification (dry weight basis) of each chlorophyll detected in *F. polita* leaves EAF by HPLC-ESI-MSⁿ: Rt, UV absorption features, molecular formula, MS features in positive ion mode with indication of main MS² detected fragments. 106

Table 14. Antifungal activity of the *F. polita* CME and its fractions, EAF and MF, against three species of filamentous fungi, assessed by MIC and MFC. 109

Table 15. Antifungal activity of *F. polita* EAF, herniarin and fluconazole against four yeast strains, assessed by MIC and MFC..... 109

List of Abbreviations

List of Abbreviations

$\Delta\Psi_m$ – mitochondrial membrane potential

A

AChE – acetylcholinesterase

ATP – adenosine triphosphate

C

C18 – octadecyl phase

C30 – triacontyl phase

CLSI – Clinical and Laboratory Standards Institute

CME – crude methanol extract

Complex I – NADH-ubiquinone reductase

Complex II – succinate-ubiquinone reductase

Complex IV – cytochrome c oxidase

CS – citrate synthase

D

DAD – diode array detector

DCF – 2',7'-dichlorofluorescein

DCFH-DA – 2',7'-dichlorodihydrofluorescein diacetate

DCPIP – 2,6-dichlorophenolindophenol

DMEM – Dulbecco's modified eagle medium

DMEM-F12 – Dulbecco's modified eagle medium/nutrient mixture F-12

DMSO – dimethyl sulfoxide

DTNB – 5,5'-dithiobis(2-nitrobenzoic acid)

E

EAF – ethyl acetate fraction

EDTA – ethylenediaminetetraacetic acid

EF – enrichment factor

ESI – electrospray ionization

F

FBS – fetal bovine serum

FCCP – carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone

FCR – Folin–Ciocalteu Reagent

FRAC – Fungicide Resistance Action Committee

H

HBSS – Hanks' balanced salt solution

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

List of Abbreviations

HIV – human immunodeficiency virus

HPLC – high performance liquid chromatography

I

IC₅₀ – half maximal inhibitory concentration

J

JC-1 – 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide

L

LC – liquid chromatography

LDH – lactate dehydrogenase

L-DOPA – L-3,4-dihydroxyphenylalanine

LOD – limit of detection

LOQ – limit of quantification

M

MF – methanol fraction

MFC – minimum fungicidal concentration

MIC – minimal inhibitory concentration

MOPS – 3-morpholinepropanesulfonic acid

MS – mass spectrometry

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N

NADH – reduced nicotinamide adenine dinucleotide

NBT – nitrotetrazolium blue

[•]NO – nitric oxide radical

O

O₂^{•-} – superoxide anion radical

P

PMS – phenazine methylosulfate

PTFE – polytetrafluoroethylene

R

R&D – research and development

ROS – reactive oxygen species

RPMI-1640 – Roswell Park Memorial Institute medium

R_t – retention time

Q

QTOF – quadrupole time of flight

S

SDA – Sabouraud dextrose agar

SDB – Sabouraud dextrose broth

SNP – sodium nitroprusside

SPE – solid-phase extraction

U

UK – United Kingdom

UPLC – ultra-performance liquid
chromatography

USA – United States of America

UV – ultraviolet

UV-Vis – ultraviolet-visible

W

WHO – World Health Organization

Thesis Outline

Thesis Outline

Chapter I – General Introduction and Objectives

This chapter comprises a general introduction concerning the challenges and opportunities of the traditional medicine of Sub-Saharan Africa in the fight against the pressing issue of fungal diseases, by gathering information about the medicinal plants used to treat fungal infections. The second part describes a four-step research strategy to identify the chemical compounds responsible for the putative antifungal activity of these plants, also applying it to the investigation of the insecticidal potential. Additionally, it introduces the plant species chosen for this work.

Chapter II – Experimental Section

This chapter describes the methodologies used to perform the work underlying this thesis.

Chapter III – Results and Discussion

This chapter presents a detailed description of the results obtained during this thesis and it is subdivided in two sub-chapters, one for each plant species.

Chapter IV – Integrated Discussion, Conclusion Remarks and Future Perspectives

This chapter brings together the main results of the experimental work carried out, presenting the conclusions and future perspectives for the research area.

Chapter V – References

This chapter contains all the bibliographic references cited throughout this thesis.

CHAPTER I: General Introduction and Objectives

1. Emerging fungal infections - an urgent public health problem

Animal and plant fungal diseases (known as mycoses) associated with the increased worldwide incidence of pathogenic fungal strains with resistance to the available drugs are a substantial threat to the global public health by their negative impact on the clinical practice, food security, crop production and food supply chains (1,2). Although fungi have colonized almost all ecosystems on Earth, it is well known that fungi kingdom presents a great diversity of species, most of them with a spatially restricted endemic distribution resulting from an adaptation to local habitats and specific hosts. However, human activities and climatic changes are modifying natural environments, creating new opportunities for fungal species evolution while intensifying the pathogenic fungal species dispersal worldwide (3). For the continual emergence of drug-resistant fungal strains, contribute not only the lack of selectivity and the environmental persistence of the used drugs but also the restricted collection of the available antifungal agents that ends in the cross-use between agriculture and health (4). Thus, over 1.5 million people die every year from fungal infections, making this one of the top global causes of death (5). It is also important to highlight that the true magnitude of the problem is underestimated due to poor diagnosis and measurement of disease morbidity in many countries (5). In fact, the treatment of fungal infections in immunocompromised patients and in people affected by malnutrition (undernutrition) remains a medical challenge (3,6). Considering the unmet research and development (R&D) needs and perceived public health importance, World Health Organization (WHO) reported in 2022 the first fungal priority pathogens list, wherein they are grouped in clusters with critical, high, and medium priority (7). Regarding the current strategy for fungal diseases, mainly supported by one target synthetic antifungal drugs, the challenge will be difficult to solve in the foreseeable future. On the one hand, the repertoire of antifungal drugs with approval for medical and agriculture uses relies on five classes of chemical compounds (azoles, allylamines, echinocandins, polyenes and the pyrimidine analogues) supported by a limited number of mechanisms of action (e.g., inhibition of ergosterol biosynthesis, inhibition of β -glucan synthesis, disruption of fungal membrane, inhibition of nucleic acid synthesis). On the other hand, the selective pressure exerted by these chemical compounds, every year used in the range of thousands of tons, led to the emergence of several pathogenic fungal strains with resistance to one or more antifungal drugs, as reported for the yeast *Candida auris* and the filamentous fungi *Aspergillus fumigatus* (8).

Although fungal diseases are a global problem, the hitches faced by developing countries are even greater and immeasurable due to the lack of access to quality health

care and the unavailability of effective antifungal drugs. In fact, the history of human immunodeficiency virus (HIV) infections and the recurrent episodes of malnutrition make the developing countries of the Sub-Saharan African region the most affected by this health problem (3). In this African region (mainly in rural areas), the medicinal plants are, most of the time, the unique resource to support the health care system, including for the treatment of fungal diseases (9). Consequently, the WHO African region recognizes the need for a holistic approach of traditional medicine to the diagnosis, prevention, and/or elimination of illness, highlighting the potential of the assessment of the antifungal activity in the medicinal plant research programs (10,11).

Many works support the idea that the botanical fungicides exhibit several advantages over synthetic ones, including its better environmental biodegradability and lower toxicity to non-target species (12). Plant extracts with antifungal activity are mixtures of compounds, most often containing bioactives from different chemical classes, thereby they can exhibit potential to control fungal pathogens by multiple mechanisms of action, hampering the emergence of new drug-resistant pathogens (13). Thus, research on plant-extracts emerges as a suitable strategy to develop new antifungal formulations with competence to address the above-mentioned fungal diseases issue, including in Sub-Saharan African region.

Driven by traditional medicine knowledge of the Sub-Saharan African region, information on the medicinal plants used to treat fungal diseases, and whenever available their putative bioactive compounds will be gathered in this chapter. Additionally, it is described a four-step research strategy to identify the chemical compounds that are responsible for the putative antifungal activity of the medicinal plants. It is a research guide to evaluate the potential of the Sub-Saharan African region medicinal plants to address the pressing issue of antifungal resistance.

2. Fungal infections and traditional medicine in Sub-Saharan Africa – Challenges and opportunities

The Sub-Saharan African region, with tropical localization, exhibits a flora biodiversity with about 45,000 different species of plants, 5,000 of them are used in traditional medicine (9,14). Therefore, the flora biodiversity of Sub-Saharan African region has a great potential to allow the discovery of new antifungal agents. Additionally, in countries of this part of the world, medicinal plants are the first choice for treating many diseases, including fungal

infections (15), thereby the antifungal drug discovery can be driven by ethnobotanical research.

According with ethnobotanical reviews and research works available in Scopus data base, the traditional medicine of the Sub-Saharan African region addresses the issue of fungal diseases with, at least, seventy-two plant species, as summarized in **Table 1**. The information was organized by plant family, in alphabetical order, along with botanical and common names of the plant species, as well as the indication of the plant parts used, fungal infections to which they are applied, and whenever available the chemical compounds reported for the plant.

The traditional medicine of the Sub-Saharan African region recognizes antifungal activity to plant species from thirty-seven families and sixty-four genera. Asphodelaceae and Asteraceae families, with, respectively, nine and six plant species, are the most represented. On the other hand, *Aloe* (Asphodelaceae family) is the gender with highest number of species. Looking at the gathered information on the plant parts used for managing fungal diseases, we can assert that it is dependent of plant species, but leaves, roots, barks, bulbs, stems, rhizomes, corms, fruits, and seeds are commonly used. However, the leaf followed by root are the most frequently referred plant parts to treat diseases caused by fungi. In fact, these plant parts are also preferred to treat several other health conditions (16). Nevertheless, eleven rural traditional healers from Venda, in South Africa, claimed to prefer using roots and barks, or even its mixture, instead of leaves in the preparation of herbal remedies, including the ones used to treat candidiasis (17). In the absence of scientific studies that connect chemical profile with antifungal bioactivity, it is not possible to select the plant part more suitable for medicinal use. It is important to highlight that in contrast to collecting leaves, harvesting roots and barks may negatively impact plant health.

The collected information indicates that seventy-two plant species are used by traditional medicine of the Sub-Saharan African region to treat candidiasis, dermatophytosis, cryptococcosis/cryptococcal meningitis, aspergillosis, mycetoma and coccidioidomycosis. According to **Table 1** data, only one plant species (*Tetradenia riparia* Benth.) is used to treat coccidioidomycosis, while to tackle mycetoma, aspergillosis, cryptococcosis/cryptococcal meningitis, dermatophytosis and candidiasis are used four, eight, twelve, twenty-two and thirty-two plant species, respectively. Additionally, several plant species seem to have a broad spectrum of application, since they are used to treat different types of fungal diseases. For six plant species, there is no information available regarding the specific type of disease for which they are used, while the plant species used

to tackle candidiasis, dermatophytosis and cryptococcosis/cryptococcal meningitis, diseases with high relevance in Sub-Saharan African region, represent around 80% of the ethnopharmacological repertoire.

Candida spp. (mainly *C. albicans*, *C. glabrata* and *C. tropicalis*) are known commensal fungi of the oral, gastrointestinal tract and urogenital mucosa that promote opportunistic infections (18). Vulvovaginal candidiasis is an ordinary infection of adult women, and it was estimated that approximately 75% of healthy adult women will suffer from at least one vaginal infection annually (19–21). On the other hand, oropharyngeal and esophageal candidiasis are very common in immunosuppressed patients resulting from HIV infection, thereby they have high relevance in Sub-Saharan Africa. *Candida* infections can evolve to more severe forms of the disease, which contribute to significant morbidity and mortality among African patients. Invasive candidiasis is a serious and progressive infection that can affect the blood, heart, brain, eyes, bones, and other human tissues. When the infection reaches the bloodstream, it is called candidemia (22). Thirty-two plant species are used to treat infections caused by the yeast *Candida* spp. (**Table 1**). However, for some plant species the available information does not allow to indicate if they are used to treat local or systemic infections and/or both forms of the disease.

Dermatophytosis are infections caused by filamentous fungi (known as dermatophytes) able to degrade keratin in skin, nails, and hair. These fungal infections affect humans, from neonates to the elderly. In people with healthy immune system, the dermatophytosis are easily managed with antifungal agents, but they are a serious problem in people under immunosuppressive condition. Although forty fungal pathogens can cause skin infections in humans, the majority are caused by dermatophytes belonging to *Epidermophyton*, *Microsporum* and *Trichophyton* spp. genera (16). Twenty-two plant species are used to treat dermatophytosis. Nevertheless, the gathered information does not allow to connect the plant species used with the pathogenic fungi that cause the disease. Some studies mention that Tinea corporis, Tinea capitis and Tinea pedis were the diseases most commonly treated by using medicinal plants (23).

Cryptococcosis is an opportunistic fungal infection caused by the species *Cryptococcus neoformans* and *Cryptococcus gattii*. In general, there are two ways for the disease to manifest itself, cryptococcal meningitis and pulmonary cryptococcosis. Lung infections can cause pneumonia-like symptoms. If the infection is disseminated to other parts of the body, it is called disseminated cryptococcosis and it usually leads to skin changes (24). Cryptococcal meningitis is the invasive form of the disease, and 70% of its deaths occur in Sub-Saharan Africa (25). According with the collected data (**Table 1**), twelve

medicinal plant species are used to treat cryptococcosis in Sub-Saharan Africa. Most of the studies report the use of the medicinal plant species to treat cryptococcal meningitis, while other studies only use the generic name of the disease (cryptococcosis) and, therefore, it is not possible to know for which form of the disease the plant is used.

Although the treatment of fungal diseases by the medicinal plants is a well-documented ethnopharmacological issue, the relationship between chemical composition and antifungal activity was not investigated for many of them. However, the research interest on these medicinal plants goes beyond their antifungal activity, thereby there is information on the chemical composition of extracts obtained from, at least, sixty-eight plant species, as included in **Table 1**. In general, the research was directed to plant secondary metabolites with well-recognized multiple bioactivities, including phenolic compounds (e.g., phenolic acids, flavonoids, tannins, stilbenes, xanthones, lignans), terpenes, alkaloids, saponins, sterols and carotenoids (**Table 1**). It is also important to highlight that the secondary metabolites-related information cannot be used for plant species prioritization, since different solvents and extraction methods were used to prepare the extracts.

Considering this scientific scenario, we can state that the rationalization of the use of the medicinal plants used in Sub-Saharan African region to treat fungal diseases requires to fulfil a big gap in scientific-based knowledge. First, it is necessary to identify, for each plant species, what are the chemical compounds responsible for antifungal bioactivity, and for what pathogenic fungal species/strains they are active. Second, to assess if the antifungal bioactivity emerges from a specific compound or from a mixture of compounds. Third, to assess the selectivity degree for the pathogenic fungi. In the next section of this thesis, it will be rationalized the basis of a research program able to pursue the above-mentioned goals and guide the pre-clinic development of formulations with therapeutic potential.

Table 1. Main plants used in the treatment of mycoses in Sub-Saharan Africa.

Plant family	Plant species used in folk medicine/Common name(s)	Plant part(s)	Mycoses	Classes of compounds detected
Aizoaceae	<i>Carpobrotus edulis</i> (L.) N.E. Br Igcukuma (Xhosa)	Leaf	Candidemia, oral candidiasis (26)	Flavonoids Phenolic Acids Tannins Carotenoids Terpenes (27–31)
Amaryllidaceae	<i>Allium sativum</i> L. Garlic	-	Candidemia, candidiasis, aspergillosis (32)	Phenolic compounds Organosulfur compounds (33)
	<i>Nerine filifolia</i> Baker Guernsey lily, itswele	Root	Candidiasis (26)	-
	<i>Tulbaghia violacea</i> Harv. Wildeknoffel, wildeknoffok (Afrikaans), utswelane (Xhosa), isihaga, incinsini, (Zulu)	Leaf	Cryptococcal meningitis (15)	Organosulfur compounds Flavonoids Phenolics Saponins (34–36)
Anacardiaceae	<i>Protorhus longifolia</i> (Bernh.) Engl. Rooiboekehout, rooimelkhout	Leaf	Dermatophytosis (37)	Terpenes Flavonoids (38,39)
	<i>Daucus carota</i> L.	Leaf	Dermatophytosis (15)	Phenolic acids Flavonoids Anthocyanins (40,41)
Apiaceae	<i>Alepidea amatymbica</i> Eckl. & Zeyh. Iqwili (Xhosa), ikhathazo (Zulu)	-	Candidiasis, aspergillosis (32)	Terpenes (42)
	<i>Cuminum cyminum</i> L. Cumin, black cumin (Nigella/kalonji), bitter cumin (Kashmiri jeera/ Shahi jeera)	-	Mycetoma (43)	Phenolic compounds Flavonoids Terpenes (44)
Apocynaceae	<i>Xysmalobium undulatum</i> L. Uzara	Root	Vaginal candidiasis (37)	Sterols (45,46)

Table 1. Main plants used in the treatment of mycoses in Sub-Saharan Africa (continued).

Plant family	Plant species used in folk medicine/Common name(s)	Plant part(s)	Mycoses	Classes of compounds detected
Araceae	<i>Zantedeschia aethiopica</i> (L.) Spreng. Wit varkoor (Afrikaans), intebe (Xhosa), ihlukwe (Zulu)	Stem, bulb/ rhizome	Dermatophytosis (47)	Flavonoids Terpenes Sterols Lignans Phenylpropanoids (48,49)
Asparagaceae	<i>Eucomis autumnalis</i> (Mill.) Chitt. Wildepynappel (Afrikaans), umathunga (Zulu)	Leaf, stem, bulb	Dermatophytosis (50)	-
Asphodelaceae	<i>Aloe arborescens</i> Mill. Kransaalwyn (Afrikaans), ikalene (Xhosa), inkalane, umhlabana (Zulu)	Leaf	Dermatophytosis (47)	Phenolics (anthraquinones, anthrones, pyrones, chromones and coumarins) (51,52)
	<i>Aloe aristata</i> Haw.	Leaf	Dermatophytosis (47)	-
	<i>Aloe ferox</i> Mill. Bitteraalwyn (Afrikaans), iNhlaba (Zulu)	Leaf	Candidemia, vaginal candidiasis, dermatophytosis (50,53)	Anthraquinones (54)
	<i>Aloe vera</i> (L.) Burm.f.	Leaf	Fungal infection not specified (15)	-
	<i>Bulbine frutescens</i> (L.) Willd. Balsam kopieva, geelkatstert (Afrikaans)	Leaf, stem, root	Dermatophytosis (47,50)	Anthraquinones Xanthenes Naphthoquinones (55–57)
	<i>Bulbine natalensis</i> Baker Ibhucu (Zulu), rooiwortel, geelkopieva (Afrikaans), incelwane (Xhosa)	Leaf, bulb, root	Vaginal candidiasis, dermatophytosis (37,47)	Anthraquinones Phenolics Flavonoids Proanthocyanidins Tannins Cardiac glycosides Saponins Alkaloids (50,55,58)

Table 1. Main plants used in the treatment of mycoses in Sub-Saharan Africa (continued).

Plant family	Plant species used in folk medicine/Common name(s)	Plant part(s)	Mycoses	Classes of compounds detected
Asphodelaceae	<i>Gasteria bicolor</i> Haw. Klein-beeston	Leaf	Fungal infection not specified (59)	Saponins Alkaloids Phenolics Proanthocyanidins Flavonoids (32)
	<i>Haworthia limifolia</i> (Marloth) G.D.Rowley Isihlalakahle, omathithibala, umathithibala	Leaf, stem, root	Dermatophytosis (47)	Phenolics Flavonoids Proanthocyanidins (50)
Asteraceae	<i>Arctotis arctotoides</i> (L.f.) O.Hoffm Gousblom	Leaf	Dermatophytosis (59)	Terpenes Sterols Flavonoids (60–62)
	<i>Artemisia afra</i> Jacq. ex Willd. Wilde Als (Afrikaans), umhlonyane (Zulu), lengana	Corm leaf	Candidemia, candidiasis (37)	Terpenes Sterols Flavonoids (63,64)
	<i>Artemisia annua</i> L.	Leaf	Dermatophytosis (15)	
	<i>Elytropappus rhinocerotis</i> Less. Renosterbos (Afrikaans)	Stem	Dermatophytosis (65)	Flavonoids Coumarins Terpenes (66)
	<i>Helichrysum cymosum</i> L. Goue tapyt, Imphepho	Root, fruits, leaves	Aspergillosis (26)	Terpenes Phenolic acids Flavonoids Chalcones (67–69)
	<i>Vernonia amygdalina</i> Del.	Leaf and root	Cryptococcal meningitis (15)	Saponins Alkaloids Tannins Phenolics Flavonoids Terpenes (70)

Table 1. Main plants used in the treatment of mycoses in Sub-Saharan Africa (continued).

Plant family	Plant species used in folk medicine/Common name(s)	Plant part(s)	Mycoses	Classes of compounds detected
Cannabaceae	<i>Cannabis sativa</i> L. Dagga	Leaf	Aspergillosis (37)	Cannabinoids Flavonoids Spiroindans Dihydrostilbenes Dihydrophenanthrenes Lignanamides Steroids Alkaloids (71)
Canellaceae	<i>Warburgia salutaris</i> (Bertol. f) Chiov Peperbasboom (Afrikaans), molaka (Sotho), mulanga (Venda), Isibaha (Zulu)	Bulb	Aspergillosis (37)	Terpenes Phenolics (72)
	<i>Warburgia ugandensis</i> Sprague Ugandan greenheart, greenheart tree	Bark, leaf and root	Fungal infection not specified (15)	Terpenes Tannins Flavonoids Saponins Sterols (73)
Caricaceae	<i>Carica papaya</i> L. Papaya, papaw, pawpaw	Leaf, root and seed	Oral candidiasis (15)	Saponins Alkaloids Flavonoids Terpenes Tannins (74)
Celastraceae	<i>Maytenus senegalensis</i> (Lam.)Exell Spike thorn	Bark, leaf and root	Oral candidiasis (15)	Benzofuranes Terpenes Xanthenes Flavonoids Tannins Polyketides Lignans Phenolics (75–78)

Table 1. Main plants used in the treatment of mycoses in Sub-Saharan Africa (continued).

Plant family	Plant species used in folk medicine/Common name(s)	Plant part(s)	Mycoses	Classes of compounds detected
Cleomaceae	<i>Cleome gynandra</i> L. Shona cabbage, African cabbage, spiderwisp, cat's whiskers, chinsaga, maman, stinkweed	Leaf	Oral candidiasis (15)	Carotenoids Tocopherols Phenolics Glucosinolates Terpenes Flavonoids Proanthocyanidins (79,80)
Clusiaceae	<i>Garcinia buchananii</i> Bak. Mutunduru	Bark and root	Cryptococcal meningitis (15)	Phenolics Flavonoids Anthocyanins Polyisoprenylated benzophenones Xanthenes (81,82)
	<i>Garcinia livingstonei</i> T. Anderson African Mangosteen, Imbé	Bark and root	Cryptococcal meningitis (15)	Benzophenones Xanthenes Flavonoids Terpenes (83–85)
Combretaceae	<i>Guiera senegalensis</i> J.F.Gmel. Gubeish	Leaf	Dermatophytosis (86)	Phenolic acids Flavonoids Alkaloids Naphthalenes Terpenes Chlorophylls (86)
	<i>Pteleopsis suberosa</i> Engl. & Diels Kpossinkpo	-	Candidemia, candidiasis	Terpenes Flavonoids (87,88)
	<i>Terminalia brownii</i> (Fresen) Alshaf	Leaf, bark and root	Candidiasis (89)	Tannins Terpenes Flavonoids Stilbenes Alkaloids Phenolic compounds (89,90)

Table 1. Main plants used in the treatment of mycoses in Sub-Saharan Africa (continued).

Plant family	Plant species used in folk medicine/Common name(s)	Plant part(s)	Mycoses	Classes of compounds detected
Combretaceae	<i>Terminalia sericea</i> Burch. ex DC. vaalboom (Afrikaans), mususu (Venda)	Bark	Cryptococcal meningitis (15)	Saponins Lignans Sterols Phenolic acids Stilbenes Amino acids Terpenes Flavonoids (91–93)
Cornaceae	<i>Curtisia dentata</i> Burm.f Assegaai (Afrikaans), umgxina (Xhosa), umagunda (Zulu)	Leaf, root	Oesopharyngeal candidiasis (37)	Terpenes Flavonoids Tannins Saponins Proanthocyanidins Alkaloids (94,95)
Cucurbitaceae	<i>Citrullus colocynthis</i> (L.) Schrad <i>Abu Jahl's melon</i>	-	Mycetoma (43)	Phenolic acids Flavonoids Terpenes Alkaloids Saponins (96)
Ebenaceae	<i>Euclea natalensis</i> A.DC. Guarri, inkunzane, inkunzemnyama	Leaf	Vaginal candidiasis (37)	Flavonoids Terpenes Naphthoquinones Tannins Naphthols Steroids Phenolic compounds Saponins Alkaloids (97,98)
Euphorbiaceae	<i>Croton gratissimus</i> Burch. Gunukira (Shona), mubangwa (Shona), mufandemengwe (Shona), mufarata (Shona)	Bark	Candidiasis (17)	Terpenes Flavonoids Alkaloids Phenolic compounds Lignans Proanthocyanidins (99,100)

Table 1. Main plants used in the treatment of mycoses in Sub-Saharan Africa (continued).

Plant family	Plant species used in folk medicine/Common name(s)	Plant part(s)	Mycoses	Classes of compounds detected
Euphorbiaceae	<i>Euphorbia hirta</i> L. Acak, akajwa, akasandasanda, akawjamate, amatu, mbabazi za ntaama	Root	Dermatophytosis (15)	Terpenes Flavonoids Phenolics Alkaloids Steroids Tannins Saponins (101,102)
	<i>Jatropha curcas</i> L.	Leaf	Oral candidiasis (15)	Terpenes Lignans Flavonoids Coumarins Alkaloids (103)
Fabaceae	<i>Acacia nilotica</i> (L.) Willd. Ex Del. <i>Isanqawe</i> (Ndebele), <i>mubayamhondoro</i> (Shona), <i>muhunga</i> (Shona), <i>muzunga</i> (Shona)	Stem bark	Mycetoma (43)	Tannins Steroids Terpenes Tannins Flavonoids Phenolic acids Alkaloids (104)
	<i>Abrus precatorius</i> L. <i>Chonjo</i> (Shona), <i>minimini</i> (Shona), <i>munhutuwaro</i> (Shona)	Bark, leaf and root	Oral candidiasis (15)	Flavonoids Phenolics Terpenes Steroids Alkaloids Esters (105)
	<i>Cajanus cajan</i> (L.) Millsp	Stem	Oral candidiasis (15)	Phenolics Lignans Stilbenes Flavonoids (106–108)

Table 1. Main plants used in the treatment of mycoses in Sub-Saharan Africa (continued).

Plant family	Plant species used in folk medicine/Common name(s)	Plant part(s)	Mycoses	Classes of compounds detected
Fabaceae	<i>Dichrostachys cinerea</i> (L.) Wight & Arn <i>Mumhangara</i> (Shona), <i>mupangara</i> (Shona), <i>muruka</i> (Shona), <i>musekera</i> (Shona), <i>ugagu</i> (Ndebele)	Leaves	Oral candidiasis (15)	Alkaloids Flavonoids Proanthocyanidins Steroids Terpenes Tannins (108–111)
Hydnoraceae	<i>Hydnora africana</i> Thunb. Jakkalskos, bobbajaankos	Bulb	Oesopharyngeal candidiasis (37)	Terpenes Alkaloids Saponins Tannins Flavonoids Sterols (112,113)
Hypericaceae	<i>Hypericum aethiopicum</i> Thunb. Unsukumbili	Leaf, stem, root	Dermatophytosis (47)	Flavonoids Proanthocyanidins (50)
	<i>Merwillia plumbea</i> (Lindl.) Speta Blouberglelie	Leaf, bulbs, roots	Dermatophytosis (47)	Saponins Terpenes (114)
Hypoxidaceae	<i>Hypoxis hemerocallidea</i> Fisch. Mey. and Avé-Lall African potato	Corm	Oesopharyngeal and vaginal candidiasis (15)	Benzoylcyclopropane derivatives Lignans Sterols Phenolic acids Flavonoids (115–118)
Lamiaceae	<i>Plectranthus amboinicus</i> (Lour.) Spreng. Afrika-tiemie, krui-spoorsalie	Leaf	Fungal infection not specified (15)	Terpenes Phenolic acids Flavonoids Tannins (119)
	<i>Plectranthus barbatus</i> Andr.	Leaf	Oral candidiasis (15)	Phenolics Flavonoids Terpenes (120–122)
	<i>Tetradenia riparia</i> Benth. Umuravumba, Iboza, ibozane	Leaf	Cryptococcosis, candidemia, candidiasis, coccidioidomycosis, dermatophytosis (122)	Terpenes Sterols Flavonoids (122)

Table 1. Main plants used in the treatment of mycoses in Sub-Saharan Africa (continued).

Plant family	Plant species used in folk medicine/Common name(s)	Plant part(s)	Mycoses	Classes of compounds detected
Limeaceae	<i>Limeum aethiopicum</i> L. Koggelmandervo, koggelmandervoetkaro, o, aarbossie, boesmandagga	Leaf	Dermatophytosis (37)	-
Loganiaceae	<i>Strychnos henningsii</i> Gilg. Umnonono	Bark, leaf	Aspergillosis (26)	Terpenes Alkaloids (123,124)
Meliaceae	<i>Azadirachta indica</i> A. Juss	Leaf	Fungal infections (15)	Flavonoids Terpenes Sterols (125)
Moringaceae	<i>Moringa oleifera</i> Lam. <i>Nèbèday, zogale, ilale</i>	Leaf	Mycetoma (43)	Phenolics Flavonoids Tannins Saponins Sterols Tocopherols Carotenoids Chlorophylls (126)
Myricaceae	<i>Morella salicifolia</i> (Hochst. ex A. Rich.) Verdc. & Polhil	Bark and root	Cryptococcal meningitis (15)	Terpenes Alkaloid Tannins Saponins Flavonoid Diarylheptanoids (127–129)
Myrsinaceae	<i>Maesa lanceolata</i> Forssk. <i>Mudovatova (Shona), musungwa (Shona), mutendekwani (Shona)</i>	Seeds	Fungal infections (15)	Terpenes Tannins Saponins Flavonoids Alkaloids Benzoquinones Sterols (130,131)

Table 1. Main plants used in the treatment of mycoses in Sub-Saharan Africa (continued).

Plant family	Plant species used in folk medicine/Common name(s)	Plant part(s)	Mycoses	Classes of compounds detected
Myrtaceae	<i>Eucalyptus</i> sp. Gums	Leaf	Oral candidiasis (37)	Alkaloids Flavonoids Propanoids Tannins Terpenes (132)
	<i>Psidium guajava</i> L. Malaka, mankala	Leaf bulb	Vaginal candidiasis (15)	Phenolics Flavonoids Terpenes Carotenoids Alkaloids Saponins Tannins Sterols (133)
Oleaceae	<i>Ximenia americana</i> L. Iholotshane (Ndebele), mutengeninyatwa (Shona), mutsanzavabere (Shona), umswanja (Ndebele)	Bark and root	Oral candidiasis (15)	Phenolics Tannins Flavonoids Anthocyanins Carotenoids Steroids Terpenes (134,135)
Oleaceae	<i>Olea europaea</i> L. Motholoari (Sotho), olienhout (Afrikaans), umquma (Xhosa and Zulu)	Bulb	Oropharyngeal candidiasis (15)	Phenolic compounds (136)
Papaveraceae	<i>Argemone mexicana</i> L.	Leaf	Cryptococcal meningitis (15)	Sterols Terpenes Quinones Flavonoids Alkaloids Phenolics (137)

Table 1. Main plants used in the treatment of mycoses in Sub-Saharan Africa (continued).

Plant family	Plant species used in folk medicine/Common name(s)	Plant part(s)	Mycoses	Classes of compounds detected
Piperaceae	<i>Piper guineense</i> Schumach & Thonn Ashanti pepper	Leaf, seed and fruit	Candidemia, candidiasis (138)	Saponins Alkaloids Tannins Flavonoids Phenolics Cyanogenic glycosides Terpenes (139,140)
Pittosporaceae	<i>Pittosporum viridiflorum</i> SimsKasuur	Leaf and bark	Cryptococcosis (32)	Terpenes Carotenoids Phenolics Flavonoids Tannins Alkaloids Saponins (141)
Poligalaceae	<i>Securidaca longipedunculata</i> Fres. Krinkhout (Afrikaans), sateen (Bambara), mamba (Tswana), mpesu (Venda), ipèta (Yoruba)	Bark, leaf and root	Cryptococcal meningitis, oral candidiasis (15)	Benzophenones Xanthenes Steroids Flavonoids Alkaloids Phenolic acids Saponins (142,143)
Rubiaceae	<i>Rubia petiolaris</i> DC Rooistam, kleeftgras	Leaf	Cryptococcal meningitis (37)	Anthraquinones (144)
Rutaceae	<i>Agathosma betulina</i> (Berg.) Pillans Buchu (Afrikaans)	Leaf	Oropharyngeal candidiasis, dermatophytosis (47)	Terpenes (145)
	<i>Citrus limon</i> (L.) Burm.f. Lemon herb	Fruit	Cryptococcosis, aspergillosis (15,37)	Polyphenols Flavonoids Limonoids Carotenoids (146)

Table 1. Main plants used in the treatment of mycoses in Sub-Saharan Africa (continued).

Plant family	Plant species used in folk medicine/Common name(s)	Plant part(s)	Mycoses	Classes of compounds detected
Rutaceae	<i>Coleonema album</i> (Thunb.) Assbossie (Afrikaans)	Leaf	Dermatophytosis (47)	Coumarins Flavonoids Terpenes (147–149)
	<i>Coleonema pulchellum</i> I. Williams Confetti buchu (Afrikaans)	Leaf	Dermatophytosis (59)	Phenolic compounds Terpenes Phenylpropenes (148,150,151)
Solanaceae	<i>Solanum aculeastrum</i> Dunal Bok-bitterappel, bitterappel, bokappel, gifappel (Afrikaans), thola (Tswana), murulwa (Venda), umthuma, itunga (Xhosa), mtuma (Zulu)	Leaf	Oral candidiasis (37)	Alkaloids Terpenes (152–154)
Talinaceae	<i>Talinum</i> sp. Osbossie, ystervarkwortel, ystervarkkos, wildevygie	Bulb	Cryptococcal meningitis (37)	Flavonoids Tannins Terpenes Saponins Polyphenols (155)
Zingiberaceae	<i>Zingiber officinalis</i> L. Common ginger	Rhizome	Aspergillosis (15,37)	Phenolic compounds Terpenes (156,157)

3. Research strategy to reveal the chemical compounds that ensure antifungal activity to the medicinal plants

Taking advantage of the ethnopharmacological knowledge of the Sub-Saharan African region summarized in **Table 1**, it is proposed a four-step research strategy. The first step is focused on crude plant extract preparation and compounds fractionation procedures to separate the compounds according with their degree of polarity. In the second one, crude

extract and fractions run on a chemical technology platform to reveal their chemical composition. Following with the assessment of the antifungal activity of the crude extract and fractions on a panel of fungal strains selected from WHO fungal priority list. Then, the results of both chemical and biological assays are used to construct an analytical model to establish the relationship between chemical composition and antifungal bioactivity.

3.1. Plant extract preparation and fractionation

The proper extraction solvent system and extraction procedure should be chosen according with target compounds considering the part of plant that will be used (158). In traditional medicine of the Sub-Saharan African region, herbal remedies are mainly prepared with fresh or dry plant parts by decoction, maceration, infusion or simply by plant burning (17,159). However, the available knowledge does not allow to identify the target compounds, but it is known that decoction and maceration in water or in water-ethanol solutions are the most used methodologies to prepare herbal remedies for oral delivery. Regarding the bioactives reported for plant species indicated in **Table 1**, maceration in a water-ethanol solution is more suitable than decoction to avoid the loss of thermolabile and volatile compounds. Setting the plant material weight/volume of solvent ratio, the water-ethanol solution allows the extraction of bioactive compounds with different degrees of polarity.

To increase the homogeneity and the reproducibility of the maceration procedure, it is also essential to define the conditions to air-dry the plant material, ground and sieve it to collect solid particles with a similar mean size (e.g. less than 910 μm) (160). In general, the finer the particle is, the better results the extraction achieves as consequence of an improved solvent penetration and solutes diffusion (161). Nevertheless, the degree of plant material fragmentation should be optimized according with the competence of centrifugation/filtration step used to remove the solid particles (162).

For traditional medicine, the solution with extractable compounds is an herbal remedy ready to use. However, the scientific-based knowledge requires to solve the 3-D puzzle underlying the relationship between chemical composition and antifungal activity calibrated by the putative interactions among the different chemical compounds present in the complex mixture. Thus, the solution with extractable compounds needs to be converted into a solid extract by solvent evaporation/sublimation. In general, the organic solvent (ethanol) is recovered by evaporation under reduced pressure and the water is discarded by freeze drying. Then, the crude extract should be split into two portions, one for chemical analysis

and to perform the biological assays, and other for compounds fractionation according with their degree of polarity. The fractionation plan should not be designed to obtain a pure compound but rather a mixture with a well-defined composition, containing compounds that, through their individual or conjugated bioactivities, exhibit antifungal activity. The crude extract fractionation on a reverse phase octadecyl (C18) solid-phase extraction (SPE) column with sequential elution by using acidic water, ethyl acetate and methanol is suitable to obtain three fractions with compounds discriminated according with their degrees of polarity. Thus, the high hydrophilic compounds without affinity for column stationary C18 phase are obtained in the water fraction, the low polarity compounds in the ethyl acetate fraction (EAF) and the remaining compounds in methanol fraction (MF) (i.e., methanol soluble compounds with affinity for column stationary C18 phase and low solubility in ethyl acetate).

3.2. Chemical analysis of the plant crude extract and fractions

The subsequent step consists in the chemical analysis of the crude extract and of the three fractions, mainly by high performance liquid chromatography-mass spectrometry (HPLC-MS)⁽ⁿ⁾ analysis, to allow: i) the identification of a set of plant species biomarkers to ensure quality control and standardization of plant extract and/or fractions; ii) to collect data required to connect the chemical composition to the antifungal activity.

The main difficulties in the standardization of herbal remedies arise from the fact that they are highly complex mixtures rich in secondary metabolites, with concentrations dependent from plant geographical location and harvest season, as well as from plant processing and extraction procedure (158). Thus, to define a set of plant species biomarkers it is important to chemically characterize plant extracts from different batches of the medicinal plant harvested at different times of the year and in different locations of Sub-Saharan African region.

The chemical analysis of the crude extract and of the three fractions requires the selection of appropriate methodologies. Liquid chromatography (LC) is a versatile and robust technique that remains the method of choice for the separation, identification and quantification of compounds in complex matrices (163). HPLC analysis using a reverse-phase column is the most common and effective mode, due to its high efficiency and reproducibility. In fact, this chromatographic technique is an effective option for the analysis of many of the bioactive compound classes present in **Table 1** (e.g., phenolic compounds, alkaloids, carotenoids, and sterols), whereby it will be appropriate for the present goal. The

chemical structure of the compounds in mixture will influence the selection of a suitable stationary phase. Generally, C18 columns are the first choice, since they allow excellent peak shape and the possibility of accommodating the most common LC and LC-MS mobile phases (164). However, when it is not possible to obtain proper compound separation, or it is previously known that the plant extract includes compounds difficult to retain and/or resolve on a C18 column, it should be considered changing the stationary phase. The triacontyl phase (C30) columns are more suitable for the separation and analysis of high hydrophobic, long-chain structural compounds, such as carotenoids, steroids, and other lipids (165). Therefore, it is proposed that crude plant extract and fractions should be analyzed by using the two types of reverse-phase columns, C18 and C30 with suitable mobile phases (e.g., acid water/methanol; methanol/tert-butyl methyl ether, respectively). For compound identification, several technologies are available, such as ultraviolet-visible (UV-Vis) absorption spectrometry by diode array detector (DAD) and electrospray ionization-mass spectrometry (ESI-MS). Compared with UV-Vis based detection, MS presents higher sensitivity and selectivity, and it is the method of detection that provides the most embracing applicability (166).

In short, to analyze the bioactive constituents of the medicinal plant extract and fractions, it is suggested, whenever possible, to use HPLC techniques coupled to ESI-MS. This methodology will allow revealing the chemical profile of the complex mixture in the crude plant extract and following the distribution of each compound in different fractions separated in both C18 and C30 columns. Another important issue is the implementation of a quantification plan for the identified compounds, which can be supported by external or internal standard approaches, each one with specific limitations and advantages (167). The internal standard approach allows the quantitative analysis to run simultaneously with the identification process, and its simplicity makes it our first choice within the main purpose of the present work. However, to reach an acceptable accuracy, the selected standards cannot overlap with endogenous molecular species of the complex mixture (crude extract or its fractions) during chromatographic separation and the concentrations of both analytes and internal standards need to fall in the linear dynamic range. Using the internal standards approach, the quantification of the identified compounds in medicinal plant extract and fractions are achieved by “ratiometric comparison”. In the next step, the concentration of the compounds in each fraction should be compared with its concentration in the crude extract, determining the enrichment factor (EF) ($\alpha_{Ci} = [Ci]_{\text{fraction}}/[Ci]_{\text{crude extract}}$). The EF will be used to gather compounds into two clusters, one containing the compounds with $\alpha \geq 1$ and other with compounds with $\alpha \leq 1$. This clusters compound organization is a tool to evaluate

if and how the identified compounds are associated with the antifungal activity of plant extracts, as will be described in the section 3.4.

3.3. Selection of the relevant pathogenic fungal species and assessment of the antifungal activity

The prioritization process followed by WHO to systematically order fungal pathogens considered their capacity of causing invasive acute and systemic fungal infections for which the treatment and management challenges exist, sometimes associated to drug resistance (7). Combining the ethnopharmacological knowledge summarized in **Table 1** with WHO fungal priority list, it is possible to select a relevant panel of pathogenic fungal species to evaluate the antifungal activity of the medicinal plant extracts (**Table 2**).

Table 2. Panel of pathogenic fungal species to assess the antifungal activity.

Fungal disease	Pathogenic fungi	WHO fungal priority list
Candidiasis	<i>Candida auris</i>	Critical priority group
	<i>Candida albicans</i>	Critical priority group
Cryptococcosis	<i>Cryptococcus neoformans</i>	Critical priority group
	<i>Cryptococcus gattii</i>	Medium priority group
Dermatophytosis	<i>Trichophyton soudanense</i>	Not applicable, but with relevance in Sub-Saharan African region
Aspergillosis	<i>Aspergillus fumigatus</i>	Critical priority group

According with **Table 1** data, many plant species are used to tackle candidiasis, dermatophytosis and cryptococcosis. Candidiasis and cryptococcosis, can evolve into life-threatening diseases with serious risk of mortality and/or morbidity and, therefore, they were considered by WHO a huge public health threat. Thus, yeasts *C. auris* and *C. albicans* for triggering candidiasis, and *C. neoformans* and *C. gatti* for cryptococcosis were included in the panel of evaluation. The pathogenic filamentous fungi *A. fumigatus*, included in WHO fungal priority list by its capacity to originate azole-resistant invasive aspergillosis treated in Sub-Saharan African region with herbal remedies from eight plant species, was also

selected. Although fungi strains underlying the dermatophytosis were not included in WHO fungal priority list, the relevance of dermatophytosis in Sub-Saharan African region justify the inclusion in the panel of evaluation of, at least, one pathogenic fungus strain responsible for skin infections. Our choice lies in *Trichophyton soudanense* by its high prevalence in the region.

The choice of suitable methodologies to screen the antifungal activity of the medicinal plant extract and fractions on the panel of pathogenic fungal strains is a critical step to pursue the goals of the research strategy. Within a work plan that handles with samples from seventy-two plant species screened on a panel of pathogenic fungal species, it is important: i) to choose cheap and high-quality methods with competence to reduce both false positives and negatives; and ii) to select the range of concentrations of plant extract/fractions to be tested.

Regarding antifungal activity assays, cell density/growth-based assays (e.g., dilution methods) are the most used to determine the lowest plant extract/compound concentration necessary to inhibit the fungal growth after long-time exposure (24-72 h), known as minimal inhibitory concentration (MIC) (168). Despite the macrodilution and microdilution methods are standardized for yeasts and filamentous fungi by the Clinical and Laboratory Standards Institute (CLSI), they only give information about the capacity of the plant extract/fraction to inhibit fungal growth (169,170). Thus, to evaluate its competence to kill the pathogenic fungi, additional assays are needed to assess the minimum fungicidal concentration (MFC), the lowest concentration of extract/compound in which no visible growth is observed. Conventionally, it is considered that an extract/fraction has fungicidal activity when both MFC and MIC has the same value, and only fungistatic activity in the cases wherein the MFC is higher than the MIC. However, it is reported that these methodologies have several disadvantages, including long-time consumption, low reproducibility and poor sensitivity (171). For instance, the assessment of the effects on filamentous fungi liquid cultures by using the optical density assays can be significantly affected by the heterogeneous distribution of cells in the liquid-culture and/or by the formation of biofilms on the surface (172). Thus, different types of metabolic-based methods, including the reduction of tetrazolium salts or resazurin and total adenosine triphosphate (ATP) quantification assays, have been proposed to cope with these drawbacks, which can be used as alternative or in association to cell density/growth-based assays (172–175). Both cellular ATP levels and the extension of tetrazolium salts or resazurin reduction are dependent not only of the number of living cells but also of the metabolic performance of individual cells. Thus, using metabolic-based assays to assess the effects of increasing concentrations of a drug or plant

extract/fraction on a fungi strain after incubation for a long time (several generation time) allows to build a dose-response curve. It is reported that these dose-response curves produce similar results to those obtained with standardized methods expressed in terms of MICs, in addition to detect antifungal activity of drugs and plant extracts/fractions that compromise the metabolism with minor effects on the fungal growth (176). Reporting changes in the metabolic performance of cells/organisms, the metabolic-based assays are also suitable to assess the antifungal activity of drugs and plant extracts/fractions after incubation times below a generation time, which allows to decrease significantly the time required for screening assays. Therefore, an affordable workplan to screen the antifungal activity of the medicinal plant extracts/fractions should combine one of the above-mentioned metabolic-based assays with standardized methods to determine MICs.

West African herbal pharmacopoeia indicates that the infusions and/or decoctions are used at bulk concentrations in the order of 50-200 $\mu\text{g/mL}$ (177). Thus, the assessment of antifungal activity of plant extracts/fractions in the concentration range of 0-1000 $\mu\text{g/mL}$, for example by using ten-fold serial dilutions, should be suitable to detect any relevant bioactivity.

To take advantage of the better sensitivity and low-time consumption of the metabolic-based assays, the antifungal activity screened on each pathogenic fungal species should start with these assays, aiming to build a dose-response curve for the plant extract and its fractions. The dose-response curves are used to determine numeric indexes (e.g., such as half maximal inhibitory concentration (IC_{50}) – concentration of crude extract or fraction that decreases in 50% the metabolic performance of a population of fungi with normalized biomass) with pharmacological value to compare the effectiveness of the crude extract with its fractions, as well as to assess the degree of selectivity for different fungal strains. Plant extracts and fractions exhibiting antifungal activity until 1000 $\mu\text{g/mL}$ will follow for cell density/growth-based assays to determine the MIC and MFC values, while the others are considered non active (bioactivity = 0).

Thus, for each fraction, the ratio between its MIC (and/or IC_{50}) and the MIC (and/or IC_{50}) of the crude extract (R_{fx}) are calculated, and its inverses ($1/R_{fx}$) are used for analytical purposes. It should be noted that $R_{fx} < 1$ indicates that the fraction fx exhibits higher activity than the crude extract ($R_{ce} = 1$). Therefore, the $1/R_f$ values are proportional to the antifungal activity. The obtained $1/R_{ce}$, $1/R_{fw}$, $1/R_{fea}$ and $1/R_{fm}$ values will be used to construct a matrix, aiming to set the relationship between the antifungal activity of medicinal plant extract and its chemical composition, as will be described below.

3.4. The challenge of the relationship between chemical profile and bioactivity

Regarding the accumulated knowledge of traditional medicines, bioactivity is, in general, assigned to the mixture of compounds, which could act together synergistically, additively, or even antagonistically. However, there are no standardized methodologies to establish the relationship between bioactivity and the chemical makeup of complex mixtures. Thus, in this section, it is proposed a simple methodology that takes advantage of the chemical characterization of the crude extract and of its fractions with compounds organized in clusters to assess if and how the revealed chemical profile is associated with the antifungal activity.

To reveal the group of compounds responsible for the antifungal activity it is constructed a matrix of multiple variables considering, for each pair medicinal plant/pathogenic fungal species, the bioactivity (i.e., $1/R_{fw}$, $1/R_{fa}$ and $1/R_{fm}$ values for fractions and 1 for crude extract) as reference variable, and as chemical profile-related variables, the concentrations of compounds, individually and in selected mixtures (e.g., grouped by chemical classes). Then, Pearson's correlation coefficients are determined by multivariate statistical analyses to identify the compounds and the chemical combinations that are positively correlated with bioactivity ($p < 0.05$), which have a higher probability to be responsible for the antifungal bioactivity. If the fraction with the highest activity has, in its cluster with $\alpha \leq 1$, compounds that, individually or in combination, are positively correlated with the bioactivity, then the fractionation process can be tuned to obtain a fraction with improved activity.

4. The growing problem of the insecticide resistance

The demand for increasing amounts of food in order to feed an expanding global population and the need to stop vector-borne diseases are the motivation for the discovery of new tools to control insect pests (178). Besides, the concerns arising from the widespread, and commonly unregulated, use of insecticides (such as pest resistance) highlight the importance to continue this battle to discover not only new molecules with potential, but also new targets of action (179). Nearly 600 insect species have developed resistance to one or more insecticides and/or genetically modified insecticidal traits (180). Pest resistance is a major concern insofar, as once established, it is not possible to restore the species susceptibility and to work with insecticides at the same levels (181). The mechanisms by which insects can offer resistance are diverse, among them are target site resistance, metabolic resistance, physical adaptation and behavioral adaptation (181).

Target site resistance happens when a mutation of the genetic code for the receptor protein occurs, changing its shape and hindering the connection between the insecticide and the target; metabolic resistance is characterized by the increase in the enzymes responsible for its breakdown or the modification of these enzymes, becoming more effective in its function of breaking down the insecticide molecule; physical adaptation is related with the acquisition by the insect of physical features that will help in the protection against the insecticide, such as thickening of the cuticle; and the behavioral adaptation, as the name implies, is the changing of the natural behaviors of the insect, which ends in the diminishing exposure of the animal to the insecticide, allowing their survival (181).

Nowadays, different techniques and technologies for pest insect management have been developed, such as biological control, transgenic plants, host plant resistance and cultural control (182). However, even the resistance to the insect toxins present in transgenic plants is emerging, which highlights the continuing importance of conventional insecticides that, due to their reliability and rapid effectiveness, remained the main method to successfully reduce pests in agricultural practices or in human residences (178,183). Thus, the need to develop new efficient, selective and less persistent insecticides remains, as well as the urgent investigation of new or undervalued modes of action capable of overcoming the challenges and problems of the insecticide field (183).

5. Research strategy to reveal the chemical compounds that ensure insecticidal activity to the medicinal plants

The research strategy behind the urgent need to find compounds with insecticidal activity in medicinal plants capable of overcoming the challenges and problems of the insecticide field should start with the same approach followed for the antifungal activity. Thus, in this section, it is necessary to focus the attention on the selection of the relevant insect species and in the techniques/methods to be used in the insecticidal activity assessment.

5.1. Selection of the relevant insect species and assessment of the insecticidal activity

According to recent data from WHO, 80% of the world's population is at risk of one or more vector-borne diseases (184). Millions of people continue to be infected by viruses, parasites and bacteria transmitted by mosquitoes, flies, and other bugs, which can cause deadly diseases, such as malaria, dengue, yellow fever and Zika virus disease. These

diseases are particularly thriving in conditions of poverty, making developing countries in the Sub-Saharan African region the main stage for this public health problem. In addition to mortality rates being very high in these countries, the ones who survive can be permanently disabled or disfigured, restricting the country's development (184,185). Thus, controlling their spread is of primary importance.

Malaria may be caused by five species of *Plasmodium* parasites. However, the most severe clinical form of the disease is caused by the tropical form of this parasite, *Plasmodium falciparum*, which is widespread in the tropics and in Sub-Saharan African region (186). This parasite is transmitted by mosquito females' bite of the genera *Anopheles*. *Anopheles stephensi* is spread across Africa and it is particularly worrying, due to its incredible ability to adapt to the environment, even at extremely high temperatures and to its exhibited resistance to pyrethroids, organophosphates and carbamates (187). Therefore, this mosquito species was included in the evaluation panel.

Infected mosquitoes belonging to the species *Aedes aegypti* and *Aedes albopictus* are the vectors of dengue virus, which is transmitted to humans typically in tropical and sub-tropical areas worldwide (188). *A. aegypti* is the primary vector of the disease and *A. albopictus*, although responsible for the transmission of the disease, is more widespread in Europe and North America. Dengue circulation has been detected in several African countries, with 171,991 cases and 753 deaths being reported in 2023 in the area. Various outbreaks began in 2023 in 15 countries, mostly belonging to the Sub-Saharan African region (188). *Aedes* mosquitoes, in addition to transmitting the dengue virus, are vectors of other diseases that continue to be top concerns for WHO, such as Zika, Chikungunya and yellow fever (189). Thus, *A. aegypti* and *A. albopictus* were also selected for the insecticidal activity assessment panel.

The selection of the methodologies to assess the insecticidal activity was made considering the assumptions used for the choice of the antifungal activity assessment methods. The most used assays to test the insecticidal activity of extracts/compounds are contact bioassays used to assess the acute toxicity, in which the administration routes are topical and/or oral. However, these methodologies have several disadvantages, such as low efficiency and high cost (190). In this work plan, it is suggested to start the assessment of the insecticidal activity of crude extract and fractions through cell-based models. Insect cell models have been increasingly used in the last decades, playing important functions in pest management. Compared to classic *in vivo* assays, *in vitro* screenings are less-time consuming, easy handling and present high reproducibility and sensitivity. There are currently thousands of insect cell lines from several species and different tissues, but the

choice falls on two common used insect cell lines in pest management, a *Drosophila melanogaster* neuronal cell line (ML-Dm-BG2-c2) and *Spodoptera frugiperda* pupa ovarian cells (Sf9) (190). Thus, the plan proposal is to begin the screening of the potential insecticidal activity of medicinal plant extract and fractions by the assessment of the cell viability of these two cell lines, in the presence of increasing concentrations of crude extract or fractions, through the reduction of tetrazolium salts or resazurin. Plant extracts and fractions exhibiting cytotoxicity until 200 µg/mL in at least one cell line will follow for *in vitro* toxicological and mechanistic studies to uncover the mode of action of insecticidal compounds/mixtures at cellular level. Insect cell-based models have extra advantages compared to the traditional *in vivo* assays, but also present false positives - extracts/compounds active *in vitro* with poor *in vivo* insecticidal ability. Therefore, an affordable robust work plan implies the combination of the two methodologies. The extracts/fractions with cytotoxicity until the referred concentration will also follow for *in vivo* studies with the above chosen species of mosquito and using acute toxicity assays with topical and oral administration. It is important to highlight that the range of concentrations used *in vivo* can start in the order of magnitude used for cell assays but must be increased if no activity is observed.

Like what was described above for the screening of the antifungal activity, the ratios between IC₅₀/EC₅₀ of each fraction and IC₅₀/ EC₅₀ of crude extract should be determined to establish a relationship between insecticidal activity and its chemical composition, as was described in the section 3.4.

6. Selection of the plant species

6.1. *Guiera senegalensis*

Guiera senegalensis J. F. Gmel., (**Figure 1**) commonly known as “Senegal Tea Plant” and “Wild Coffee”, is a medicinal shrub from Combretaceae family that typically grows 1-3 m high. It is widely distributed in African countries, particularly in semi-desert areas of the Sudano-Sahelian region, with a banded vegetation pattern known as “tiger bush” (86). This plant species presents small, lance-shaped leaves organized in clusters along the branches and fragrant flowers that are usually white or pale yellow. In terms of ecological role, *G. senegalensis* supports local ecosystems by providing habitat and food for insects and other animals (191).



Figure 1. *Guiera senegalensis* J. F. Gmel in Eticoga, Guinea-Bissau (photographs by Nelson Gomes).

In addition to its ecological importance, *G. senegalensis* has gained popularity among several African nations, due to its use by traditional healers for the treatment of many diseases. Thus, the whole plant is sold in African markets, but its bitter leaves are considered the most important part of the plant, being known as a “cure-all” medicine. This plant is commonly used for the treatment of several chronic diseases, colds, bronchitis, stomach pain, vomiting, jaundice, kidney stones, wounds, skin ulcers and infections (86).

Due to its ethnopharmacological relevance, several studies concerning the chemical composition of different *G. senegalensis* plant parts were performed (86). In fact, compounds belonging to different phytochemical classes were isolated and identified, such as phenolic acids, flavonoids, terpenes, steroids, alkaloids, and naphthalene derivatives. Galloylquinic acid derivatives, simple phenolic acids and flavonoids are the most reported groups of compounds (86). Furthermore, various studies reported different biological activities of extracts and isolated compounds from *G. senegalensis*, such as cytotoxicity against cancer cells, enzyme inhibitory, antiprotozoal, and antimicrobial activities (192–195). Indeed, a decoction of *G. senegalensis* leaves has been used in the treatment of venereal diseases as an antimicrobial agent (194). Bosisio and colleagues (196) reported the antifungal properties of aqueous and methanol leaves extracts, assessing their effects on six species of yeast and twelve species of filamentous fungi. Other parts of *G.*

senegalensis were studied concerning their antifungal potential, with the finding that the extracts from galls and roots have activity against five serotype strains of fungi and three strains of yeast, respectively (197,198). While the relationship between the antifungal activity and the chemical profile of the above-mentioned plant extracts was not established, another study supports the idea that the antifungal activity of *G. senegalensis* extracts can be attributed to guieranone A (199). However, the mechanisms of action underlying guieranone A antifungal activity remain to be clarified. Furthermore, the presence of toxic alkaloids has been identified in different parts of *G. senegalensis*, including in the leaves and roots (194). Thus, the valorization of *G. senegalensis* for new antifungals development requires the establishment of clear relationships between the chemical composition of the plant extracts and the antifungal activity.

6.2. *Ficus polita*

Ficus polita Vahl. (**Figure 2**), also called heart-leaved fig, is an endemic evergreen tree of Africa, growing about 15 m high in lowland rainforests, coastal, dry and gallery forests, up to an altitude of 1200 m (200–202). Its stem is straight, and its crown is round; its ovate leaves present a glossy green color above and light green beneath; the fruits called figs are smooth, and purple-green when ripe in summer (203). Both leaves and fruits are edible. This plant species starts as a non-parasitic plant growing on another plant (epiphyte) and then its aerial roots from the lower branches reach the ground and develop into trunks, causing the death of the tree where it is growing. Furthermore, the wasp *Courtella bekiliensis* Risbec subsp. *bispinosa* Wiebes and *F. polita* present a mutualistic relationship, once the first can only reproduce inside the fig, helping the last with propagation and survival (203).

A few studies aiming its chemical characterization report the occurrence of terpenes, cerebrosides and phytosterols in different *F. polita* plant parts collected in Nigeria and Cameroon (201,204,205). In addition, (E)-3,5,4'-trihydroxy-stilbene-3,5-O- β -D-diglucopyranoside is the only phenolic compound described in this plant species (201).



Figure 2. *Ficus polita* Vahl. in Varela, Guinea-Bissau (photographs by Nelson Gomes).

In African traditional medicine, its edible fruits are chewed for dyspepsia, whereas bark, root or leaf infusions are administered for the management of infectious diseases, abdominal pain, and diarrhea (201,206). The antimicrobial activity of methanol, ethanol:water (80:20) and ethanol extracts of *F. polita* roots and its fractions were previously reported in several bacterial strains and in the yeast strain *C. albicans* (201,202,207). Acetone and ethanol leaves extracts have also been studied concerning their antibacterial potential, showing effectiveness against four bacterial strains (200). While most studies do not establish a correlation between the chemical profile and the antimicrobial activity, Kuete and colleagues attributed this ability of root extracts to the presence of terpenoid-like compounds, namely triterpenes, such as lupeol and euphol 3-O-cinnamate (201). However, no mechanism of action was studied or described in these studies.

7. Objectives

The main goal of this PhD thesis is to develop multi-target formulations for addressing the current challenges associated with resistance to antifungal drugs and insecticides. This will be achieved through a comprehensive research approach focusing on plant species native to Guinea-Bissau designed to unveil the compounds responsible for bioactivity and to elucidate their mechanism of action. Thus, the specific objectives are:

- 1) Samples preparation to guide the chemical and biological characterization of *G. senegalensis* and *F. polita* leaves, which involves the preparation of crude methanol extracts and a fractionation procedure to discard the water-soluble components and to separate the remaining compounds based on their polarity into ethyl acetate or methanol fractions;
- 2) Chemical characterization of *G. senegalensis* and *F. polita* crude methanol extracts and fractions, combining chemical assays with HPLC-DAD-ESI/MSⁿ analysis;
- 3) To characterize the antifungal activity of crude methanol extracts and corresponding fractions against selected pathogenic filamentous fungi and yeasts, using the standard methodologies to determine MIC and MFC values, as well as effects on specific growth rate;
- 4) To investigate the mechanism of action of crude methanol extracts and fractions with antifungal activities, combining their competence to inhibit key enzymes with *in vivo* effects on the bioenergetic metabolism of the *Saccharomyces cerevisiae*;
- 5) To establish the relationship between the chemical profile of crude methanol extracts and fractions with their antifungal activity with identification of the bioactive compounds;
- 6) To characterize the insecticidal potential of crude methanol extracts and corresponding fractions, by using cellular assays with *Drosophila melanogaster* neuronal cells;
- 7) To investigate the mechanisms underlying the toxicity on *D. melanogaster* neuronal cells, considering effects on reactive oxygen species (ROS) generation, mitochondria functionality, activity of the mitochondrial redox chain complexes, and acetylcholinesterase (AChE) enzyme;

- 8) To establish the relationship between the chemical profile of crude methanol extracts and fractions with their toxicity on *D. melanogaster* neuronal cells, with identification of the bioactive compounds;
- 9) To evaluate the toxicological profile of the crude methanol extracts and fractions on cellular models of human neuronal and skin cells, in order to determine the selectivity degree for fungal and insect cells and rationalize the safety profile.

CHAPTER II: Experimental Section

1. Standards and reagents

Methanol, acetonitrile, perchloric acid and 2-propanol were obtained from Merck (Darmstadt, Germany). Formic acid and petroleum ether was acquired from Carlo Erba Reagents S.A.S. (Val de Reuil, France). Dimethyl sulfoxide (DMSO) was purchased from Fischer Scientific (Loughborough, United Kingdom (UK)). Ethyl acetate was acquired from Valente e Ribeiro, Lda. (Belas, Portugal). Acetic acid and anhydride acetic were acquired from Atom Scientific (Manchester, UK). Ethanol and tris-hydrochloride was acquired from VWR Chemicals (Radnor, PA, United States of America (USA)). *tert*-Butyl methyl ether, ammonia and sulfuric acid were obtained from Honeywell (Charlotte, NC, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), tungstosilicic acid hydrate, phosphoric acid, glycerol, dichloromethane, trypan blue, gallic acid, 3-O-Caffeoylquinic acid, 5-O-caffeoylquinic acid, *p*-coumaric acid, ferulic acid, myricetin-3-O-glucoside, myricetin-3-O-rhamnoside, quercetin-3-O-galactoside, quercetin-3-O-glucoside, quercetin-3-O-arabinoside, quercetin-3-O-rhamnoside, luteolin-7-O-glucoside, myricetin, kaempferol-3-O-glucoside, quercetin, hesperetin, isorhamnetin, rotenone, potassium cyanide (KCN), antimycin A, reduced nicotinamide adenine dinucleotide (NADH), decylubiquinone, oxaloacetate, succinate, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) probe, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probe, 3,4-dihydroxy-L-phenylalanine (L-DOPA), acetylthiocholine iodide, insulin solution human, select yeast extract and Shields and Sang M3 Insect Medium, penconazole, mushroom tyrosinase, decylubiquinone, 2,6-dichlorophenolindophenol (DCPIP), cytochrome C (Cyt c), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), acetyl-CoA, chlorophyll a, chlorophyll b, 3-morpholinepropanesulfonic acid (MOPS), phenazine methylsulfate (PMS), nitrotetrazolium blue (NBT), sulphanilamide, naphthylethylenediamine, potassium phosphate monobasic, potassium chloride, ethylenediamine tetraacetic acid (EDTA), sucrose, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), xanthine oxidase, xanthine, pyruvate, bovine serum albumine, sodium nitroprusside (SNP), ammonium molybdate, sodium carbonate, sodium sulfate, magnesium chloride, dextrose, ascorbic acid, Triton X-100, and Folin-Ciocalteu reagent (FCR) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fluconazole was acquired from Alfa Aesar (Haverhill, MA, USA). Roswell Park Memorial Institute medium (RPMI-1640, with L-glutamine, without bicarbonate and with the pH indicator phenol red) was purchased from Biochrom AG (Berlin, Germany). Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) were purchased from Bio-

Mèrieux (Marcy L'Etoile, France). Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM-F12), heat inactivated fetal bovine serum (FBS), trypsin-EDTA, and Pen-Strep solution (penicillin 5000 units/mL and streptomycin 5000 mg/mL) were acquired from Gibco Invitrogen TM (Grand Island, NY, USA). Bactopeptone was purchased at BD Difco™ (Michigan, United States). Mangiferin, stigmasterol was purchased from Extrasynthese (Genay, France). Citric acid was acquired at Labkem (Dublin, Ireland).

2. Sampling, preparation of extracts and fractionation

2.1. Sampling

G. senegalensis and *F. polita* leaves were collected in Orango Island (Guinea-Bissau) in June 2017, February 2020, and November 2021. Voucher specimens were deposited at LAQV/REQUIMTE, University of Porto. Samples of leaves were air-dried, grounded to a fine powder, and sieved to collect solid particles with a mean size lower than 910 µm.

2.2. Preparation of leaves crude methanol extracts

For each collected leaves sample, a crude methanol extract (CME) was obtained through the maceration of 3 g of powdered plant material in 500 mL of methanol under stirring (200 rpm) for 2 h, followed by 30 min of sonication and an additional step of stirring for 1 h. The solid material was removed by centrifugation (1792 g, 4 min), and the liquid fraction was filtered through a Büchner funnel to obtain the CME. The obtained methanol solution was submitted to reduced pressure in a Rotavapor R-215, Büchi Labortechnik (Flawil, Switzerland) at 40 °C to obtain a dried CME. The dry weight of the extract was determined, extraction yield was calculated, and the samples preserved in vials protected from light for further studies.

2.3. Fractionation of leaves crude methanol extracts

The SPE clean-up procedure using Hypersep C18 non-end-capped columns (10 g bed weight; 75 mL column volume; 40 - 60 µm particle size) was applied to CME to obtain two fractions, the EAF and the MF, and to discard the water-soluble compounds without affinity for the column. The activation of the SPE column was performed by adding 20 mL of ethyl acetate, 20 mL of methanol and 20 mL of acidified water (pH 2). Then, the column

was loaded with the CME and subsequently washed with 20 mL of acidified water. To obtain the different fractions, 30 mL of ethyl acetate and 40 mL of methanol were eluted in the column. EAF and MF were dried by a nitrogen stream at room temperature, weighted to determine the fractionation yield, and preserved in vials protected from light for further studies. At least, three independent fractionations were performed.

2.4. Alkaloid-targeted extraction

The alkaloid-target extract from *G. senegalensis* leaves was prepared according to (194) with some modifications. The powdered plant (5 g) was defatted with 20 mL of petroleum ether through maceration under stirring (200 rpm) for 1 h. The plant material was air-dried, and ammonia (40 mL) was added during 1 h. After this time, it was subjected to a maceration with dichloromethane for 12 h and filtered through a Whatman no 1 filter paper in a Büchner funnel. A first liquid-liquid partition was performed with water acidified with sulfuric acid (pH 1-2). After the alkalization of the resulting solution (pH 9-10), dichloromethane was added to perform the second liquid-liquid partition. The dichloromethane layer was collected and concentrated under reduced pressure in a Rotavapor R-215, Büchi Labortechnik (Switzerland).

3. Chemical characterization

3.1. Total phenolic compounds

The quantification of phenolic compounds in CME and its fractions was performed using the Folin-Ciocalteu method, which is based in the oxidation of these compounds, in acidic medium, into blue molecules that can be quantified by spectrophotometry (208).

To prepare standards with different concentrations, increasing volumes of gallic acid were prepared in methanol:water to construct a calibration curve (range 0 – 14.2 µg/mL). At the same time, CME and fractions were diluted in the same solvent (1 mg/mL). Afterwards, 1 mL of FCR, 2 mL of Na₂CO₃ 25% (m/v) and 3 mL of water were added. The mixtures were vortexed each time a new reagent was added. Standards and samples were incubated in a water bath at 70 °C for 30 minutes and after cooling at room temperature, the absorbance was determined at 750 nm. Phenolic compounds concentration of each sample was calculated by interpolation in the external standard curve of gallic acid (0 – 14.2 µg/mL).

3.2. Sugars

Total sugars can be assessed spectrophotometrically after the reaction with a solution of phenol in concentrated sulfuric acid. Sulfuric acid allows the dehydration and hydrolyzation of sugars that will react with phenol forming orange-yellow compounds with maximum absorption at 490 nm (**Figure 3**) (209).

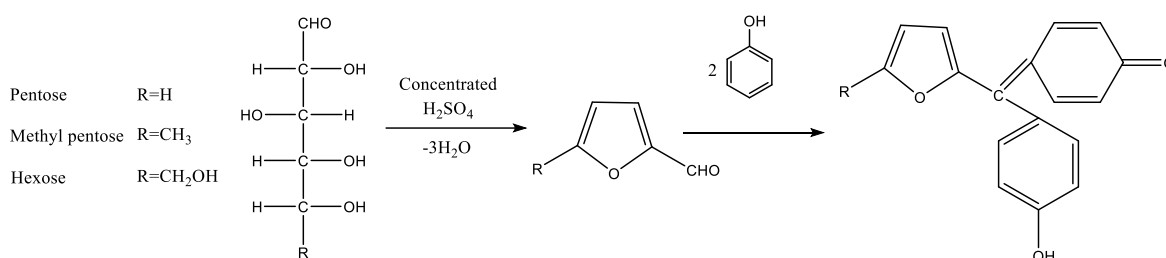


Figure 3. Hydrolyzation of sugars that will react with phenol forming orange-yellow compounds.

Therefore, glucose standards (range 0 – 25 nmol) were prepared in water with a final volume of 100 μ L, the volume used for CME and fractions. Then, 100 μ L of phenol (6.5% m/v) was added to standards and samples, and the mixtures were stirred in a vortex. To add pure sulfuric acid (500 μ L), the mixtures were maintained in stirring. Afterwards, small portions of each CME and each fraction were transferred to a microplate and the absorbance was read at 490 nm. Thus, total sugars levels of CME and fractions were obtained through the interpolation of the obtained absorbance in the external standard curve of glucose (0 – 25 nmol).

3.3. Phospholipids

Phospholipids were quantified by the Bartlett and Lewis procedure after samples acid hydrolysis, and phosphatidylcholine C16:0/18:1 was used as standard (0 – 250 nmol) (210).

To perform an acidic hydrolysis, 250 μ L of each CME and fraction were transferred into a test tube. Then, 650 μ L of perchloric acid were added and the mixtures were incubated at 180 $^{\circ}$ C for 2 h. After cooling, 3.3 mL of water, 0.5 mL of ascorbic acid (10% m/v) and 5 mL of ammonium molybdate (2.5% m/v) were added, and the mixtures incubated in a water bath at 100 $^{\circ}$ C. Phosphatidylcholine standards were prepared exactly in the same conditions (0 – 250 nmol). 250 μ L of extracts and fractions were transferred to a microplate

and the absorbance was determined at 800 nm. Phospholipids quantification was obtained by interpolation in the standard curve of phosphatidylcholine (0 – 250 nmol).

3.4. Liebermann-Burchard reactive compounds

Liebermann–Burchard reactive compounds (e.g. steroids and triterpenes) levels were determined using stigmasterol as standard (0 – 250 µg) (211).

Thus, 100 µL of each CME and fraction were placed into test tubes and were dried under a nitrogen stream. The same volume of acetic acid (100 µL) and 5 mL of Liebermann–Burchard reagent (120 mL anhydride acetic, 60 mL acetic acid, 20 mL sulfuric acid and 4 g of sodium sulfate) were added. At the same time, different concentrations of stigmasterol were prepared in the same conditions. After 30 min of room temperature incubation, 250 µL of each CME and fraction were placed in a microplate and the absorbance was read at 630 nm. Liebermann–Burchard reactive compounds (e.g. steroids and triterpenes) quantification was determined by interpolation in the standard curve of stigmasterol (0 – 250 µg).

3.5. Chlorophylls

Chlorophylls were quantified by their UV-Vis spectrum signatures, at 665 nm, using chlorophyll *a* as standard (0 – 25 µg/mL) (212). CME and fractions were diluted in ethanol and different concentrations of chlorophyll *a* were also prepared in the same solvent. The UV-Vis spectrum of the samples was plotted and the absorbances of the external standards at 665 nm were used to construct a standard curve and to interpolate the absorbance of CME and fractions, allowing to obtain their levels of chlorophylls.

3.6. Alkaloids

The presence of alkaloids in *G. senegalensis* CME were screened by the general alkaloid precipitation tests, using Dragendorff's (solution of potassium bismuth iodide), Mayer's (potassium mercuric iodide solution) and Bertrand's (silicotungstic acid solution) reagents (213).

3.7. Compounds identification by HPLC-DAD-ESI(Ion Trap)-MSⁿ and UPLC-ESI-QTOF-MS² analyses

3.7.1. Phenolic compounds

HPLC-DAD-ESI(Ion Trap)-MSⁿ analyses were carried out as previously described (214), solely varying the gradient; mobile phase consisted of two solvents, water-formic acid (1%) (A) and acetonitrile (B), starting with 5% B and using a gradient to obtain 20% B at 10 min and 50% B at 25 min for the analysis of *G. senegalensis* CME; and starting with 10% B and using a gradient to obtain 25% B at 30 min and 70% B at 35 min for *F. polita* CME. The flow rate was 0.8 mL/min and the injection volume was 20 µL. Chromatograms were recorded at 250 and 280 nm for the two plant species extracts, 340 nm for *G. senegalensis* and 335 nm for *F. polita*. The mass full scan covered the range from *m/z* 100 up to 1500.

Determination of the exact mass was carried out using an Agilent 1290 Infinity LC system coupled to the 6550 Accurate-Mass quadrupole time of flight (QTOF) (Agilent Technologies, Waldbronn, Germany) with an electrospray interface (Jet Stream Technology). Samples (2 µL) were injected onto a reverse phase Kinetex column (1.7 µm, C18, 100 Å, 50 × 2.1 mm; Phenomenex, Macclesfield, UK) with a SecurityGuard ULTRA Cartridge of the same material operating at 30 °C and a flow rate of 0.5 mL/min. The mobile phase was a mixture of acidified water (0.1 % formic acid) (A) and acidified acetonitrile (0.1 % formic acid) (B). Compounds were separated using the following gradient conditions: 0 min 5% B, obtaining 50% B at 20 min and 70% B at 22 min (*G. senegalensis*) and 0 min 5% B, obtaining 40% B at 12 min and 70% B at 15 min (*F. polita*). The optimal conditions for the electrospray interface were the following: gas temperature 280 °C, drying gas 11 L/min, nebulizer pressure 45 psi, sheath gas temperature 400 °C, sheath gas flow 12 L/min. The MS system was operated in negative ion mode (*G. senegalensis*) or in negative and positive ion modes (*F. polita*) with the mass range set at *m/z* 100–1500 in full scan resolution mode. Further conditions as described in (215).

3.7.2. Chlorophylls

Dried samples of EAF were redissolved in ethyl acetate, filtered through 0.45 µm pore size membrane and analyzed on a HPLC-DAD unit (Gilson Medical Electronics, Villiers le Bel, France) equipped with a C30 YMC carotenoid column (5 µm, 250 × 4.6 mm; YMC, Kyoto, Japan). A gradient elution was conducted, using a flow rate of 0.9 mL/min and an injection volume of 20 µL. The mobile phase constituted by two solvents, methanol (A) and

tert-butyl methyl ether (B), started with 95% A and used a gradient to obtain 70% at 30 min, 50% at 50 min, 0% at 60 min, 0% isocratic at 65 min and 95% at 70 min. Chromatograms were recorded at 420 and 450 nm and spectral data from all peaks were collected in the range of 190-700 nm.

Analysis was performed in an Orbitrap Exploris 120 mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) controlled by Orbitrap Exploris Tune Application 2.0.185.35 and Xcalibur 4.4.16.14 softwares. Samples (2 μ L) were injected onto a reverse phase C30 YMC carotenoid column (5 μ m, 250 $\times$ 4.6 mm; YMC, Japan) with a SecurityGuard ULTRA Cartridge of the same material operating at 30 °C and a flow rate of 0.3 mL/min. The mobile phase constituted by two solvents, methanol (A) and *tert*-butyl methyl ether (B), started with 100% A and used a gradient to obtain 30% at 90 min, 30% isocratic from 90 to 12min, and 100% A at 135 min. The capillary voltage of the ESI source was set to 3.5 and 2.5 kV for positive and negative modes. The capillary temperature was 350 °C. The sheath, auxiliary and sweep gases flow rates were 50, 10 and 1 (arbitrary units, as provided by the software settings) and 70% tube lens. The resolution of MS scan was 60000. Data dependent MS/MS was performed on HCD using nitrogen as gas with collision energy settings of 35 V at 1500 of resolution. The range was set from 150 to 2000 Da. The MS system was operated in both positive and negative ion modes with the mass range set at m/z 100–1500 in full scan resolution mode.

3.7.3. Alkaloids

Chromatographic analyses were carried out on a Luna C18 column (250 $\times$ 4.6 mm, 5 μ m, Phenomenex, Torrance, USA), using a gradient elution as described in (194), solely varying the gradient. Solvents used were water with 0.5% of acetic acid (A) and methanol (B). The elution gradient started with 85% A for 25 min, from 85% A to 0% A from 25 to 35 min, 0% A from 35 to 45 min, 0% A to 85% A from 45 to 50 min and 85% A for 5 min. The flow rate was 0.5 mL/min, and the injection volume was 20 μ L. Chromatograms were recorded at 240 nm and spectral data were collected in the range 190-700 nm.

3.8. Quantification of compounds by HPLC-DAD

3.8.1. Phenolic compounds

Quantitative analysis was attained using the gradient and the mobile phases described for HPLC-DAD-ESI (Ion Trap)-MSⁿ analyses. CME/fractions were dissolved in

methanol and filtered through a polytetrafluoroethylene (PTFE) (0.45 μm pore size) membrane (Millipore, Bedford, USA), 20 μL being injected. Triplicate analysis proceeded on a Gilson HPLC-DAD unit (Gilson Medical Electronics, Villers le Bel, France) equipped with a reverse phase Kinetex column (5 μm , C18, 100 Å, 150 $\times$ 4.6 mm; Phenomenex, Torrance, USA). Detection was achieved with an Agilent 1260 series DAD (Agilent Technologies, Waldbronn, Germany). Spectral data from all peaks were collected in the range of 190-700 nm and chromatograms were recorded at 280, 320, 350, and 370 nm. Data were processed on Clarity software system, version 5.04.158 (DataApex, Ltd., Prague, Czech Republic).

Calibration curves were built with six concentrations of the external standards injected in triplicate (**Table 3**). Regarding the compounds present in *G. senegalensis*, myricetin-3-O-glucoside (**14**), myricetin-3-O-rhamnoside (**19**), quercetin-3-O-galactoside (**20**), quercetin-3-O-glucoside (**21**), quercetin-3-O-arabinoside (**23**), quercetin-3-O-rhamnoside (**24**), myricetin (**25**), quercetin (**28**), hesperetin (**32**) and isorhamnetin (**33**) were quantitated as themselves. Compounds **1-10** were quantitated against the standard curve of gallic acid, **11-13**, **15** and **18** were quantitated as myricetin-3-O-glucoside, **16** and **17** as quercetin-3-O-galactoside, **22** as quercetin-3-O-arabinoside, and **26**, **27**, **29-31** as kaempferol-3-O-glucoside. Guieranone A (**34**) was quantified as mangiferin.

Table 3. Linear regression equation analysis, limit of detection (LOD) and limit of quantification (LOQ) for external standards used in the quantification of *G. senegalensis* phenolic compounds.

Standard	Regression Equation			Linearity range ($\mu\text{g/mL}$)	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
	Slope (σ)	Intercept (b)	R^2 ($n = 3$)			
Gallic acid	63.644	-156.840	0.9998	6.25 - 200	0.116	0.352
Myricetin-3-O-glucoside	16.314	-79.310	0.9997	12.5 - 400	2.515	7.621
Myricitrin	38.099	-376.870	0.9999	25 - 800	0.973	2.949
Quercetin-3-O-galactoside	57.521	-431.470	0.9953	6.25 - 200	0.122	0.371
Quercetin-3-O-glucoside	25.381	-46.102	0.9996	4.69 - 150	0.469	1.422
Quercetin-3-O-arabinoside	20.192	20.251	0.9998	2.5 - 80	0.158	0.479
Quercetin-3-O-rhamnoside	28.511	-38.083	0.9997	2.81 - 90	0.398	1.205

Table 3. Linear regression equation analysis, limit of detection (LOD) and limit of quantification (LOQ) for external standards used in the quantification of *G. senegalensis* phenolic compounds (continued).

Standard	Regression Equation			Linearity range ($\mu\text{g/mL}$)	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
	Slope (σ)	Intercept (b)	R^2 ($n = 3$)			
Myricetin	43.683	-149.500	0.9996	6.25 - 200	0.311	0.943
Kaempferol-3-O-glucoside	29.610	-26.566	0.9999	6.25 - 200	1.099	3.330
Quercetin	39.213	-18.603	0.9985	0.78 - 25	0.085	0.257
Hesperetin	63.191	0.981	0.9991	1.56 - 50	0.089	0.271
Isorhamnetin	14.284	0.782	0.9992	0.94 - 30	0.078	0.237
Mangiferin	19.527	1394.000	0.9961	500 - 2000	370.744	1123.466

Concerning the compounds present in *F. polita* (**Table 4**), 3-O-caffeoylquinic acid (**1**), 5-O-caffeoylquinic acid (**4**) and herniarin (**11**) were quantitated as themselves. 3-*p*-Coumaroylquinic (**2**) and 4-*p*-coumaroylquinic acids (**6**) were quantitated against the standard curve of *p*-coumaric acid; 4-O-caffeoylquinic acid was quantitated as 5-O-caffeoylquinic acid; ferulic acid hexoside isomers (**5** and **7**) as ferulic acid and luteolin-7-(6-pentoside)hexoside (**8**), luteolin-7-(6-rhamnoside)hexoside (**9**) and luteolin-7-(2-pentoside)hexoside (**10**) were quantitated as luteolin-7-O-glucoside.

Table 4. Linear regression equation analysis, LOD and LOQ for external standards used in the quantification of *F. polita* phenolic compounds.

Standard	Regression equation			Linearity range ($\mu\text{g/mL}$)	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
	Slope (σ)	Intercept (b)	R^2 ($n = 3$)			
3-O-Caffeoylquinic acid	78.871	-95.31	0.9999	7.81 - 250	0.607	1.840
5-O-Caffeoylquinic acid	77.218	49.413	0.9999	6.25 - 200	1.163	3.524
<i>p</i> -Coumaric acid	137.760	-17.060	0.9999	1.56 - 50	0.059	0.178
Ferulic acid	119.980	198.3	0.9996	5.625 - 180	0.326	0.987
Luteolin-7-O-glucoside	70.369	-4.505	0.9992	9.38 - 300	0.287	0.870
Herniarin	127.320	990.920	0.9987	14.84 - 475	1.161	3.518

Linearity was assessed from the coefficients of determination (R^2) of the calibration curves (**Tables 3 and 4**). LOD and LOQ were determined from the residual standard deviation (σ) of the regression curves and the slopes (S), according to the equations $\text{LOD} = 3.3\sigma/S$ and $\text{LOQ} = 10\sigma/S$.

3.8.2. Chlorophylls

For quantification, calibration curves were built with six concentrations of the external standards injected in triplicate (**Table 5**). Chlorophyll *a* and chlorophyll *b* were quantitated as themselves. Chlorophyll *a* derivatives were quantitated as chlorophyll *a*, and chlorophyll *b* derivatives were quantitated as chlorophyll *b*. Linearity was assessed as described above in the previous section.

Table 5. Linear regression equation analysis, LOD and LOQ for chlorophylls *a* and *b*.

Standard	Regression equation			Linearity range ($\mu\text{g/mL}$)	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
	Slope (σ)	Intercept (b)	R^2 ($n = 3$)			
Chlorophyll <i>a</i>	7.292E-05	8.000E+06	0.9991	6.25 - 1600	2.181	6.609
Chlorophyll <i>b</i>	9.610E-05	2.000E+06	0.9976	6.25 - 200	1.426	4.321

4. Antioxidant activity

4.1. Nitric oxide radical scavenging activity

At physiological pH, SNP is capable of spontaneously generate the nitric oxide radical ($\cdot\text{NO}$), which interacts with oxygen to produce nitrite that can be determined by the Griess reaction (**Figure 4**) (216). The absorbance of the chromophore formed by the diazotization reaction of nitrite with sulphanilamide and subsequent binding with N-(1-naphthyl)ethylenediamine can be determined at 562 nm.

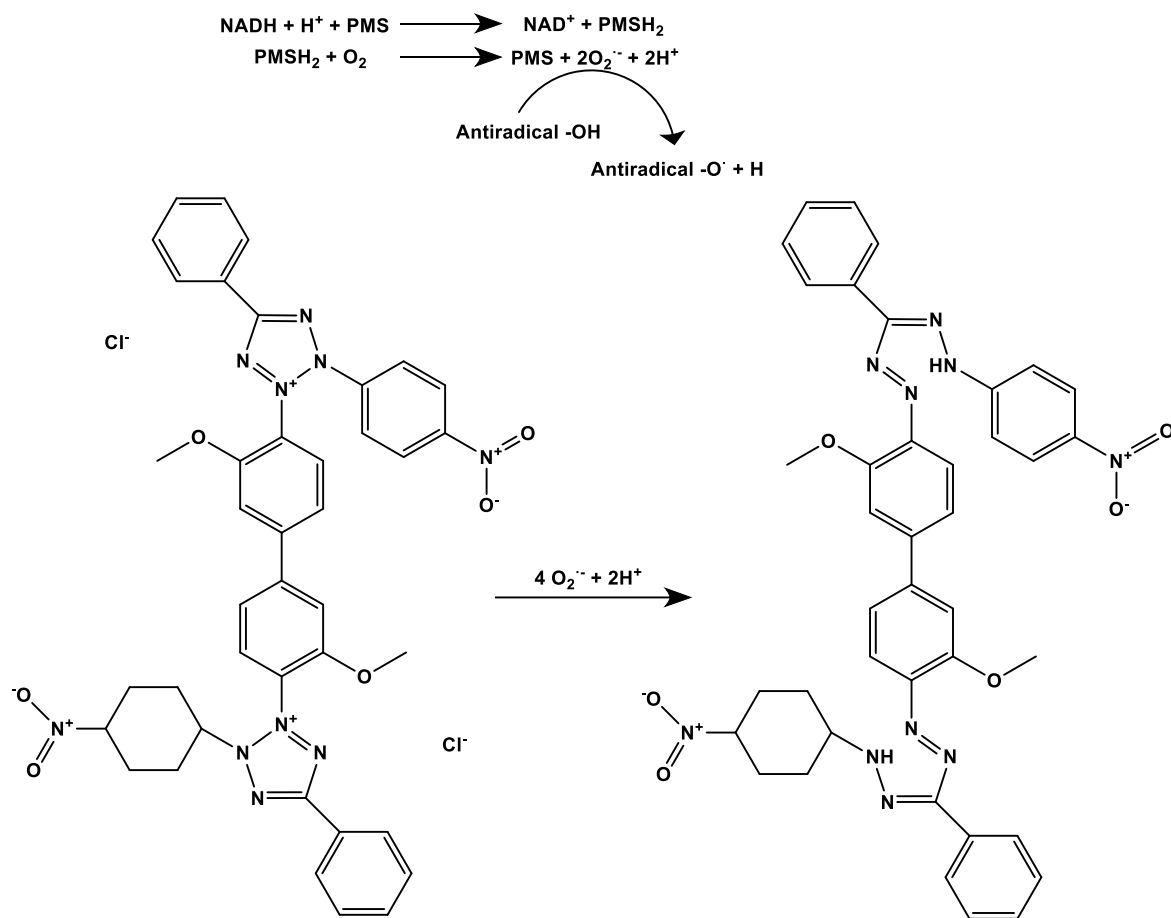


Figure 5. $\text{O}_2^{\cdot-}$ is formed by the NADH/PMS system and reacts with NBT to form a diformazan product.

5. Antifungal activity assessment

5.1. Selection of fungal species

Four yeast strains [*C. albicans* (ATCC 10231), *C. krusei* (ATCC 6258), *C. neoformans* (CECT1078), *S. cerevisiae* (FF180)] and three filamentous fungi [*A. fumigatus* (ATCC 204305) *F. oxysporum* (FF/T-9) and *C. gloeosporioides* (FF/T-6)] were used in the present work. Yeast strains and filamentous fungi were stored in SDB with 20% glycerol at -80°C . Before each test, a sub-culture in SDA of 1-5 days was prepared to achieve optimal growth conditions and purity.

5.2. *In vivo* and *ex vivo* assays

5.2.1. Effects on growth

MICs were determined by broth microdilution method according with the CLSI reference documents M27-A3, S3, and M38-A2, for yeasts and filamentous fungi, respectively.

Fungal organisms previously cultured in SDA were suspended in RPMI-1640 broth buffered with MOPS (pH 7.0). Two-fold serial dilutions of CME and of the respective fractions were prepared in RPMI-1640 broth. The solutions and cells suspensions were distributed into sterile 96-well plates that were incubated aerobically in a humid atmosphere, without agitation, at 35 °C and 25 °C, for yeasts and for filamentous fungi, respectively. Time of incubation was 48 h for *S. cerevisiae*, *C. albicans* and *C. krusei*, and 72 h for *C. neoformans* and for filamentous fungi. MIC was defined as the lowest concentration showing 90 – 100% of absence of growth on 96-well plates. MFCs were determined with the results obtained with the subculturing of 20 µL of all MIC plate wells without visible growth in SDA plates after 48 or 72 h of incubation, at 35 or 25 °C, according with the fungi strain described above. The MFC was defined as the lowest concentration showing a total absence of growth on SDA plates. For each assay condition, at least three independent experiments were performed. The medians of the obtained results in each type-test were used as MIC and MFC values.

The effects of CME, EAF and MF on time-dependent growth of *S. cerevisiae* and *C. neoformans* liquid cultures were also assessed to evaluate the antifungal activity by changes on the specific growth rate. *S. cerevisiae* and *C. neoformans* cultured in SDA were subcultured in lactone-rich media and liquid SDA, respectively, and grew overnight, at 30 °C, under orbital shaking (200 rpm) to obtain an active inoculum. Then, test tubes with the suitable yeast strain culture medium without or supplemented with CME, EAF or MF at 50 µg/mL were inoculated with the active inoculum and incubated for 60 h under the conditions mentioned above. The yeast growth was followed by changes of the optical density at 610 nm measured at different time points. For both yeast strains, the effects of increasing concentrations of *G. senegalensis* and *F. polita* EAF were also tested.

5.2.2. Effects on the mitochondrial respiratory function

S. cerevisiae yeasts oxygen consumption was monitored with a Clark-type electrode, at 30 °C, in a 2.5 mL thermostatic chamber with continuous agitation using an Oxygraph+ system (Hansatech Instruments, King's Lynn, UK) (218). *S. cerevisiae* liquid cultures grown in 250 mL flasks with 50 mL of glycerol-rich media for 8 h were used to assess the effects on the mitochondrial respiratory function. Yeasts were exposed to increasing concentrations of *F. polita* EAF or to herniarin for 4 h before the assessment of the mitochondrial respiratory function. Thus, ca. 2 million yeasts were diluted in 1 mL of reaction medium (citric acid 100 mM, EDTA 10 mM) supplemented with glycerol-rich media and recorded by the Clark-type electrode during ca. 4 min, at 30 °C. The oxygen concentration and flux were recorded and analyzed by Oxytrace+ v.1.0.48 by Hansatech Instruments. Four minutes after starting the record, KCN (2 mM) was added to stop mitochondrial respiration, allowing to determine the rate of oxygen consumption connected with mitochondrial respiration, expressed by nmol/mL.

5.2.3. Preparation of mitochondria-enriched fractions from *S. cerevisiae* yeasts

Yeast cultures of *S. cerevisiae* grown in 250 mL flasks with 50 mL of glycerol-rich media in the absence (control) or presence of 50 µg/mL of *G. senegalensis* EAF for 8 h were used to isolate mitochondria. *S. cerevisiae* yeasts were collected from the culture medium by centrifugation (1000 *g* for 4 min) and resuspended in 2 mL of buffer (250 mM sucrose, 5 mM HEPES buffer, pH = 7.4) at 4 °C. The cell wall of the yeast cells was mechanically disrupted by zirconia beads (1 g, 0.5 mm of diameter) under vortex stirring (five cycles, 1 min of stirring followed by a resting step for 2 min at 4 °C). Cell suspensions were filtered through a standard surgical gauze to remove zirconia beads and the filtered was homogenized, at 4 °C, in a Glass-Teflon Potter Elvehjem (clearance of 0.004–0.006 in.) using 20 up and downstrokes at 500 rpm to promote the cell lysis. Then, cell lysates were centrifuged at 1000 *g* for 10 min, at 4 °C, and the supernatant collected. The supernatant was centrifuged at 12000 *g* for 15 min (4 °C), the mitochondria-rich fraction being collected as pellet. The pellet was resuspended in 250 µL of buffer medium (130 mM sucrose, 50 mM KCl, 5 mM MgCl₂, 5 mM KH₂PO₄, 5 mM HEPES, pH = 7.4). An aliquot was used to determine the mitochondrial protein concentration by biuret method, using bovine serum albumin as standard reference (219). Samples were frozen in liquid nitrogen and stored at -80 °C for further studies.

5.2.4. Effects on mitochondrial respiratory chain complexes

S. cerevisiae mitochondria-enriched fractions were submitted to three cycles of freezing/thawing before being used to assess the activity of mitochondrial NADH-ubiquinone reductase (complex I), succinate-ubiquinone reductase (complex II), cytochrome c oxidase (complex IV) and citrate synthase (CS) at 30 °C, in 250 μ L of reaction medium with a volume containing between 25-50 μ g of protein (219). The activity of the mitochondrial redox chain complexes was measured using a microplate reader (Synergy H1 Biotek) and normalized by the activity of CS.

The assessment of complex I was followed spectrophotometrically at 340 nm following NADH ($\epsilon_{\text{DCPIP}} = 6220 \text{ M}^{-1} \text{ cm}^{-1}$) oxidation, detected by color change from yellow to colorless. The assay was performed in the absence and presence of a complex I inhibitor, rotenone (600 μ M), with phosphate buffer (KH_2PO_4 25 mM, MgCl_2 5 mM, pH 7.5), KCN (100 mM), antimycin A (2 mM) and decylubiquinone (38.75 mM). The difference between the slopes with and without rotenone allows the determination of the complex I activity.

The assessment of complex II activity was based on the color change from blue to colorless resulting from the reduction of DCPIP, used as an exogenous acceptor of electrons (219,220). Thus, complex II promotes the oxidation of succinate (100 mM), providing electrons to ubiquinone, which in turn facilitates the reduction of DCPIP (25 mM). The assay is conducted in phosphate buffer (25 mM KH_2PO_4 , pH 7.5) containing decylubiquinone (38.75 mM), rotenone (600 μ M), antimycin (2 mM), and KCN (100 mM). The decrease in absorbance intensity at 600 nm (with $\epsilon_{\text{DCPIP}} = 20700 \text{ M}^{-1} \text{ cm}^{-1}$) over time is monitored using a microplate reader, both in the absence and presence of oxaloacetate (500 mM, a complex II inhibitor). Finally, complex II is determined by the difference between the slopes with and without oxaloacetate.

The evaluation of complex IV activity was based on the color change from light red to dark red resulting from the oxidation of reduced Cyt c (200 μ M) (221). The solution of Cyt c is prepared in a spectrophotometer cuvette (Helios Alpha, UNICAM) by adding an appropriate volume of dithionite saturated solution, necessary to elevate the absorbance of the Cyt c solution by 0.6 units at 550 nm, following the saturation of nitrogen gas. The decrease in absorbance intensity, spectrophotometrically measured at 550 nm, in the absence and presence of the complex IV inhibitor, KCN (100 mM), was recorded for 10 min using a microplate reader. The experiment was conducted in the presence of phosphate buffer (KH_2PO_4 25 mM, pH 7.5), antimycin A (2 mM) and rotenone (600 μ M). Complex IV activity is revealed by the difference between the slopes with and without KCN.

The activity of CS was assessed spectrophotometrically by observing the color change associated with the reduction of DTNB by coenzyme A, a byproduct of the condensation reaction catalyzed by the CS enzyme. This reaction involves the conversion of acetyl-CoA and oxaloacetate into citrate and coenzyme A (219,222). The assay was conducted in a microplate reader, with buffer (Tris-HCl 20 mM, pH 8, Triton X-100 0.02%), DTNB (10 mM), oxaloacetate (100 mM) and acetyl-CoA (6.1 mM). The activity of CS is determined by DTNB ($\epsilon_{\text{DTNB}} = 13600 \text{ M}^{-1} \text{ cm}^{-1}$) reduction rate.

Mitochondria obtained from yeasts cultured in control condition were also used to evaluate the direct effects of increasing concentrations of *G. senegalensis* and *F. polita* EAF on the activity of mitochondrial complexes I and II, after 5 min of incubation. Results are expressed as percentage of control.

5.3. *In vitro* assays

5.3.1. Tyrosinase activity

The activity of mushroom tyrosinase was assessed by the oxidation of L-DOPA (substrate), by recording over 3 min the increase of absorbance at 475 nm resulting from dopachrome generation (**Figure 6**) (223).

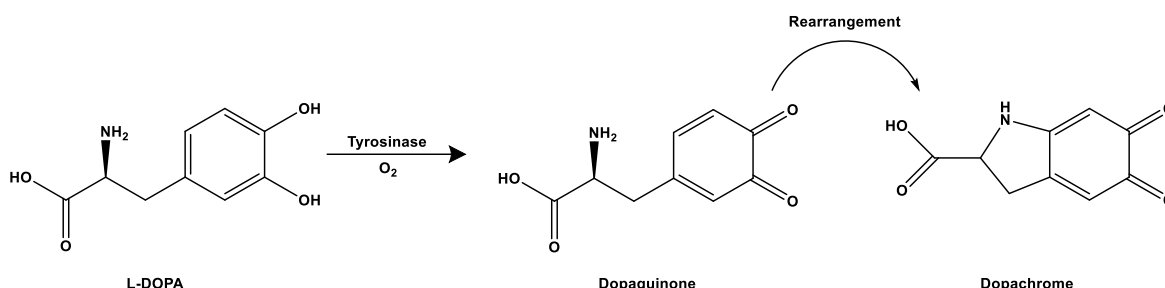


Figure 6. Metabolism of L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosinase and formation of dopachrome.

Assays, in the absence or presence of increasing concentrations of CME/fraction, were carried out at 30 °C, in 200 μL of 50 mM potassium phosphate monobasic buffer (pH 6.8) supplemented with 5 U/mL of enzyme, and 90 or 900 μM L-DOPA to assess the IC_{50} , or with seven different substrate concentrations for enzyme kinetic studies. Enzyme activity was expressed in nmol/min, using $3600 \text{ M}^{-1} \text{ cm}^{-1}$ as extinction coefficient of dopachrome or as percentage of activity compared to control.

5.3.2. Xanthine oxidase activity

Xanthine oxidase is responsible by the conversion of xanthine into uric acid, which generates hydrogen peroxide and the superoxide radical from molecular oxygen (**Figure 7**) (224). The rate reduction of the superoxide radical should not be used to assess xanthine oxidase activity, since the ability of scavenging these radicals may lead to an overestimated percentage of inhibition. Therefore, the rate of uric acid, detectable by changes in the absorption at 295 nm, must be determined.

The effects of increasing concentrations of *F. polita* CME, MF and EAF in the xanthine oxidase activity were assessed by incubating the CME or its fractions in 250 μ L buffer (100 mM phosphate, 5 mM EDTA, pH 7.4) with 1.12 U/mL of enzyme, and 200 μ M of xanthine, at 37 °C. The reaction was initiated by the addition of xanthine. Results are expressed as percentage of inhibition compared to control.

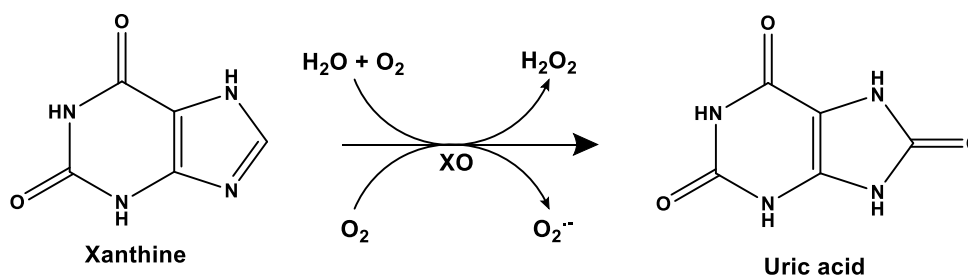


Figure 7. Reduction of xanthine to uric acid by xanthine oxidase.

6. Insecticidal activity assessment

6.1. Assays with ML-DmBG2-c2 *Drosophila melanogaster* cell line

Neuronal *D. melanogaster* ML-DmBG2-c2 cells (Drosophila Genomics Resource Center, Indiana University, USA) were cultured at 25 °C in T25 flasks containing Shields and Sang M3 insect medium supplemented with 10% FBS, 0.5% penicillin/streptomycin, 1000 μ g/mL yeast extract, 2500 μ g/mL bactopectone, and 10 μ g/mL insulin. After reaching a confluence of 80% (cell density between 5×10^6 and 1×10^7 cells/mL), cells were harvested with a cell scraper, and then used to preserve the cell cultures, seeding a new T25 flask with 5×10^6 cells in 5 mL of culture medium or to perform the assays, as will be described below.

6.1.1. Effects on cell viability

The viability of neuronal *D. melanogaster* cells (ML-DmBG2-c2) was assessed by the MTT reduction assay (**Figure 8**) (225). Cells were seeded in 96-well plates at a density of 7.5×10^4 cells/well. After 24h, cells were incubated with extract/fraction or vehicle (control) for 48 h. The assays were performed by removing the culture medium and by incubating with the MTT at a final concentration of 0.5 mg/mL for 4 h, at 25 °C. Formazan crystals were dissolved with 100 μ L of a DMSO:isopropanol mixture (3:1, V:V) and quantified spectrophotometrically at 560 nm using a microplate reader, Multiscan GO (Thermo Fisher Scientific, Massachusetts, USA).

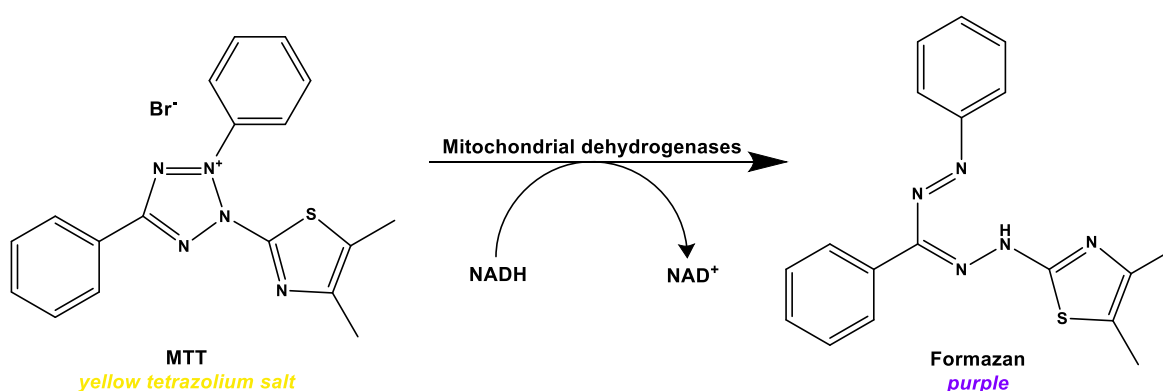


Figure 8. Reduction of MTT to formazan by mitochondrial dehydrogenases of metabolically active cells.

The doubling-time of ML-DmBG2-c2 cells was assessed in 96-well plates seeded with a cell density of 3.5×10^4 or 7.5×10^4 cells/well, and the cell proliferation curves built with the values of MTT reduction assay obtained at 24, 48, 72, 96 and 120 h incubation time.

6.1.2. Effects on membrane integrity

The effects on the membrane integrity were assessed by lactate dehydrogenase (LDH) release assay (**Figure 9**) (225). Cells were seeded in 96-well plates at a density of 7.5×10^4 cells/well, as previously mentioned for the MTT assay. After the incubation period of 48 h, 50 μ L of cell-free supernatant of extracellular medium were removed from the plate and mixed with 25 μ L pyruvate (10 mM) and 200 μ L NADH (0.3 mM). LDH activity was assessed spectrophotometrically following NADH oxidation at 340 nm for 3 min, using a microplate reader, Multiscan GO (Thermo Fisher Scientific, Massachusetts, USA).

The total LDH activity was assessed in control cells treated with lysis solution (1% Triton X-100 in 10 mM phosphate buffer, pH = 7.2). Results are expressed as percentage

of control for MTT reduction assay and as fold increase relative to control cells for LDH activity.

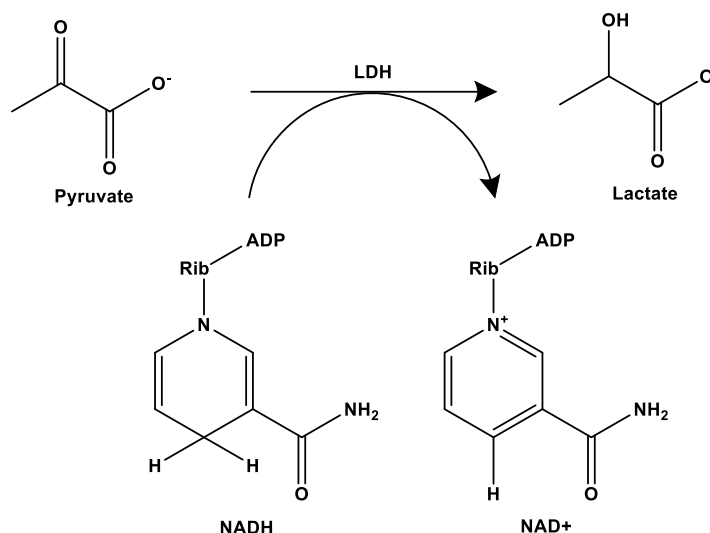


Figure 9. Reduction of pyruvate to lactate by LDH.

6.1.3. Effects on the generation of intracellular reactive oxygen species

The generation of ROS by ML-DmBG2-c2 cells was evaluated in 96-well black plates, using cellular cultures seeded at 7.5×10^4 cells/well. For ROS generation ML-DmBG2-c2, cells were treated with increasing concentrations of *G. senegalensis* CME or vehicle for 48 h. After the incubation period, the medium was removed, cells were washed with Hanks' balanced salt solution (HBSS) and then incubated with DCFH-DA (10 μ M) fluorescent probe in HBSS for 30 min at 25 °C. DCFH-DA enters the cells through passive diffusion, where intracellular esterase enzymes cleave its acetate groups (**Figure 10**). Its subsequent oxidation results in the formation of a fluorescent adduct known as 2',7'-dichlorofluorescein (DCF) (225,226). The time-dependent changes in fluorescence intensity of the DCF resulting from probe oxidation of control and treated ML-DmBG2-c2 cells were recorded with excitation/emission settings at 490/520 nm in a microplate reader (Synergy H1, Biotek). The results were expressed as percentage of control.

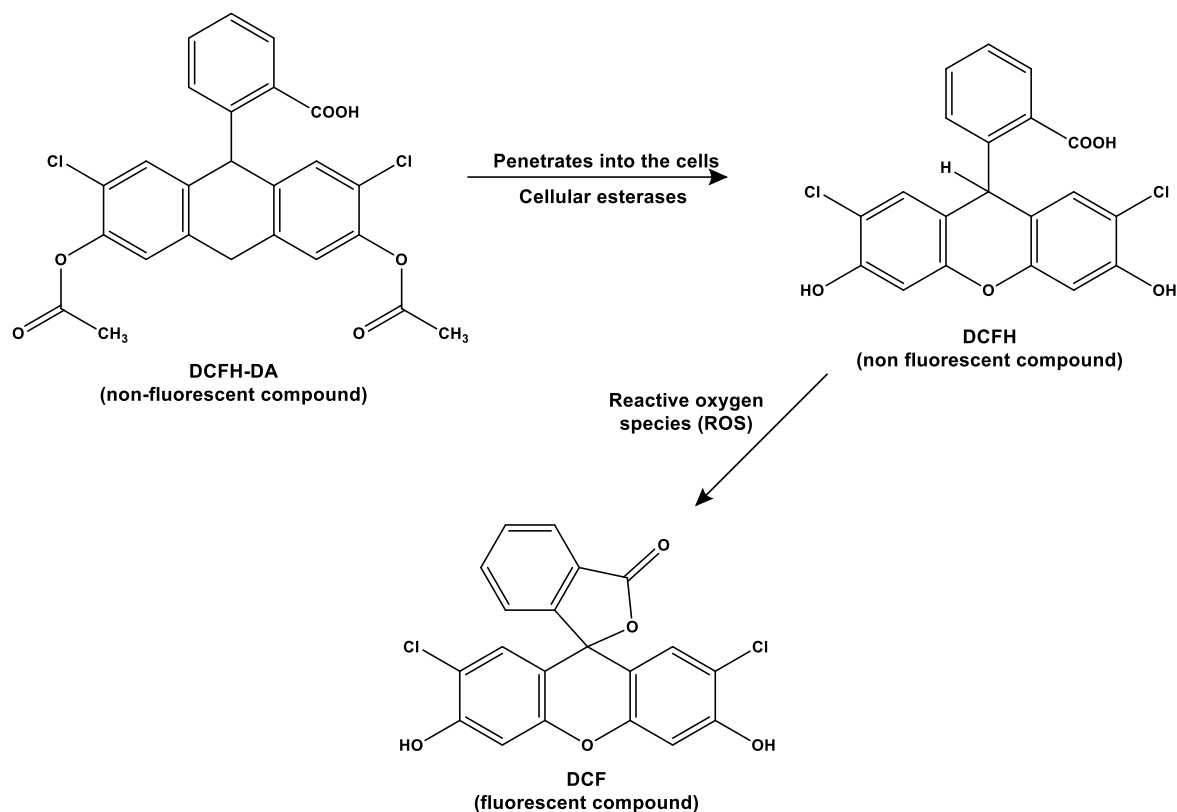


Figure 10. DCFH-DA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation yields the fluorescent adduct DCF.

6.1.4. Effects on mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\Psi_m$) was assessed by using JC-1 probe (225). Cells were seeded in 96-well black plates, using cell cultures of 7.5×10^4 cells/well, and incubated with increasing concentrations of *G. senegalensis* CME for 48 h. After the incubation period, 4 μ M of JC-1 probe in culture medium was added for 30 min. Cells were washed three times with HBSS before used to assess the JC-1 aggregate to monomer ratio, by reading the plate every five minutes for 30 min. A microplate reader (Synergy H1, BioTek) was used to measure the probe fluorescence intensity with excitation/emission settings at 485/535 and 560/595 nm to assess probe fluorescence as monomer and aggregates, respectively. The $\Delta\Psi_m$ was determined by the ratio aggregate/monomer (595:535 ratio).

6.1.5. Preparation of mitochondria-enriched fractions from ML-DmBG2-c2 cells

The activity of mitochondrial respiratory chain complexes and CS were assessed in mitochondria isolated from ML-DmBG2-2 cells. Briefly, ML-DmBG2-2 cells were grown in T25 flasks until a confluence of 70%, and then treated with 25 µg/mL of *G. senegalensis* CME or vehicle for 48 h. Cell metabolism was stopped by removing the cell culture medium, washing with HBSS, freezing, and storing the cells in liquid nitrogen at -80 °C until required for further studies. Afterwards, cells were collected in HBSS, and used to obtain a mitochondria-enriched fraction. Cells were centrifuged and 2 mL of buffer (sucrose 2000 mM, EDTA 0.5 mM and HEPES 10 mM, pH 7.4) were added at 4 °C. Then, cells were homogenized by using a Glass-Teflon Potter Elvehjem using 20 up- and downstrokes at 500 rpm. Mitochondria-enriched fractions were obtained by sequential differential centrifugation steps, using standard procedures (226). The concentration of mitochondrial protein was determined through the biuret method, using bovine serum albumin as standard reference.

6.1.6. Effects on mitochondrial respiratory chain complex activities

The activities of mitochondrial complex I, complex II, complex IV and CS were assessed in mitochondria-enriched fractions from ML-DmBG2-c2 cells submitted to three cycles of freezing/thawing. Assays were conducted in a 96-well plates at 25 °C, with a final volume of 250 µL, using the procedures described above for mitochondria isolated from yeasts (219). The activity of the mitochondrial redox chain complexes was normalized by the activity of CS.

The mitochondria-enriched fractions obtained from control ML-DmBG2-c2 cells were also used to evaluate the direct effects of *G. senegalensis* CME on the activity of mitochondrial complex II after 5 min of incubation. Results are expressed as percentage of control, i.e., the activity of the mitochondrial complex II in the absence of the extract.

6.2. Effects on acetylcholinesterase activity

The activity of AChE enzyme was assessed based on Ellman's method, using acetylthiocholine as substrate (**Figure 11**) (227). Assays were performed in the absence or presence of increasing concentrations of *G. senegalensis* and *F. polita* CME in 250 µL of phosphate buffer (100 mM, pH 7.4), supplemented with 1 U/mL AChE enzyme and 75 µM of Ellman's reagent (DTNB). After an incubation period of 2 min, the reaction was started

with the addition of acetylthiocholine at a final concentration in the well of 0.625 and 20 mM. The increase in absorbance was measured at 412 nm for 3 minutes. Results are expressed as percentage of control.

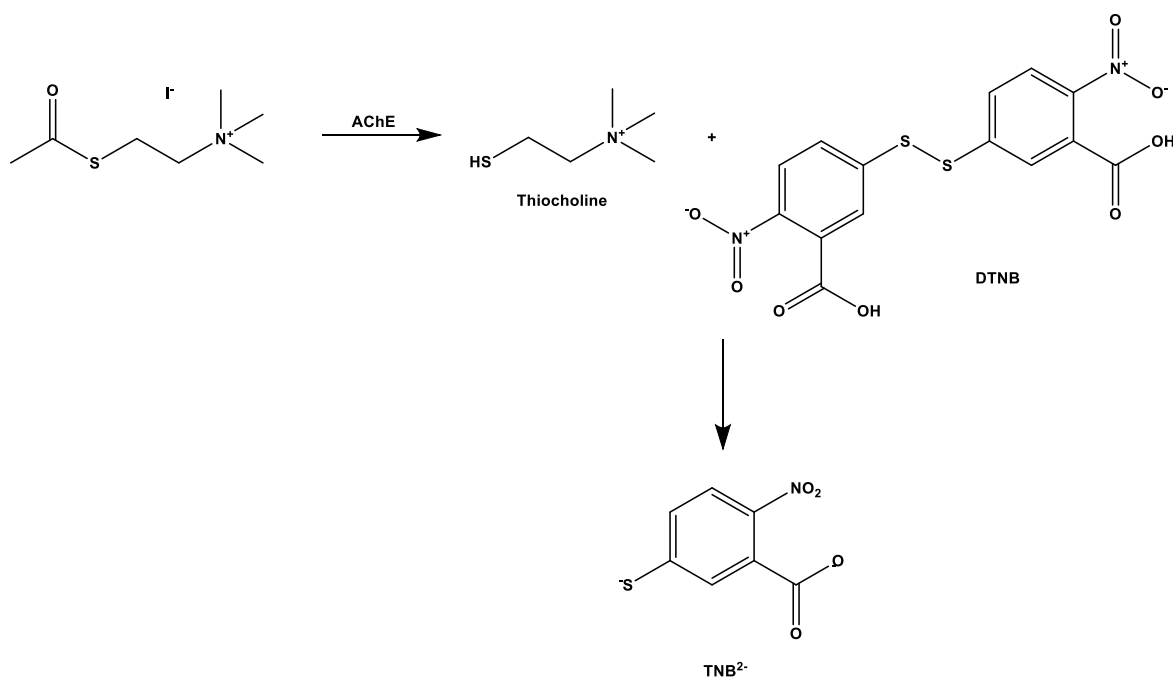


Figure 11. Reactions underlying Ellman's method. Thiocholine results from the hydrolysis of acetylthiocholine iodide by AChE. Then, thiocholine reacts with DTNB to generate the anion TNB with yellow color.

7. Toxicological profile

7.1. Cell conditions

Human keratinocytes HaCaT (ATCC, Barcelona, Spain) and human neuroblastoma SH-SY5Y cells (ATCC, Barcelona, Spain) were cultured in T75 flasks with glutamine-enriched DMEM and DMEM-F12, respectively, supplemented with 1% penicillin/streptomycin and 10% FBS, in a humidified incubator operating with 5% carbon dioxide and a temperature of 37 °C. After reaching a confluence of 70-80%, cells were washed with HBSS, collected with trypsin-EDTA, and used to preserve the cell cultures and to perform the assays, as will be described below.

7.2. Effects on cell viability

The effects of CME and its fractions on the cell viability of HaCaT and SH-SY5Y cells were evaluated by using the MTT reduction assay previously described for ML-DmBG2-c2 with slight modifications. Cells, seeded in 96-well plates at a density of 2.0×10^4 cells/well and 3.0×10^4 cells/well for HaCaT and SH-SY5Y cells, respectively, were incubated with extract/fraction or vehicle (control) for 48 h. Afterwards, the medium was removed and MTT was added to each well at a final concentration of 0.5 mg/mL, and the plate was incubated for 90 and 120 min, for HaCaT and SH-SY5Y, respectively, at 37 °C. Formazan crystals were dissolved with 200 μ L of a DMSO:isopropanol mixture (3:1, V:V) and quantified spectrophotometrically at 560 nm using a microplate reader Multiscan GO (Thermo Fisher Scientific, Massachusetts, USA). Results are expressed as percentage of control for MTT reduction assay.

7.3. Effects on membrane integrity

To assess the release of the cytosolic enzyme LDH, cells were seeded in 96-well plates at a density of 2.0×10^4 cells/well and 3.0×10^4 cells/well for HaCaT and SH-SY5Y cells, respectively. After 24 h, cells were incubated as previously mentioned for the cell viability assay. The followed procedure of the LDH release assay was the same as that used for insect cells. Thus, after the incubation time, 50 μ L of cell-free supernatant of extracellular medium were removed and mixed with 25 μ L pyruvate (10 mM) and 200 μ L NADH (0.3 mM). LDH activity was assessed spectrophotometrically following NADH oxidation at 340 nm for 3 min, using a microplate reader, Multiscan GO (Thermo Fisher Scientific, Massachusetts, USA). The total LDH activity was assessed in control cells treated with lysis solution (1% Triton X-100 in 10 mM phosphate buffer, pH = 7.2). Results are expressed as fold increase relative to control cells for LDH activity.

7.4. Effects on the generation of intracellular reactive oxygen species

The generation of ROS was assessed in 96-well black plates, using similar conditions of cell seeding and incubation described for cell viability and membrane integrity assays. The protocol used for the assessment of the generation of ROS was identical to that employed for insect cells. Briefly, after the treatment period, cells were incubated with the DCFH-DA fluorescent prepared in HBSS (10 μ M) probe for 30 min. The time-dependent changes in fluorescence intensity of the DCF resulting from probe oxidation of control and

treated cells were recorded with excitation/emission settings at 490/520 nm in a microplate reader (Synergy H1, Biotek). The results were expressed as percentage of control.

7.5. Effects on mitochondrial membrane potential

To assess the $\Delta\Psi_m$, human cells were seeded with the same cell density and culture conditions used for the previously described assays with these cells and by using JC-1 probe according with the described for insect cells (225). Briefly, after the incubation period with increasing concentrations of extract, cells were incubated with 4 μ M of JC-1 probe in culture medium for 30 min. Then, cells were washed three times with HBSS and used to assess the JC-1 aggregate to monomer ratio in the conditions previously described.

7.6. Effects on cell viability and intracellular reactive oxygen species after UVA irradiation

The impact of *G. senegalensis* CME on the cell viability and on the intracellular ROS generation of HaCaT cells exposed to UV radiation (365 nm) was also determined, by exposing the cells to the UVA radiation for 5 min. To evaluate the viability of skin cells exposed to radiation, HaCaT were seeded as previously mentioned for these assays. After the incubation period of 48 h, the culture media was removed, cells were washed and HBSS was added to allow penetration of UV rays. Some cells were used as a control without irradiation being covered with aluminum foil, and the others were irradiated with a Vilber Lourmat™ UV lamp 230 V/50 Hz using a wavelength of 365 nm for 15 min. The HBSS was removed, and the cells were incubated with the MTT, following the previously established procedure for the evaluation of cell viability.

The assessment of the intracellular ROS generation after UVA irradiation was performed by seeding HaCaT cells in a 6-well plate at a density of 2.5×10^5 cells/well, incubated in the conditions described above. After 24 h, cells were washed with HBSS and incubated with 50 μ g/mL of *G. senegalensis* CME and a fluorescent probe, DCFH-DA (10 μ M) dissolved in HBBS with HEPES 1% for 30 min. Following 15 min of exposure to UVA radiation, cells were frozen for 1 h, at -80 °C, to stop metabolism and then collected in HBSS with a scraper. The fluorescent decay was recorded in a 96-well black plate with a final volume of 200 μ L and excitation/emission settings at 490/520 nm in a microplate reader (Synergy H1, Biotek).

CHAPTER III: Results and Discussion

1. *G. senegalensis* leaves: from the chemical characterization to the antifungal and insecticide potential

1.1. Chemical characterization

1.1.1. Chemical composition of crude methanol extract and fractions

The maceration of *G. senegalensis* leaves in methanol allowed to prepare the CME with an extraction yield of $17.58 \pm 5.43\%$. The SPE on Hypersep C18 non-end-capped column was used for CME fractionation, allowing to discard the water-soluble compounds, and to collect the lipophilic components with ethyl acetate, the EAF, and the remaining compounds with methanol, the MF. The obtained fraction yields for EAF and MF were $24.15 \pm 16.43\%$ and $11.44 \pm 6.89\%$, respectively (**Table 6**). The general chemical characterization of the CME and of its fractions followed a work plan to assess the total levels of Folin-Ciocalteu reactive phenolic compounds, sugars, phospholipids, Liebermann-Burchard reactive compounds, chlorophylls and alkaloids, with subsequent determination of the EF for these parameters in each fraction (**Table 6**).

Reduction of the Folin–Ciocalteu reagent (FCR) in the presence of phenolic compounds results in the production of molybdenum-tungsten blue that is measured spectrophotometrically at 760 nm (208). Considering this methodology, the concentration of Folin-Ciocalteu reactive phenolic compounds in *G. senegalensis* CME is 37.63 ± 3.48 g/Kg dry extract, expressed as gallic acid equivalents. The compounds with this type of antioxidant activity are mainly retained in EAF, since this fraction exhibits a concentration of 46.12 ± 5.98 g/Kg dry extract, EF of 1.23, and the MF only of 17.7 ± 1.13 g/ Kg dry extract, EF of 0.47. These data indicate that many phenolic compounds present in *G. senegalensis* CME exhibit lipophilic properties. Previously, it was shown that the poly-methoxylated flavonoids are dragged from the Hypersep C18 column by low polar solvents such as ethyl acetate (228), suggesting that EAF may contain poly-methoxylated compounds.

Total sugars content was determined spectrophotometrically at 490 nm after a reaction with phenol in the presence of sulfuric acid (229). The levels of total sugars are higher in the EAF (76.45 ± 20.90 g glucose equivalents per Kg dry extract) than in CME (58.91 ± 4.56 g glucose equivalents per Kg dry extract), with an EF of 1.30. On the other hand, MF, with 33.70 ± 3.44 g glucose equivalents per Kg dry extract, has a lower content than the CME (EF of 0.57). It is important to highlight that a significant portion of the sugars (close to 50%) present in *G. senegalensis* CME were discarded by water used in the clean-up procedure

step of the SPE. Thus, the sugars quantified in the two fractions may include free sugars that were not removed by the water, as well as the sugars naturally attached to phenolic compounds that are released by reaction with sulfuric acid.

Table 6. Extraction yields of *G. senegalensis* leaves with methanol, CME, and of its solid phase fractionation obtained with ethyl acetate, EAF, and methanol, MF, and their general characterization in terms of total levels of Folin-Ciocalteu reactive phenolic compounds, sugars, phospholipids, Liebermann-Burchard reactive compounds, chlorophylls and alkaloids indicating the EF for each one.

(g compound equivalents per Kg extract)	CME	EAF	EF	MF	EF
Extraction/fraction yield (%)	17.58 ± 5.43	24.15 ± 16.43	n. a.	11.44 ± 6.89	n. a.
Folin-Ciocalteu reactive phenolic compounds	37.63 ± 3.48	46.12 ± 5.98	1.23	17.7 ± 1.13	0.47
Total Sugars	58.91 ± 4.56	76.45 ± 20.90	1.30	33.70 ± 3.44	0.57
Phospholipids	37.70 ± 6.02	22.38 ± 6.20	0.59	8.45 ± 6.38	0.22
Liebermann-Burchard reactive compounds	67.39 ± 6.30	193.65 ± 20.38	2.87	17.17 ± 8.16	0.25
Chlorophylls	5.83 ± 1.00	28.63 ± 1.36	4.91	Not detected	n. a.
Alkaloids	Not detected	Not detected	n. a.	Not detected	n. a.

n. a. - not applicable

Phospholipids were quantitated spectrophotometrically at 800 nm by phosphorous assay, after acidic hydrolysis (219). Total phospholipid content in *G. senegalensis* CME, EAF and MF is 37.70 ± 6.02, 22.38 ± 6.20 and 8.45 ± 6.38 g PC (C16:0/18:1) equivalents per Kg dry extract, respectively. Thus, the EF in both fractions is less than unity, indicating that most phospholipids are retained by silica in column.

Liebermann–Burchard reactive compounds (e.g. steroids and triterpenes) were quantified by taking advantage of the colorful products generated through the reaction of these compounds with acetic anhydride in the presence of concentrated sulfuric acid (211). The products resulting from the Liebermann-Burchard reaction, with blue green color, revealing the predominance of steroids, were quantified in stigmasterol equivalents.

Results of **Table 6** show that *G. senegalensis* EAF presents a phytosterol content of 193.65 ± 20.38 g stigmasterol equivalents per Kg dry extract, an EF of 2.87, which represents 69% of the total steroids and triterpenes present in the *G. senegalensis* CME (67.39 ± 6.30 g stigmasterol equivalents per Kg dry extract). With 17.17 ± 8.16 g stigmasterol equivalents per Kg dry extract, *G. senegalensis* MF only retains 2.8% of sterols present in CME. These data indicate that a significant part of the steroids and triterpenes remains in SPE column.

Chlorophylls content of *G. senegalensis* CME, EAF and MF were assessed by chlorophylls UV-Vis spectrum signature. The chlorophylls content in CME is 5.83 ± 1.0 g chlorophyll a equivalents per Kg dry extract, while in MF they were not detected. In fact, all chlorophylls were dragged with ethyl acetate, thereby their content in EAF is 28.63 ± 1.36 g chlorophyll a equivalents per Kg dry extract, corresponding to an EF of 4.92.

Several studies report the presence of β -carboline alkaloids in different parts of *G. senegalensis*, including on the leaves (194,230,231). Thus, a preliminary screening for the presence of this class of compounds was carried out with the alkaloids reagents (Dragendorff, Mayer and Bertrand) in *G. senegalensis* CME, EAF and MF, and the results were negative. Typically, extracting *G. senegalensis* alkaloids requires the use of solvents with lower polarity than methanol, such as dichloromethane or chloroform, after releasing the alkaloidal bases from their salts by treating them with a mineral base (194,230). In fact, HPLC analysis of the alkaloid-directed extract obtained from *G. senegalensis* leaves or of the solid residue of CME has confirmed the presence of four alkaloid compounds in the leaves and their absence in CME (**Figure 12**).

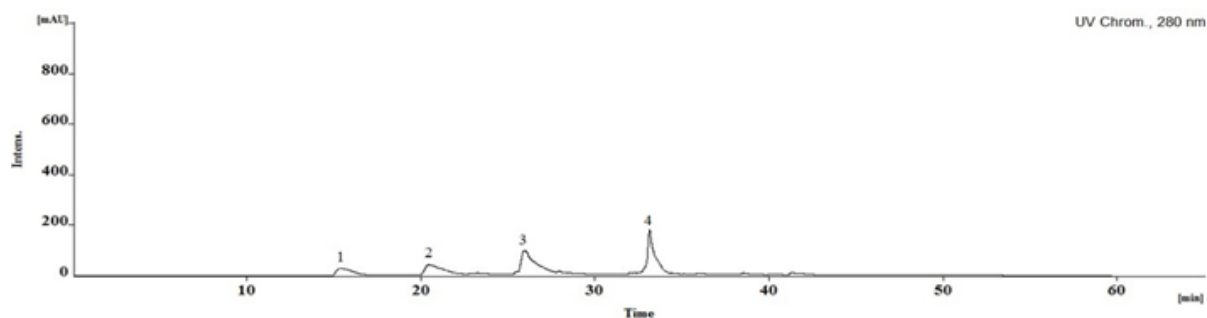


Figure 12. HPLC-UV profile of the alkaloid-targeted *G. senegalensis* leaves extract, using a reverse phase Luna C18 (Phenomenex, California, USA) with spectra acquisition in continuous scan mode from 200 to 600 nm, and the chromatogram recorded at 280 nm.

1.1.2. Characterization of the phenolic profile of *G. senegalensis* CME

HPLC-DAD-ESI/MSⁿ analysis of the CME obtained from the leaves of *G. senegalensis* evidences a rich phenolic profile, as shown in UV chromatograms recorded at 280 and 340 nm (**Figure 13**) with peak identification in **Table 7**. Combining UV spectrum features of each chromatogram peak with its MS data, it was possible to identify ten galloyl derivatives (**1-10**), three flavonol glycosides-cinnamoyl derivatives (**29-31**), seventeen flavonoid glycosides and three aglycones (**11-28**, **32** and **33**). Additionally, guieranone A was identified as peak **34**, while two chromatogram peaks (**35**, **36**) were not identified (**Table 7** and **Figure 13**).

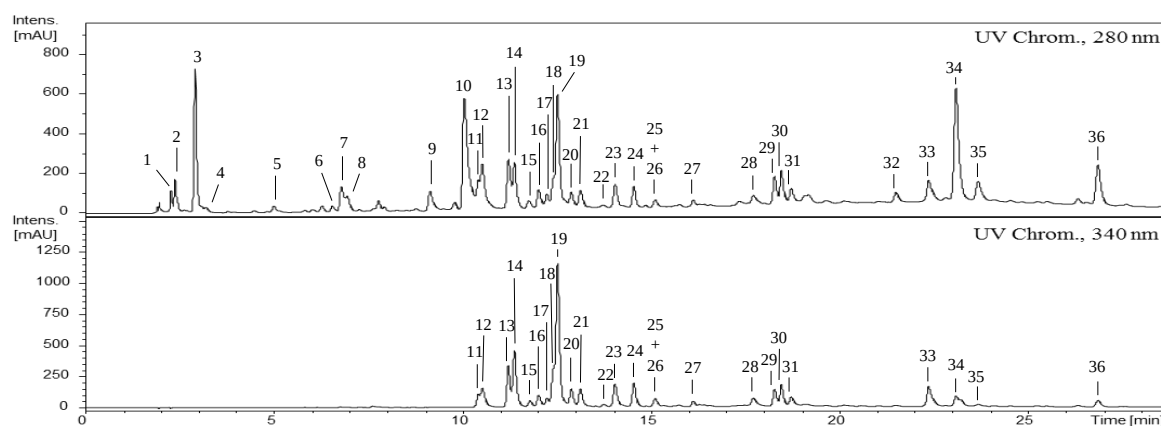


Figure 13. HPLC-UV profile of CME obtained from the leaves of *G. senegalensis*. Polyphenolic compounds were analyzed by reverse-phase HPLC-DAD-ESI-MSⁿ using a C18 column with spectra acquisition in continuous scan mode from 200 to 600 nm. The chromatograms were recorded at 280 and 340 nm. Compound identifications are indicated in **Table 7**.

Regarding galloyl derivatives, compounds **1-10** present a similar UV spectrum (~272-276 nm) and specific MS signatures that allow their identification, as will be described below. Compounds **1** and **4** both with [M-H]⁻ at m/z 343.0673 are isomers (C₁₄H₁₅O₁₀) that exhibit MS fragmentation pattern with ions at m/z 191.0565 and 169.0145, corresponding to the deprotonated molecular ions of quinic and gallic acids, respectively. As such, it is suggested that they should be galloylquinic acid isomers. Compounds **2**, **5**, **6** and **8** with ([M-H]⁻) at m/z 495.0780 are isomers (C₂₁H₁₉O₁₄) and their MS fragmentations revealed ions at m/z 343.0669, 191.0568 and 169.0149, corresponding to deprotonated molecular ions of galloylquinic, quinic and gallic acids, respectively.

Table 7. Identification and quantification (dry weight basis) of each phenolic compound detected in *G. senegalensis* leaves CME by HPLC-DAD-ESI-MSⁿ: Rt (retention time), UV-Vis absorption features, molecular formula, MS features in negative or positive (for guaianone A) ion modes with indication of main MS² detected fragments.

Compounds ^b		Rt (min)	UV (nm)	Molecular Formula	[M-H] ⁻ , m/z	MS ² fragments ^c , m/z(%)	g/Kg dry extract
Galloyl derivatives							
1	Gall-quinc ac (GQA)	2.2	272	C ₁₄ H ₁₅ O ₁₀	343.0673	191.0565(Quinc-H), 169.0145(GA-H)	1.33 ± 0.01
2	digall-Quinc ac (dGQA)	2.4	272	C ₂₁ H ₁₉ O ₁₄	495.0780	343.0669(GQA-H), 191.0568(QA-H), 169.0149(GA-H)	2.06 ± 0.02
3	Gallic ac (GA)	2.9	273	C ₇ H ₆ O ₅	169.0147	125.0244(GA-H-44)	11.64 ± 0.25
4	Gall-quinc ac (GQA)	3.1	274	C ₁₄ H ₁₅ O ₁₀	343.0675	191.0563(Quinc-H), 169.0146(GA-H)	0.37 ± 0.004
5	digall-Quinc ac (dGQA)	5.0	273	C ₂₁ H ₁₉ O ₁₄	495.0782	343.0670(GQA-H), 191.0569(QA-H), 169.0152(GA-H)	0.46 ± 0.02
6	digall-Quinc ac (dGQA)	6.6	275	C ₂₁ H ₁₉ O ₁₄	495.0779	343.0668(GQA-H), 191.0569(QA-H), 169.0150(GA-H)	0.59 ± 0.03
7	trigall-Quinc ac (tGQA)	6.8	272	C ₂₈ H ₂₃ O ₁₈	647.0892	495.0774(dGQA-H), 343.0670(GQA- H), 169.0147(GA-H)	2.25 ± 0.05
8	digall-Quinc ac (dGQA)	6.9	275	C ₂₁ H ₁₉ O ₁₄	495.0780	343.0671(GQA-H), 191.0570(QA-H), 169.0146(GA-H)	1.60 ± 0.04
9	trigall-Quinc ac (tGQA)	9.1	275	C ₂₈ H ₂₃ O ₁₈	647.0895	495.0770(dGQA-H), 343.0672(GQA- H), 307.0270, 169.0147(GA-H)	1.98 ± 0.006
10	tetragall-Quinc ac	10.1	276	C ₃₅ H ₂₇ O ₂₂	799.1000	647.0894(tGQA-H), 629.0768(tGQA- H-W), 601.0818(tGQA-H-W-28), 495.0780(dGQA-H), 343.0665(GQA- H), 169.0145(GA-H)	11.48 ± 0.07
						Σ	33.76 ± 0.16

Table 7. Identification and quantification (dry weight basis) of each phenolic compound detected in *G. senegalensis* leaves CME by HPLC-DAD-ESI-MSⁿ: Rt (retention time), UV-Vis absorption features, molecular formula, MS features in negative or positive (for guieranone A) ion modes with indication of main MS² detected fragments (continued).

Compounds ^b		Rt (min)	UV (nm)	Molecular Formula	[M-H] [−] , m/z	MS ² fragments [−] , m/z(%)		g/Kg dry extract
flavonoid glycosides and aglycones								
						-152		[M-H/2H] [−]
11	My-3-(gall)galc	10.4	264, 300sh, 356	C ₂₈ H ₂₄ O ₁₇	631.0917	479.0843 (100)		316.0228(15)
12	My-3-(gall)glc	10.5	264, 300sh, 355	C ₂₈ H ₂₄ O ₁₇	631.0911	479.0845 (100)		316.0226(15)
13	My-3-galc	11.2	254sh, 265, 300sh, 356	C ₂₁ H ₂₀ O ₁₃	479.8361			316.0229 (100)
14	My-3-glc	11.4	253, 264, 302, 356	C ₂₁ H ₂₀ O ₁₃	479.0834			316.0230 (100)
15	My-3-xyl	11.8	255sh, 265, 302sh, 354	C ₂₀ H ₁₈ O ₁₂	449.0716			316.0225(100)
16	Qct-3-(gall)galc	12.0	254sh, 266, 300sh, 356	C ₂₈ H ₂₄ O ₁₆	615.1025	463.0877 (100)		301.0350(20)
17	Qct-3-(gall)glc	12.2	254sh, 265, 300sh, 355	C ₂₈ H ₂₄ O ₁₆	615.1018	463.0881 (100)		301.0352(30)
18	My-3-arab	12.4	254sh, 264, 302sh, 356	C ₂₀ H ₁₈ O ₁₂	449.0720			316.0227(100)
19	My-3-rh	12.5	254, 264sh, 300sh, 352	C ₂₁ H ₂₀ O ₁₂	463.0882			316.0228(100)
20	Qct-3-galc	12.9	256, 266sh, 300sh, 354	C ₂₁ H ₂₀ O ₁₂	463.0876			301.0349(100)
21	Qct-3-glc	13.1	255, 266sh, 302sh, 354	C ₂₁ H ₂₀ O ₁₂	463.0880			301.0353(100)
22	Qct-3-xyl	13.7	255, 266sh, 302sh, 352	C ₂₀ H ₁₈ O ₁₁	433.0782			301.0350(100)
23	Qct-3-arab	14.0	255, 266sh, 300sh, 352	C ₂₀ H ₁₈ O ₁₁	433.0780			301.0351(100)

Table 7. Identification and quantification (dry weight basis) of each phenolic compound detected in *G. senegalensis* leaves CME by HPLC-DAD-ESI-MSⁿ: Rt (retention time), UV-Vis absorption features, molecular formula, MS features in negative or positive (for guaianone A) ion modes with indication of main MS² detected fragments (continued).

Compounds ^b		Rt (min)	UV (nm)	Molecular Formula	[M-H] ⁻ , m/z	MS ² fragments ⁻ , m/z(%)			g/Kg dry extract
24	Qct-3-rh	14.5	255, 265sh, 302sh, 352	C ₂₁ H ₂₀ O ₁₁	447.0938			301.0348(100)	8.66 ± 0.07
25	My	15.2	---- ^c	C ₁₅ H ₁₀ O ₈	317.0307				11.40 ± 0.04
26	Kpf-3-pt	15.2	---- ^c	C ₂₀ H ₁₈ O ₁₀	417.0831			284.0338(100)	
27	Kpf-3-rh	16.1	266, 288sh, 348	C ₂₁ H ₂₀ O ₁₀	431.0975			285.0397(100)	1.87 ± 0.09
28	Qct	17.7	256, 265sh, 300sh, 368	C ₁₅ H ₁₀ O ₇	301.0357				0.37 ± 0.02
32	Hespt	21.5	285, 232sh	C ₁₆ H ₁₄ O ₆	301.0728				0.07 ± 0.002
33	Isorhmn	22.4	256, 266sh, 298sh, 370	C ₁₆ H ₁₂ O ₇	315.0536				1.05 ± 0.03
								Σ	187.37 ± 1.19
Flavonols glycosides-cinnamoyl derivatives									
						-146			
29	Kpf-3-(<i>p</i> -coum)hx	18.3	266, 296sh, 314, 350sh	C ₃₀ H ₂₅ O ₁₃	593.1301	447.0935(15)		284.0338(100)	2.16 ± 0.10
30	Kpf-3-(<i>p</i> -coum)hx	18.5	265, 295sh, 314, 352sh	C ₃₀ H ₂₅ O ₁₃	593.1302	447.0935(10)		284.0340(100)	5.35 ± 0.23

Table 7. Identification and quantification (dry weight basis) of each phenolic compound detected in *G. senegalensis* leaves CME by HPLC-DAD-ESI-MSⁿ: Rt (retention time), UV-Vis absorption features, molecular formula, MS features in negative or positive (for guieranone A) ion modes with indication of main MS² detected fragments (continued).

Compounds ^b		Rt (min)	UV (nm)	Molecular Formula	[M-H] ⁻ , m/z	MS ² fragments ⁻ , m/z(%)			g/Kg dry extract
31	Kpf-3-(<i>p</i> -coum)hx	18.7	266, 294sh, 316, 350sh	C ₃₀ H ₂₅ O ₁₃	593.1299	447.0935(25)	284.0339(100)		1.11 ± 0.04
							Σ		8.62 ± 0.15
Other compounds – positive ion mode									
							[M+H] ⁺ , m/z		
34	Guieranone A	23.1	266sh, 274, 312, 326, 380	C ₁₈ H ₂₀ O ₅	317.1290	287.0913 (60)	275.0913 (100)	69.0335 (40)	36.46 ± 2.87
							Σ		266.21 ± 2.05

¹ my: myricetin; qct: quercetin; hespt: hesperetin; kpf: kaempferol; isorhmn: isorhamnetin; galc: galactoside; glc: glucoside; pt: pentoside; xyl: xyloside; arab: arabinoside; rh: rhamnoside; gall: gallic acid.

² UV spectra could not be properly observed due to the co-elution of **25** and **26**.

³ Results correspond to mean ± standard deviation (n = 3).

The deprotonated molecular ion of **3** at *m/z* 169.0147 (C₇H₆O₅) exhibits a MS fragmentation with a carbonyl loss (-44 amu, CO₂) detected by the ion at *m/z* 125.0244 ([C₆H₅O₃]⁻), thereby corresponding to gallic acid. Compounds **7** and **9** have deprotonated molecular ions with *m/z* of 647.0892, 152 amu higher than compounds **2**, **5**, **6** and **8**. They exhibit a MS fragmentation pattern with *m/z* species resulting from the losses of one and two gallic acid units (152), with or without water accompaniment, in addition to the deprotonated gallic acid ion; therefore, they will be tri-galloylquinic acid isomers. Compound **10** ([M-H]⁻ at *m/z* 799.1000) presents another additional gallic acid, since its MS fragmentation profile exhibits ions resulting from the losses of different fragments of gallic acid and as base peak the ion at *m/z* 601.0818 [(tGQA-H)-(W + 28)]⁻, so it will be tetra-galloylquinic acid. Many of the ions of the MS² fragmentations of **7**, **9** and **10** resulting from

losses of one or more galloyl radicals accompanied by water are not indicated in **Table 7** due to the large number and variability of their presence.

Regarding flavonols glycosides and aglycones, compounds **11-28** and **33** exhibit UV spectra characteristic of flavonols and **32** of a flavanone (**Table 7**). Compounds **28** and **33** have a UV band I (~370 nm) typical of flavonols with a free 3-hydroxyl, and their masses correspond to quercetin (3,5,7,3',4'-pentahydroxyflavone) and isorhamnetin (3,5,7,4'-tetrahydroxy-3'-methoxyflavone), respectively. Additionally, UV spectrum of **25** could not be properly resolved due to the co-elution with **26**, but its mass ($[M-H]^-$ at m/z 317.0307) corresponds to myricetin (3,5,7,3',4',5'-hexahydroxyflavone). Remaining constituents that display UV spectra characteristic of flavonols with a substituted 3-hydroxyl (**Table 7**) are characterized below. In the MS fragmentation profile of **11**, **12**, **16** and **17** is observed a loss of a 152 amu fragment (radical galloyl) to give base peaks, whose masses, in **11** and **12** correspond to myricetin-3-O-hexoside, i.e., the $[M-H]^-$ of the **13** and **14**, and in **16** and **17** to quercetin-3-O-hexoside, i.e., the $[M-H]^-$ of the **20** and **21** (**Table 7**). Thus, these compounds can be identified as myricetin-3-O-(galloyl)galactoside (**11**), myricetin-3-O-(galloyl)glucoside (**12**), quercetin-3-O-(galloyl)galactoside (**16**) and quercetin-3-O-(galloyl)glucoside (**17**).

Compounds **13-15**, **18-24**, **26** and **27** exhibit deprotonated molecular ions of monoglycosides, being observed base peaks of $[myricetin-2H]^-$ (**13-15**, **18** and **19**), $[quercetin-H]^-$ (**20-24**) and $[kaempferol-H/2H]^-$ (**26** and **27**) in their MS fragmentations (**Table 7**). Therefore, **13** and **14** correspond to myricetin-3-O-hexoside isomers and, considering their elution order in RP, they can be labelled as myricetin-3-O-galactoside and myricetin-3-O-glucoside, respectively; **15** and **18** are myricetin-3-O-pentoside isomers: myricetin-3-O-xyloside (**15**) and myricetin-3-O-arabinoside (**18**); **19** corresponds to myricetin-3-O-rhamnoside; quercetin-3-O-hexoside isomers (**20** and **21**) are quercetin-3-O-galactoside and quercetin-3-O-glucoside; quercetin-3-O-pentoside isomers (**22** and **23**) are quercetin-3-O-xyloside and quercetin-3-O-arabinoside; **24** is quercetin-3-O-rhamnoside; **26** and **27** are kaempferol-3-O-pentoside and kaempferol-3-O-rhamnoside.

Compounds **29-31** are isomers with the characteristic UV spectrum of flavonoids acylated with cinnamoyl acids, since UV overlap deriving from the flavonoid moiety and the cinnamoyl acid (UV: 266, 296sh, 314, 350sh nm) is observed (**Table 7**). In the MS fragmentations of their deprotonated molecular ions at m/z 593.1301 ($[C_{30}H_{25}O_{13}]^-$), loss of a *p*-coumaroyl radical (-146 amu) is observed to give place to an ion at m/z 447.0935 ($[C_{21}H_{19}O_{11}]^-$) and the deprotonated ion of the aglycone at m/z 284.0338 ($[M-2H, C_{15}H_8O_6]^-$).

), thus matching with kaempferol. As such, **29-31** can be labelled as kaempferol-3-O-(*p*-coumaroyl)hexoside isomers.

The UV spectrum of **32** suggests the presence of a flavanone backbone (UV: 285, 332sh nm), its deprotonated molecular ion at m/z 301.0728 ($[C_{16}H_{14}O_6]^-$) matching with the one of hesperetin (5,7,3'-trihydroxy-4'-methoxy-flavanone) (**Table 7**).

Compounds **34-36** are very lipophilic, eluting at the end of the chromatogram, and display similar UV spectra (266sh, 274, 312, 326, 380 nm; 276, 306, 360 nm and 268, 278sh, 312, 326 nm). Compound **34** ionize in positive mode $[M+H]^+$ ion at m/z 317.1290 ($C_{18}H_{20}O_5$) and exhibits a MS fragmentation pattern compatible with the previously reported for guieranone A (199), since its characteristics acylium ion fragments are detected at m/z 287 $[C_{17}H_{19}O_4]^+$, 275 $[C_{15}H_{15}O_5]^+$ and m/z 69 $[C_4H_5O]^+$ as shown in insert of **Figure 14**.

As will be described below, this compound is also detected in EAF and its MS² spectrum is displayed in the insert of **Figure 14**. Compounds **35** and **36** also ionize in positive mode with $[M+H]^+$ ions at m/z 301.1069 ($C_{17}H_{16}O_5$) and at m/z 335.1486 ($C_{18}H_{22}O_6$), respectively. However, their weak MS fragmentation do not allow structure elucidation.

Regarding the phenolic profile of *G. senegalensis* leaves, the identification of galloyl derivatives (**1-10**) (**Table 7**) on CME is not surprising as the occurrence of gallic acid and a series of galloylquinic acid isomers was previously reported (195,232–234). Myricetin, quercetin and kaempferol derivatives are also known to occur in the species, as reported in collections of leaves and galls of plant samples obtained in Burkina Faso, Senegal, and Sudan (195,233,235–237). However, the present work reports a detailed phenolic characterization of samples collected in Guinea-Bissau, that confirm the previously known phenolic profile of the species, allowing its enlargement with the identification, for the first time, of two myricetin derivatives (**11, 15**), two quercetin derivatives (**16, 17**) and, at least, two kaempferol derivatives (**29-31**).

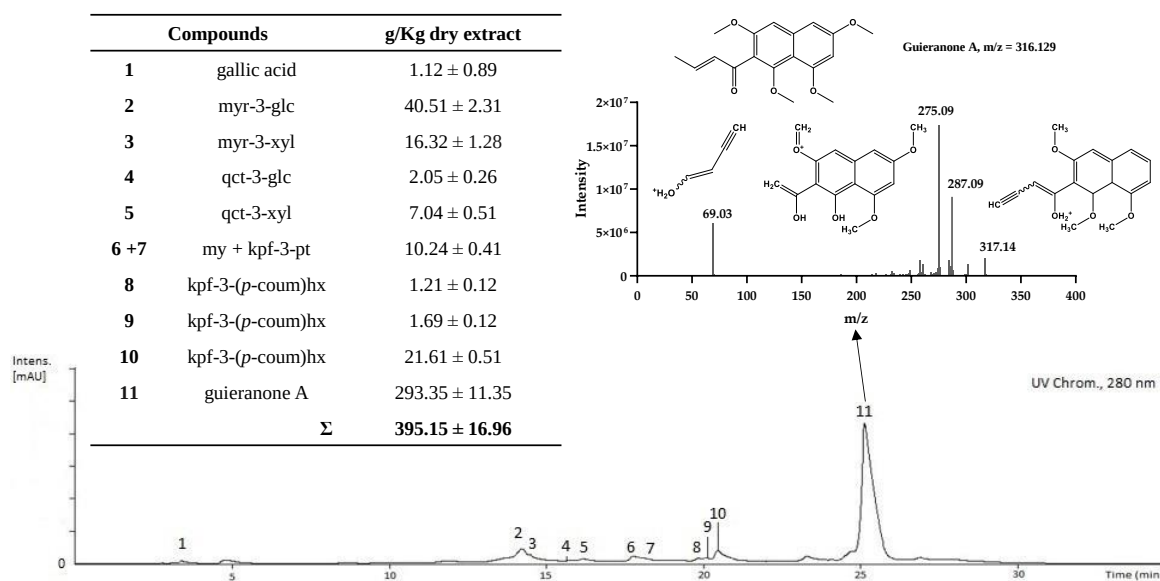


Figure 14. HPLC-UV profile of *G. senegalensis* EAF obtained by SPE (Hypersep C18) from the *G. senegalensis* leaves CME. Polyphenolic compounds were analyzed by reverse-phase HPLC-DAD using a C18 column with spectra acquisition in continuous scan mode from 200 to 600 nm. The chromatogram was recorded at 280 nm.

Compounds quantification was also performed by HPLC-DAD using the reference compounds and standard curves indicated in **Table 3**. The concentration of each identified compound in *G. senegalensis* CME is displayed in the last column of **Table 7**. The quantitative analysis revealed that flavonol-3-*O*-glycosides are predominant in *G. senegalensis* CME, comprising ca. 85% of the total quantifiable phenolic content. Among flavonol-3-*O*-glycosides, myricetin-3-*O*-rhamnoside (**19**: 36.94 ± 1.21 g/Kg dry extract) and myricetin-3-*O*-glucoside (**14**: 29.4 ± 1.39 g/Kg dry extract) are the major compounds, accounting for ca. 27% of the total phenolic content. Worth to highlight the significant content in galloyl derivatives (**1-10**: 33.76 ± 0.50 g/Kg dry extract), constituting ca. 9% of the total quantifiable phenolic content, being gallic acid (**3**) and the tetra-galloyl derivative (**10**) the most abundant compounds of the phenolic acid class. Additionally, flavonols glycosides-cinnamoyl derivatives (**29-31**) represent about 4% of the total phenolic content, being kaempferol-3-*O*-(*p*-coumaroyl)hexoside isomer (**30**) the most representative. Guieranone A, a naphthyl butanone, is, after myricetin-3-*O*-rhamnoside, the major compound with 36.46 ± 2.87 g/Kg dry extract and representing ca. 14% of the total content (**Table 7**).

1.1.3. Characterization of the phenolic and chlorophyll profiles of *G. senegalensis* EAF

Following the above-mentioned strategy, *G. senegalensis* EAF was analyzed to identify and quantify the phenolic compounds retained in this fraction. HPLC-DAD analysis, with UV chromatogram recorded at 280 nm, is displayed in **Figure 14**, as well as the concentration determined for the identified compounds and the MS/MS fragmentation pattern obtained for peak **11**, identified as guieranone A. Eleven compounds, all identified in *G. senegalensis* CME, were detected in EAF: gallic acid (**1**), five flavonoid glycosides (**2-5, 7**), one aglycone (**6**), three flavonol glycosides-cinnamoyl derivatives (**8-10**) and guieranone A (**11**), a naphthyl butanone. Concerning the quantification, guieranone A (**11**) is the major compound (293.35 ± 11.35 g/Kg dry extract) in *G. senegalensis* EAF, representing ca. 76% of the total compounds identified under these conditions. Myricetin-3-O-glucoside (**2**: 40.51 ± 2.31 g/Kg dry extract), the flavonol glycoside-cinnamoyl derivative (**10**: 21.61 ± 0.51 g/Kg dry extract) and myricetin-3-O-xyloside (**3**: 16.32 ± 11.35 g/Kg dry extract) also exhibit significant relative abundance. The other seven compounds identified appear in minor concentrations.

G. senegalensis EAF was also analyzed by means of HPLC-DAD-ESI/MSⁿ to promote the separation and identification of compounds with higher lipophilicity like chlorophylls (C30 YMC column, using as mobile phases *tert*-butyl methyl ether and methanol). The HPLC chromatogram recorded at 420 nm is displayed in **Figure 15**. Combination of UV spectrum features with MS data enabled to identify chlorophyll *a* (**4**) and six compounds that are derivatives of chlorophyll *a* (**1, 3, 5, 7, 10, 11**), as well as chlorophyll *b* (**2**) and three additional structurally related compounds (**6, 8, 9**), as indicated in **Table 8**. Thus, the compounds were quantified by HPLC-DAD using chlorophyll *a* and *b* as reference compounds and standard curves indicated in **Table 5**. As will be described below, the diversity of chlorophyll-related compounds detected in *G. senegalensis* EAF may result from the expected chlorophyll catabolism during leaf senescence triggered by the drying process.

Chlorophylls and their derivatives, analyzed in positive ion mode, exhibit a well-defined fragmentation pattern with product ions resulting from unimolecular heterolytic decomposition of peripheral substituents (238). Thus, MS signatures used to identify the compounds are the product ions that emerge from: i) the replacement of central magnesium by two protons, ii) the loss of the phytol chain followed by the total or partial fragmentation of the propionic chain, and iii) the fragmentation of the β -keto ester group.

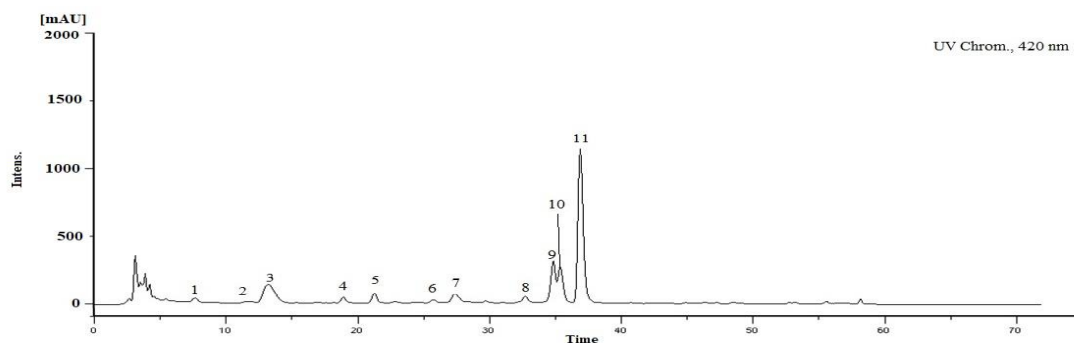


Figure 15. HPLC-UV profile of *G. senegalensis* EAF obtained by solid-phase extraction (Hypersep C18) from the *G. senegalensis* leaves CME, using a C30 column with spectra acquisition in continuous scan mode from 200 to 600 nm and the chromatogram recorded at 420 nm. Chlorophylls were also analyzed by reverse-phase HPLC-ESI-MSⁿ. The compound identification is indicated in **Table 8** and text.

Accordingly, compounds **2** and **4** with $[M+H]^+$ ions at m/z 907.55 and 893.70 were identified, respectively, as chlorophylls *b* and *a*, since they exhibit a MS fragmentation with a loss of the phytol group (-278 amu), followed by a propionic acid (-74 amu), detected by the ions at m/z 553.1992 ($C_{32}H_{25}MgN_4O_4$) and 539.2418 ($C_{32}H_{27}MgN_4O_3$), respectively (**Table 8**). For both, the detected ion with m/z at 227.1603 can be assigned to an additional phytol fragmentation. Derivatives of the chlorophylls *b* and *a*, resulting from the replacement of central magnesium by two protons (pheophytins) are detected as compounds **9** and **11** with $[M+H]^+$ ions at 885.55 and 871.57, respectively. Pheophytins exhibit a characteristic fragmentation pattern of chlorophylls. For example, pheophytin *a*, detected by ion with m/z at 871.57, produce MS² fragmentations with ions at m/z 593.2754 (chlorophyll *a* - Mg - phytol), 533.2546 (chlorophyll *a* - Mg - phytol - CH_3COOH) and at 519.2418 (chlorophyll *a* - Mg - phytol - CH_3CH_2COOH). In pheophytin *b*, an additional fragmentation pathway that starts with the loss of β -keto ester group and follows with fragmentation of phytol chain is detected, as suggested by ions with m/z at 826.5379 (chlorophyll *b* - Mg - CH_3COO) and 547.2350 (chlorophyll *b* - Mg - CH_2COOH - phytol). As previously reported, the replacement of the central magnesium atom of the tetrapyrrole ring of chlorophylls by reactions involving a metal-chelating substance is a very common catabolic process during leaf senescence (239). Thus, the concentrations of the pheophytins *a* and *b* in EAF are significantly higher than in the native compounds (chlorophyll *a* 1.07 ± 0.18 , pheophytin *a* 44.76 ± 1.78 g/Kg dry extract; chlorophyll *b* 0.67 ± 0.03 , pheophytin *b* 12.67 ± 1.82 g/kg dry extract).

Native chlorophylls and pheophytins are sensitive to oxidation processes, mainly originating two types of products. The main modification involves the replacement of the H

atom at C13² by a hydroxyl group, producing the hydroxyl derivatives (238). In fact, hydroxyl derivatives of chlorophyll *a* and pheophytin *a* are detected, respectively, as compounds **3** and **10**, with [M+H]⁺ at *m/z* 909.53 and 887.65. Compound **8**, with *m/z* at 901.65, was identified as HO-pheophytin *b*. As indicated in **Table 8**, the concentrations of these oxidized compounds decrease in the series HO-chlorophyll *a* > HO-pheophytin *a* > HO-pheophytin *b*. The other type of oxidation reactions occurs at C15¹ with the formation of a lactone group and formation of hydroxy-lactone derivatives. Compounds **1** (*m/z* at 925.00), **6** (*m/z* at 917.70) and **7** (*m/z* at 903.65) were identified as HO-lactone-chlorophyll *a*, HO-lactone-pheophytin *b* and HO-lactone-pheophytin *a*, respectively. The concentration of HO-lactone-pheophytin *a* is higher than the other HO-lactone derivatives, but significantly lower than hydroxyl derivatives (**Table 8**). Compound **5**, *m/z* at 593.27, exhibits MS² fragmentation ions compatible with a structure of the pheophorbide *a*, as suggested by the ion with *m/z* at 533.2544 (pheophytin *a* - phytol - CH₂COOH). The concentration of this dephytylated catabolite of chlorophyll *a* in EAF is 1.52 ± 0.40 g/Kg of dry extract.

Table 8. Identification and quantification (dry weight basis) of each chlorophyll detected in *G. senegalensis* leaves EAF by HPLC-ESI-MSⁿ: Rt, UV absorption features, molecular formula, MS features in positive ion mode with indication of main MS² detected fragments.

	Compounds	Rt (min)	UV (nm)	Molecular Formula	[M+H] ⁺ (m/z)	MS ² [M+H] ⁺ , m/z (%)	g/Kg dry extract ¹
1	HO-lactone-Chlorophyll <i>a</i>	7.71	407, 664	C ₅₅ H ₇₂ MgN ₄ O ₇	925.00	647.2269(60) 588.2128(20) 553.1991(35)	1.78 ± 0.34
2	Chlorophyll <i>b</i>	11.91	435	C ₅₅ H ₇₀ MgN ₄ O ₆	907.55	553.1992(30) 227.1603(100)	0.67 ± 0.03
3	HO-Chlorophyll <i>a</i>	13.44	407, 665	C ₅₅ H ₇₂ MgN ₄ O ₆	909.53	555.2154(100) 227.1608(20)	13.08 ± 1.63
4	Chlorophyll <i>a</i>	18.84	418, 442, 470	C ₅₅ H ₇₂ MgN ₄ O ₅	893.70	539.2418(100) 227.1598(30)	1.07 ± 0.18
5	Pheophorbide <i>a</i>	21.19	418, 442, 471	C ₃₅ H ₃₆ N ₄ O ₅	593.27	533.2544(80)	1.52 ± 0.40
6	HO-lactone-pheophytin <i>b</i>	25.68	434, 410	C ₅₅ H ₇₂ N ₄ O ₈	917.70	830.5706(5) 639.2801(2) 579.2597(6) 551.2652(100)	0.63 ± 0.03
7	HO-lactone-pheophytin <i>a</i>	27.27	408, 664	C ₅₅ H ₇₄ N ₄ O ₇	903.65	625.2648(20) 607.2546(20) 565.2444 (80)	2.50 ± 1.77
8	HO-pheophytin <i>b</i>	32.60	435, 412, 654	C ₅₅ H ₇₂ N ₄ O ₇	901.65	623.2682(5) 563.2349 (5) 227.1575(100)	5.28 ± 2.89
9	Pheophytin <i>b</i>	34.88	434, 412, 655	C ₅₅ H ₇₂ N ₄ O ₆	885.55	826.5379(100) 547.2350(30) 535.2348(80)	12.67 ± 1.82
10	HO-Pheophytin <i>a</i>	35.38	407, 666	C ₅₅ H ₇₄ N ₄ O ₆	887.65	609.2703(40) 591.2598(60) 531.2389(100)	11.23 ± 0.72
11	Pheophytin <i>a</i>	36.95	407, 665	C ₅₅ H ₇₄ N ₄ O ₅	871.57	593.2754(100) 533.2546(80) 519.2385(5)	44.76 ± 1.78
						Σ	95.19 ± 2.67

1.2. Antifungal activity

1.2.1. Fungal susceptibility to *G. senegalensis* CME, MF and EAF

The antifungal activity of *G. senegalensis* CME and its fractions (MF and EAF) on filamentous fungi was investigated by using species that affect agricultural crops and/or cause humans and animals' mycoses. The effects of *G. senegalensis* CME, MF and EAF on *F. oxysporum*, *A. fumigatus*, *Colletotrichum gloeosporioides*, assessed by the MIC and MFC are displayed in **Table 9**. Despite CME and MF do not reveal antifungal activity against these filamentous fungi at concentrations until 2000 µg/mL, the EAF shows antifungal activity against all species. Moreover, EAF is particularly active against *C. gloeosporioides* since a MIC value of 500 and a MFC value of 1000 µg/mL were determined (**Table 9**). These results allow assigning biological relevance to *G. senegalensis* EAF, since *C. gloeosporioides* is a prevalent pathogen of many economically important crops, including cashew, mango, papaya, avocado, citrus (240,241), and this pathogen has developed resistance against many of the available fungicides (242).

Table 9. Antifungal activity of *G. senegalensis* CME and its fractions, EAF and MF, against three species of filamentous fungi, assessed by MIC and MFC.

Filamentous fungi	MIC ¹ (µg/mL)			MFC (µg/mL)
	CME	EAF	MF	EAF
<i>Aspergillus fumigatus</i>	> 2000	2000	> 2000	> 2000
<i>Fusarium oxysporum</i>	> 2000	2000	> 2000	> 2000
<i>Colletotrichum gloeosporioides</i>	> 2000	500	> 2000	1000

¹Concentration that inhibited at least 90% of growth.

Considering the positive results of EAF towards filamentous fungi, the panel was broadened to include the human pathogenic yeasts (*C. neoformans*, *C. albicans*, *C. krusei*) and *S. cerevisiae*, a non-pathogenic species used to allow the investigation of its mode of action with safety. MIC and MFC values of *G. senegalensis* EAF on the above-mentioned yeasts are displayed in **Table 10**, as well as the results obtained with penconazole and fluconazole, used as reference fungicide and antifungal, respectively. Data of **Table 10** show that *G. senegalensis* EAF exhibit a strong antifungal activity against the pathogenic

strains *C. neoformans* (MIC = 8 µg/mL, MFC = 125 µg/mL) and *C. krusei* (MIC = 16 µg/mL, MFC = 500 µg/mL), as well as against *S. cerevisiae* (MIC = 8 µg/mL, MFC = 500 µg/mL). Under the same assay conditions, the antifungal activities of penconazole and fluconazole against *C. neoformans* are characterized by MIC values of 16 and 8 µg/mL and MFC values of > 16 and > 32 µg/mL, respectively. Therefore, the antifungal power of *G. senegalensis* EAF against *C. neoformans* is in the same order of magnitude of the penconazole, an available fungicide used in pest management, and of fluconazole, an antifungal drug used in clinical settings.

Table 10. Antifungal activity of *G. senegalensis* EAF against four yeast strains, assessed by MIC and MFC. The fungicide penconazole and the antifungal fluconazole were used as positive controls.

Yeast	MIC (µg/mL)			MFC (µg/mL)		
	EAF	Penconazole	Fluconazole	EAF	Penconazole	Fluconazole
<i>Saccharomyces cerevisiae</i>	8	1	8	500	1	16
<i>Candida albicans</i>	250	32	2	> 1000	> 32	> 128
<i>Candida krusei</i>	16	16	2	500	≥ 32	> 128
<i>Cryptococcus neoformans</i>	8	16	8	≥ 125	> 16	> 32

The antifungal activity of *G. senegalensis* CME and its fractions was also evaluated using cultures of *S. cerevisiae* and *C. neoformans* in liquid medium supplemented with 50 µg/mL of *G. senegalensis* leaves CME (filled squares), MF (open triangles) or EAF (open circles) (**Figure 16A and B**).

As a control, yeast cultures were grown in medium without extracts, and the increase of the optical density at 610 nm was used to follow the time-dependent growth of yeast strains at 30 °C during 60 h. The long-time intervals without recording the optical density correspond to the night cycles. According to the results of **Figure 16A and B**, the growth of *S. cerevisiae* and *C. neoformans* in control condition can be described by an exponential function, with a specific growth rate of 0.0447 and 0.0315 h⁻¹, respectively. While *G. senegalensis* MF does not induce detectable effects, exposure to CME or EAF, at 50 µg/mL, lead to strong inhibitory effects on the growth of both yeast strains. For example, *G. senegalensis* CME promotes a decrease of the specific growth rate of *S. cerevisiae* to 33% of control and of *C. neoformans* to 40% of control. On the other hand, in the presence of

the same concentration of EAF the specific growth rate of *S. cerevisiae* decreases to 30% of control and of *C. neoformans* to 33% of control. Therefore, the effects on growth curves of yeast strains are in line with the results obtained in MIC and MFC susceptibility tests (**Table 10**). These results confirm that the compounds with lipophilic properties underlie the antifungal activity of the methanol extract obtained from *G. senegalensis*, thereby they are obtained in the EAF during fractioning procedure. **Figure 16C** and **D** show the effects of increasing concentrations of EAF on the growth of *S. cerevisiae* and *C. neoformans* cultures, with the values of specific growth rates being indicated in the inserts. As detected by the decrease of specific growth rate (*S. cerevisiae*: $EC_{50} = 37.44 \mu\text{g/mL}$; *C. neoformans*: $EC_{50} = 39.00 \mu\text{g/mL}$), as well as by the low values of optical density in the end of the assay, the inhibitory effects of *G. senegalensis* EAF are concentration-dependent towards both yeast strains.

In a previous study, it is reported that the ethanol extract of *G. senegalensis* leaves exhibit antifungal activity against *Cladosporium cucumerinum*, this antifungal activity being ascribed to guieranone A, as revealed by assays with the isolated compound (199). Thus, the antifungal activity of *G. senegalensis* leaves CME detected in the present work, which is conserved and/or amplified in *G. senegalensis* EAF (**Tables 9** and **10**; **Figure 16**), suggests that the antifungal activity against the filamentous fungi and yeast strains can be also assigned to guieranone A. Moreover, the fraction with the weakest antifungal activity is *G. senegalensis* MF, where guieranone A is not detected, which allow to discard the phenolic compounds from this activity. However, the contribution of other compounds to the antifungal activity revealed by methanol *G. senegalensis* leaves extract cannot be ruled out, since the *G. senegalensis* EAF is rich in chlorophyll-related compounds, which have well-known antifungal properties (243,244).

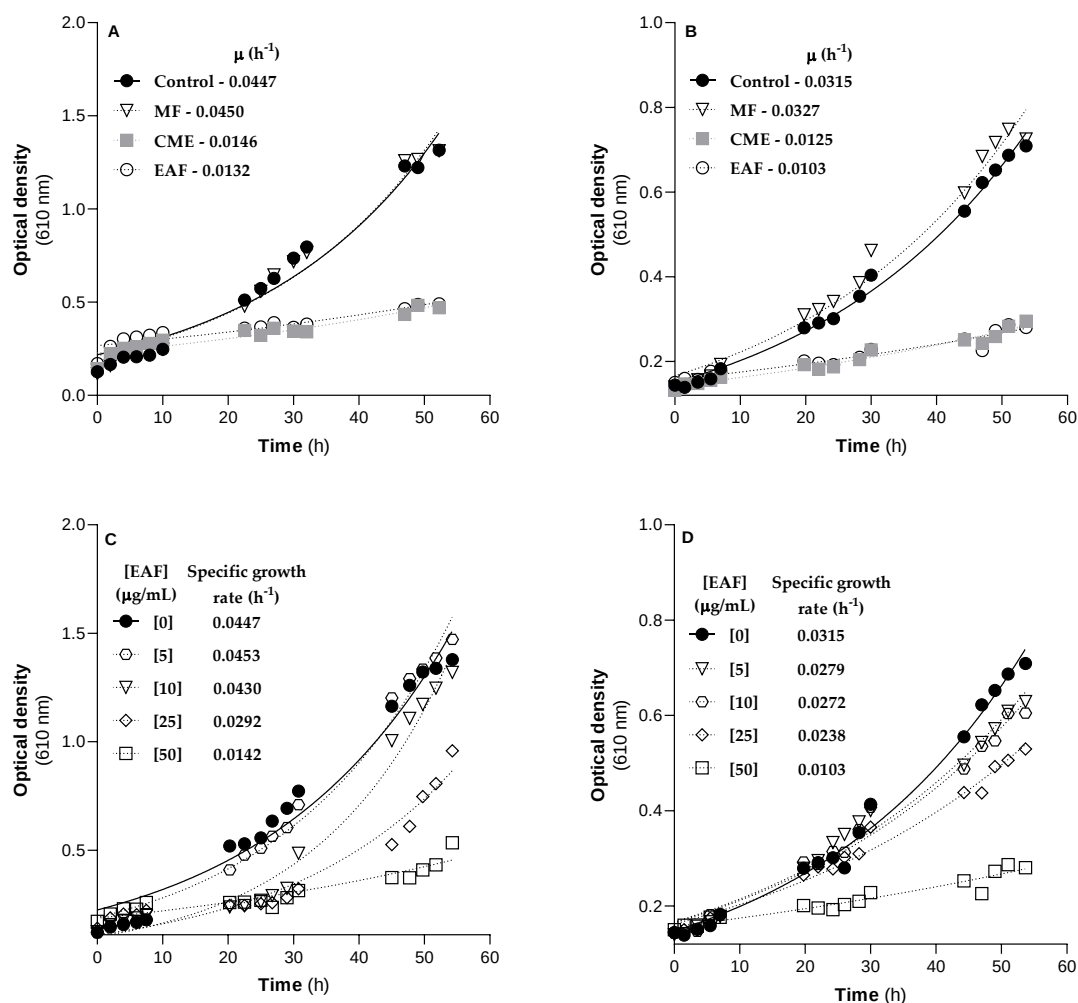


Figure 16. Effects of *G. senegalensis* leaves CME and of its MF and EAF on the growth of *S. cerevisiae* (A,C), and *C. neoformans* (B,D), at 30 °C, in the respective standard media. Time-dependent growth of *S. cerevisiae* (A) and *C. neoformans* (B) cultures in the absence and presence of 50 µg/mL of *G. senegalensis* leaves CME or in the presence of its fractions, MF and EAF, at 50 µg/mL. Effects of increasing concentrations of EAF on *S. cerevisiae* (C) and *C. neoformans* (D) growth. The cell number was measured by the optical density at 610 nm. The results shown are representative of three independent experiments using three started yeast cultures.

1.2.2.G. *senegalensis* EAF exhibits antifungal activity related with mitochondrial dysfunction

Many of the commercially profitable fungicides exhibit mechanisms of action that compromise the functionality of fungi mitochondria. In general, these compounds work as inhibitors of the mitochondrial redox chain complexes or as lipophilic compounds that affect the functional organization of the inner membrane, promoting disruption of $\Delta\Psi_m$ (245).

Thus, the mechanisms underlying the antifungal activity of *G. senegalensis* EAF were investigated, considering the effects on the functionality of the mitochondrial redox chain complexes, using *S. cerevisiae* as model organism. Yeast cultures of *S. cerevisiae* non-treated (control) and treated with 50 µg/mL of *G. senegalensis* EAF for 8 h were used to isolate mitochondria, which allowed to assess the activity of mitochondrial complexes I, II and IV. The effects of 50 µg/mL of EAF on the growth of yeast cultures (growth curves in **Figure 16**) suggest that an exposure over a period of 8 h should promote toxic effects without significant cell death, which would be detectable by a decrease of values of optical density. The results obtained with mitochondria isolated from non-treated and treated yeast cultures are displayed in **Figure 17A-D**. To discard the influence of putative differences in the purity degree of the mitochondria obtained from different yeast cultures, the activity of mitochondrial complexes was normalized by the CS activity. As shown in **Figure 17D**, mitochondria obtained from non-treated and treated yeast cultures exhibit similar activity of CS, expressed by nmol of substrate/min/mg of protein. Since CS is, many times, considered a rate limiting enzyme of citric acid cycle, thereby these data also suggest that *G. senegalensis* EAF does not impact on the ability of yeast mitochondria to process pyruvate into citric acid cycle. Data of **Figure 17A, B and C** show that yeast exposure to EAF significantly decreases the activity of mitochondrial respiratory complexes I and II without detectable effects on the activities of complex IV.

To evaluate if the decreased complexes I and II activities detected in mitochondria isolated from yeasts exposed to *G. senegalensis* EAF can emerge from inhibition of these mitochondrial complexes, direct effects on their activity were assessed in mitochondria isolated from yeast control cultures. The obtained results (**Figure 17E, F**) show that *G. senegalensis* EAF decreases the activities of both complexes I and II in a concentration-dependent manner. However, the minimal concentration of EAF required to promote a significant decrease of the complex I activity (25 µg/mL) is lower than that is required to inhibit complex II (50 µg/mL). Although these *in vitro* results are qualitatively in line with those obtained by *ex vivo* assays (using mitochondria isolated from yeast cultures exposed to 50 µg/mL of EAF - **Figure 17A, B**) their quantitative effects are divergent since the *ex vivo* assays show that complex II is more sensitive.

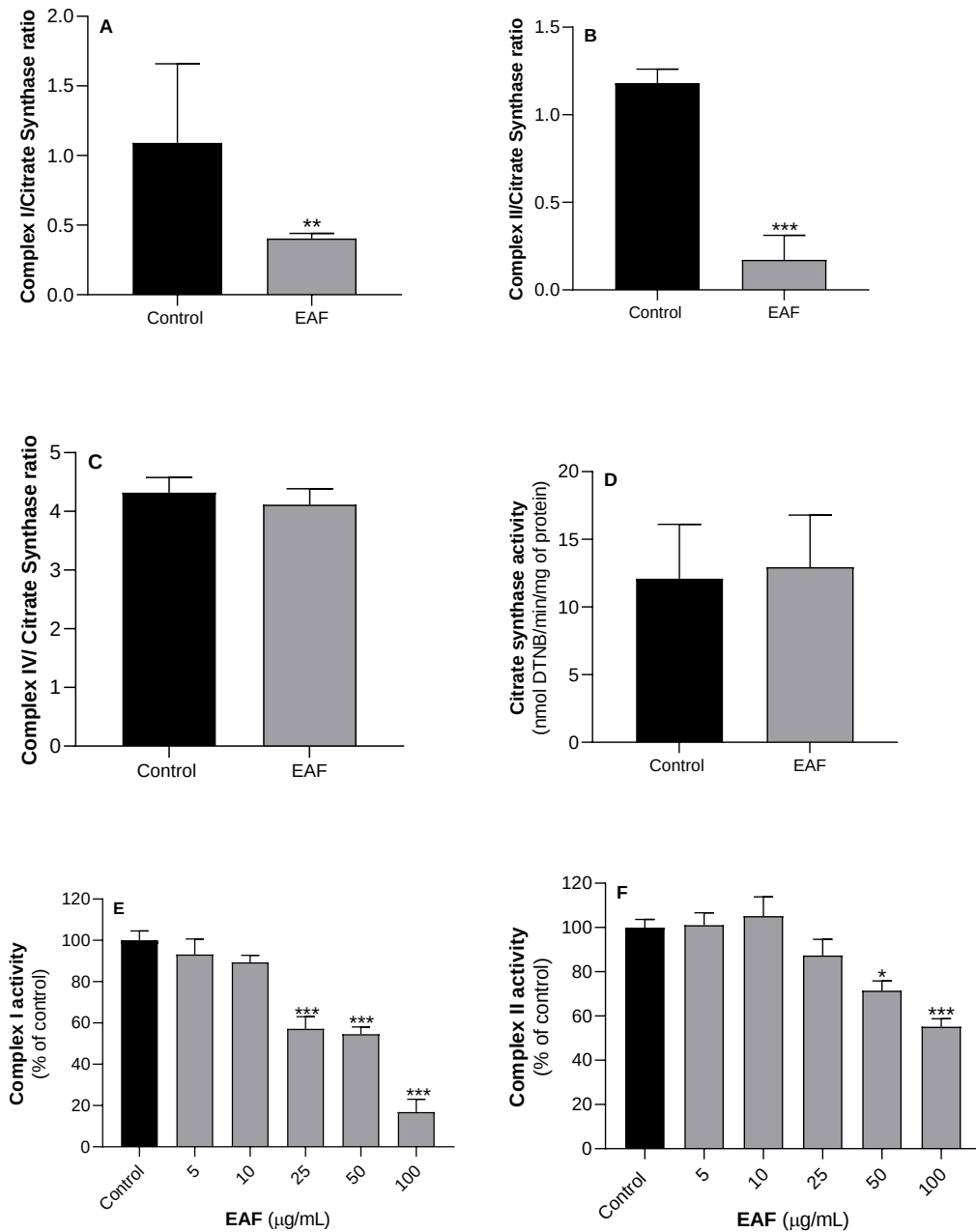


Figure 17. Effects of the exposure of *S. cerevisiae* cultures to 50 $\mu\text{g/mL}$ of *G. senegalensis* EAF, for 8 h, on the activity of the mitochondrial redox complexes I, II, and IV and citrate synthase (**A–D**). The activity of mitochondrial redox chain complexes was normalized by citrate synthase activity. *In vitro* effects of *G. senegalensis* EAF on the activity of complexes I and II of mitochondria isolated from *S. cerevisiae* cultures grown in control condition, i.e., in the absence of EAF (**E**, **F**). Results are presented as mean $\pm$ SD of three independent assays. *, **, *** Significantly different from control condition, with $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Black bars—control; gray bars—*G. senegalensis* EAF treatment.

Combining the results from **Figure 17** with those from **Figure 16**, it can be suggested that *G. senegalensis* EAF have compounds with capability to cross several biological barriers to reach the mitochondria, promoting specific inhibition at levels of complexes I and II. Considering *G. senegalensis* EAF chemical profile, it is expected that the lipophilic compounds that dominate this fraction, namely guieranone A and chlorophyll-related compounds, can be absorbed by yeasts and target the mitochondria via passive diffusion through the lipid membranes. It is well known that the mitochondrial redox chain is supplied by complex I NADH oxidation and by succinate processing at level of complex II activity (246). Therefore, with the inhibition of both complexes I and II, EAF may deteriorate the coupling efficiency between carbon flux (citrate cycle in mitochondrial matrix) and electron flux, through the respiratory chain in inner mitochondrial membrane, worsening mitochondrial functionality and the yeast ability to generate ATP by oxidative phosphorylation. Thus, the antifungal activity exhibited by *G. senegalensis* EAF can emerge, total or partially, by mitochondrial intoxication.

1.2.3. *G. senegalensis* EAF inhibits the activity of tyrosinase

Tyrosinase, a key enzyme in melanin biosynthesis, plays an important role in fungal pathways underlying the spores formation as well as in mechanism of defense against external factors, including UV radiation, toxic oxidant agents, dehydration and extreme temperatures (235). Thus, this enzyme is considered a valuable target in fungicide development programs (247). According to the *Fungicide Resistance Action Committee* (FRAC), the risk of drug resistance development of fungi pathogens with a single-site mechanism are considered significantly higher than to molecules or mixtures of molecules with multiple sites of action (248). Since resistance development has been reported for many antifungal agents that work as mitochondrial toxics (249,250), the EAF ability to inhibit tyrosinase enzyme was assessed in order to enlarge its potential as a multi-target fungicide.

Figure 18A shows the activity of mushroom tyrosinase in the absence and presence of increasing concentrations of EAF, using L-DOPA as substrate at low (close of enzyme K_m) and high concentrations.

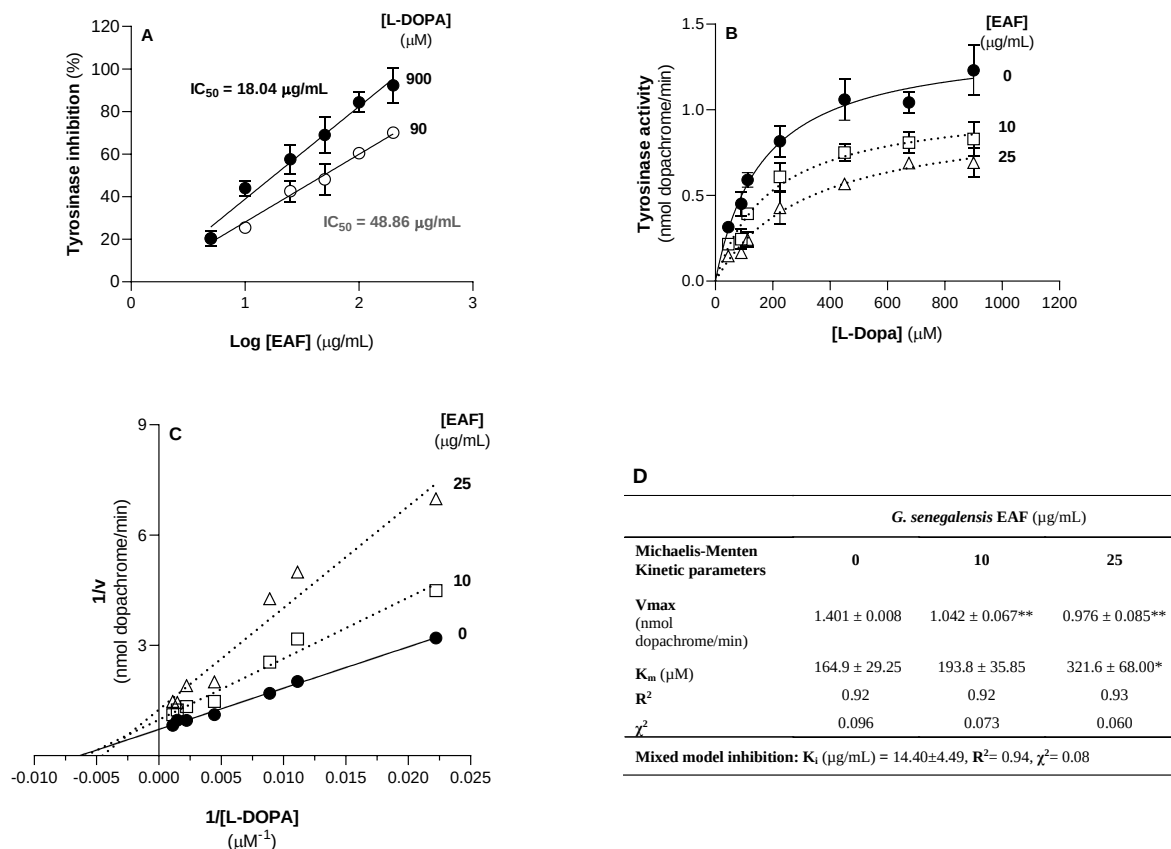


Figure 18. Effects of increasing concentrations of *G. senegalensis* EAF on the activity of pure mushroom tyrosinase assessed in the presence of low (90 μM – white circles) and high (900 μM – black circles) L-DOPA (enzyme substrate) concentration (**A**). Kinetics of tyrosinase activity in the absence (black circles) and presence of 10 (squares) or 25 (triangles) μg/mL of *G. senegalensis* EAF using seven concentrations of substrate (**B**). Data were fitted into a Michaelis-Menten kinetic equation and used to construct the double-reciprocal plot analysis (**C**). Table with the apparent Michaelis–Menten kinetic parameters and K_i values obtained with a noncompetitive inhibition model using GraphPad Prism software (**D**). *, ** Significantly different from control condition, with $p \leq 0.05$, $p \leq 0.01$, respectively.

At low substrate concentration (90 μM of L-DOPA), EAF decreases the activity of the enzyme in a concentration-dependent manner, with an IC_{50} of 48.86 ± 4.40 μg/mL. At high substrate concentration (900 μM – a concentration that saturate the enzyme), the IC_{50} decreases to 18.04 ± 2.83 μg/mL. Thus, *G. senegalensis* EAF is an effective inhibitor of mushroom tyrosinase. Although the IC_{50} value is a parameter largely used to screen enzyme inhibitors, it is highly dependent on the assay conditions, hindering the comparison

among laboratories. Thus, the assessment of IC_{50} values at low and high enzyme substrate concentrations aims to improve the potential of this parameter to identify and compare enzyme inhibitors. In fact, previous works have reported that extracts of *G. senegalensis* leaves obtained with water, ethanol-water mixtures, methanol, or acetone have competence to inhibit tyrosinase (251,252). This bioactivity was assigned to phenolic compounds since the extract obtained with acetone exhibits strong tyrosinase inhibition and greater richness in phenolic compounds (252). However, *G. senegalensis* acetone extract may have many other compounds in addition to phenolic compounds, weakening the reported relation. Comparison of the present IC_{50} values with those previously reported is also difficult since different enzyme concentrations were used and the previous IC_{50} values were evaluated at a single substrate concentration.

To investigate the kinetic model of enzyme inhibition and confirm the effective ability of *G. senegalensis* EAF to inhibit the enzyme, tyrosinase activity supported by seven substrate concentrations was assessed in the absence (control) and presence of EAF, at 10 and 25 $\mu\text{g/mL}$. These concentrations were selected, since they are in the range where *G. senegalensis* EAF exhibits mitochondrial toxicity and antifungal activity. As shown in **Figure 18B** and **C**, the enzyme activity as function of substrate concentration was analyzed by Michaelis-Menten kinetic model. In fact, the Michaelis-Menten kinetic equation fits reasonably to the experimental data, allowing to determine enzyme V_{max} and K_m , the apparent kinetic parameters (**Figure 18D**). Thus, a value of K_m of $164.9 \pm 29.25 \mu\text{M}$ was found for mushroom tyrosinase at 30 °C, which is in line with those previously reported (253). Under our assay conditions, an enzyme V_{max} value of $1.401 \pm 0.008 \text{ nmol dopachrome/min}$ was calculated. *G. senegalensis* EAF, at 10 or 25 $\mu\text{g/mL}$, significantly decreases the enzyme V_{max} ($p \leq 0.01$), and increases enzyme K_m , but for this kinetic parameter the differences only reach statistical significance for 25 $\mu\text{g/mL}$ of *G. senegalensis* EAF. A decrease in V_{max} and an increase of K_m suggests that the tyrosinase inhibition promoted by EAF can be described by a mixed model inhibition, with a predicted K_i value of $14.40 \pm 4.49 \mu\text{g/mL}$ by using the equation of GraphPad Prism software for this type of inhibition (**Figure 18D**). The K_i is close to the IC_{50} value obtained with a substrate concentration that saturates the active center of the enzyme (i.e., 900 μM), suggesting predominance of the non-competitive inhibition. Thus, the first tyrosinase inhibitory effect of *G. senegalensis* EAF can be associated to allosteric modulation of enzyme activity by compounds that do not compete with substrate by active site.

1.3. Insecticidal activity

1.3.1. *G. senegalensis* CME and its fractions, MF and EAF, exhibit toxicity for insect cells

To investigate the potential insecticidal activity of *G. senegalensis* leaves CME and of its fractions, a toxicological screening on a neuronal *D. melanogaster* cell line (ML-DmBG2-c2) was performed, as first approach.

Considering the scarce information on ML-DmBG2-c2 cells proliferation, the growth curves of cell cultures seeded with 3.5×10^4 or 7.5×10^4 cells/well were built by using MTT reduction assay data assessed at different time-points over 120 h of culture (**Figure 19**). Although cell cultures have not reached the stationary phase, the recorded exponential phase data allowed to calculate the cell culture doubling-time for both experimental conditions. Thus, cell doubling-time values of 41 and 50 h were found for cultures started with 3.5×10^4 and 7.5×10^4 cells/well, respectively. Thus, a toxicological screening of *G. senegalensis* CME and its fractions in ML-DmBG2-c2 cells was evaluated using cell cultures with 7.5×10^4 cells/well, considering their effects at 48 h of exposure.

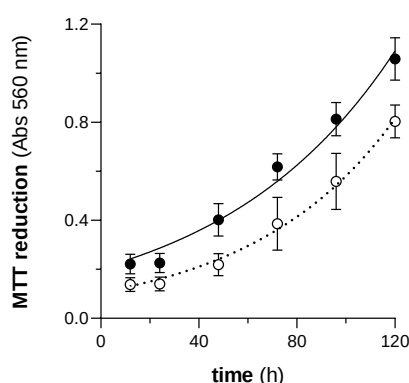


Figure 19. Proliferation curves of ML-DmBG2-c2 cells built with MTT reduction assay data obtained at different time-points, over 120 h, using cultures seeded in 96-well plates with 3.5×10^4 (white symbols) or with 7.5×10^4 (black symbols) cells/well.

The cytotoxic profile of *G. senegalensis* CME (0 – 250 $\mu\text{g/mL}$) on ML-DmBG2-c2 cells, assessed by the MTT reduction (cell viability) and by LDH release (membrane integrity) assays, is displayed in **Figure 20**. Data show the cell viability decreases with increasing CME concentration, with significant differences detected for concentrations equal to or greater than 5 $\mu\text{g/mL}$ and an estimated EC_{50} value of 47.9 $\mu\text{g/mL}$ (**Figure 20A**). The LDH leakage assay data indicate that this toxicity for insect cells does not involve cell death by necrosis since plasma membrane rupture is not detected (**Figure 20B**).

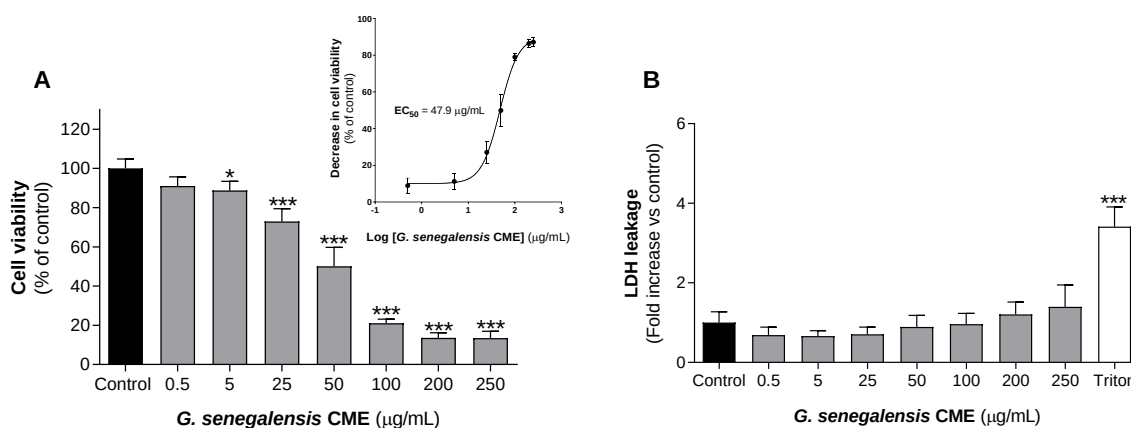


Figure 20. Effect of increasing concentrations of *G. senegalensis* leaves CME on the viability (A) and membrane integrity (B) of ML-DmBG2-c2, evaluated, respectively, by the MTT reduction and the LDH leakage assays, after a 48 h incubation period. The decrease in cell viability as function of extract concentration is shown in the insert A. Results are expressed as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of five independent assays, each one performed in triplicate. *, *** Significantly different from the control cells, with $p \leq 0.05$ and $p \leq 0.001$, respectively.

With the aim of establishing a relationship between chemical composition and bioactivity, increasing concentrations of *G. senegalensis* MF and EAF were also tested on ML-DmBG2-c2 cells (Figure 21). Data displayed in Figure 21 show that the insecticidal potential of *G. senegalensis* leaves is clearly enhanced by EAF, as revealed by higher toxicity of this fraction comparing to *G. senegalensis* CME. Although *G. senegalensis* MF displays lower toxicity against neuronal insect cells when compared to CME and EAF, the results of Figure 21A reveal activity for this fraction, allowing to suggest that not only the compounds enriched in EAF are responsible for the insecticidal activity.

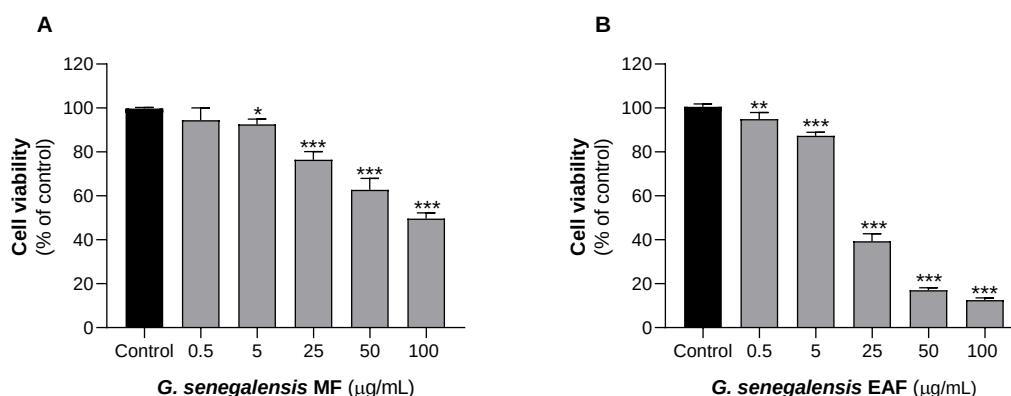


Figure 21. Effect of increasing concentrations of *G. senegalensis* MF (A) and EAF (B) on ML-DmBG2-c2 cells, evaluated by the MTT reduction, after a 48 h incubation period. Results are expressed as percentage of control (cells treated with vehicle) and presented as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of three independent assays, each one performed in triplicate. *, **, *** Significantly different from the control cells, with $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

1.3.2. *G. senegalensis* extract triggers toxicity for neuronal insect cells related with mitochondrial dysfunction

It is well-known that the overproduction of ROS can promote a decline of cell viability linked to cell death by apoptosis (254,255) and the results of LDH leakage assay indicate that the toxicity of *G. senegalensis* CME is not associated with cell death by necrosis. Thus, the effects of increasing CME concentrations on ROS production by insect ML-DmBG2-c2 cells were assessed. *G. senegalensis* CME was tested until 50 µg/mL, a concentration close to the EC₅₀ value. Results indicate that the cellular ROS generation is not affected at low concentrations of the *G. senegalensis* CME, but a significant enhancement of ROS production is detected in cells exposed at 50 µg/mL of extract (**Figure 22**), indicating pro-oxidant effects. The pro-oxidant effects of polyphenol-rich plant extracts on insect cells, in line with the reported here, are used to explain the anti-herbivore activity of the phenolic compounds. (256). However, it was revealed that an imbalance between the cellular ROS production and antioxidant defense system of insect cells can be induced by different classes of plant compounds, including Liebermann–Burchard reactive compounds and chlorophyll-related compounds (257,258). Thus, different compounds of *G. senegalensis* CME can contribute for the small enhancement of ROS generation.

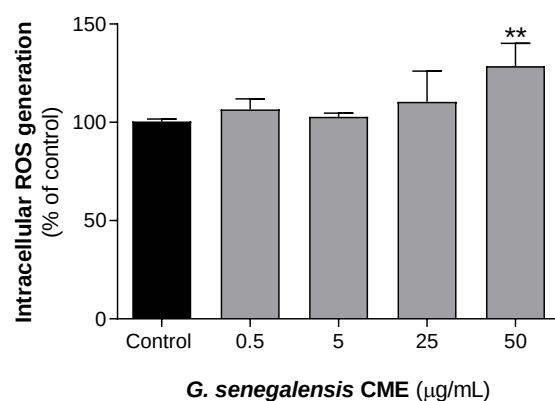


Figure 22. Effect of increasing concentrations of *G. senegalensis* leaves CME on the cellular ROS generation of ML-DmBG2-c2 assessed by using DCFH-DA probe. Results are expressed as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of four independent assays, each one performed in triplicate. ** Significantly different from the control cells, with $p \leq 0.01$.

The mechanisms underlying the toxicity of *G. senegalensis* CME for insect cells were investigated considering effects on mitochondrial functionality since the decreased cellular capacity for MTT reduction (**Figure 20A**) and the increased ROS generation (**Figure 22**) suggest mitochondrial dysfunction (259,260). Thus, ML-DmBG2-c2 cells were exposed to 25 µg/mL of *G. senegalensis* CME for 48 h and mitochondria was isolated. The impact on mitochondria functionality, assessed by effects on the activity of mitochondrial redox complexes (I, II and IV) normalized by CS activity, are displayed in **Figure 23**. The activity of CS was assessed to discard the influence of putative differences in the mitochondria content of the isolated mitochondria preparations (**Figure 23D**). Cell exposure to *G. senegalensis* CME significantly decreases the activity of complex II without effects on the activities of complexes I and IV.

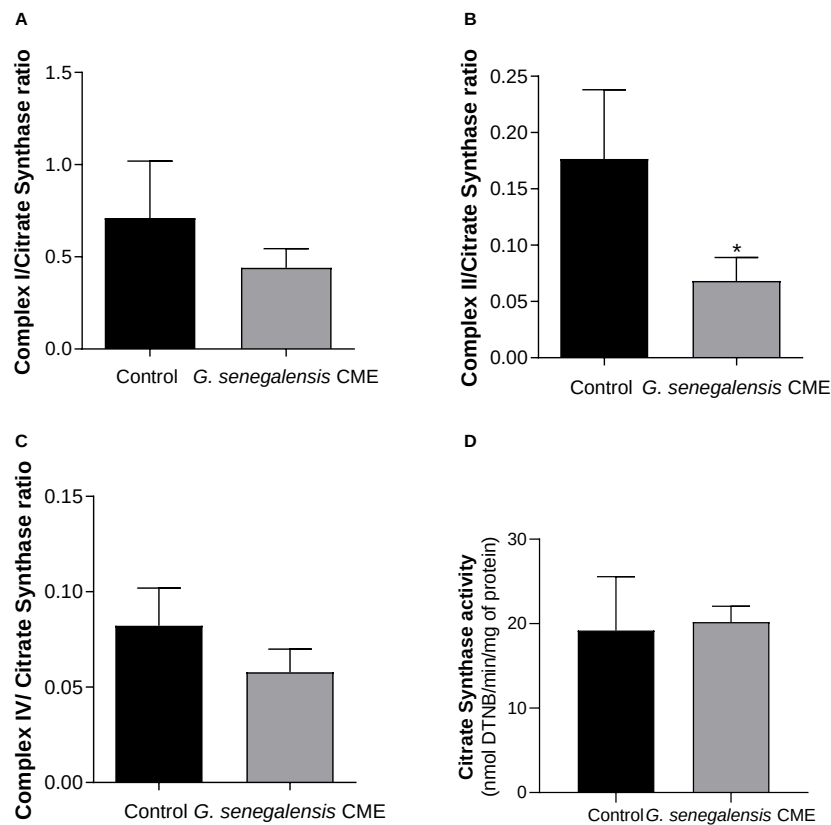


Figure 23. Effect of 25 µg/mL of *G. senegalensis* leaves CME on the activity of the mitochondrial complexes I (**A**), II (**B**), and IV (**C**). The activity of mitochondrial redox chain complexes was assessed in mitochondria-enriched fraction obtained from untreated or treated ML-DmBG2-c2 cells and normalized by citrate synthase activity (**D**). Results are presented as mean $\pm$ SD of four independent assays. * Significantly different from untreated cells, with $p \leq 0.05$.

The decreased complex II activity detected in mitochondria isolated from cells exposed to *G. senegalensis* CME can emerge from changes in protein expression or from direct effects on the protein complex activity (inhibition). To discriminate from these two hypotheses, mitochondria isolated from control cells were exposed for 5 min to increasing concentrations of extract and the complex II activity assessed. The obtained results (**Figure 24A**) show that the extract decreases the activity of complex II in a concentration-dependent manner, with an IC_{50} of 97.3 $\mu\text{g}/\text{mg}$ protein. In fact, these results are consistent with the decreased complex II activity detected in cells exposed to *G. senegalensis* CME (**Figure 23**), thereby putative changes in pattern of expression of the different polypeptides of the complex II were not investigated.

It is well known that the electron flow through the mitochondrial redox chain is supported by complex I NADH oxidation and by complex II activity (246). Therefore, the inhibition of complex II may deteriorate the coupling efficiency between carbon flux (Krebs cycle in mitochondrial matrix) and electron flux through the respiratory chain, decreasing the cell ability to generate ATP by oxidative phosphorylation, which is also dependent of the $\Delta\Psi_m$.

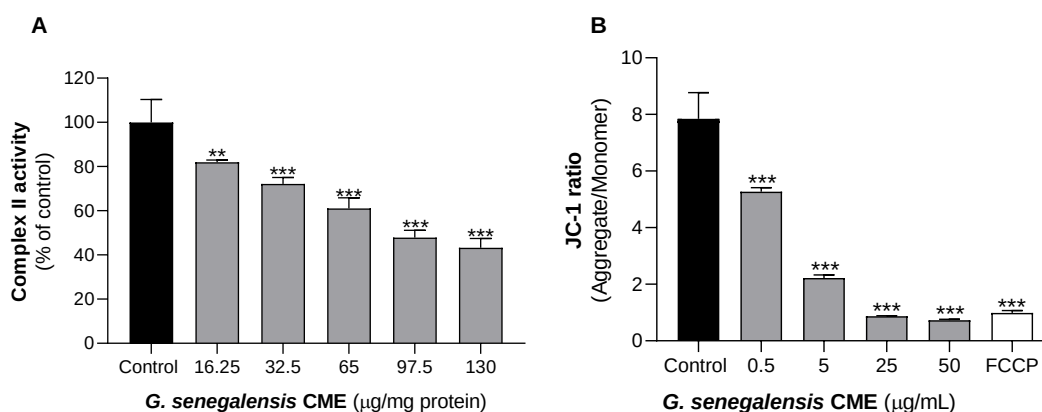


Figure 24. (A) Direct effects of *G. senegalensis* leaves CME on the activity of the complex II of mitochondria isolated from ML-DmBG2-c2 cells. (B) $\Delta\Psi_m$ of the ML-DmBG2-c2 cells exposed for 30 minutes to increasing concentrations of *G. senegalensis* leaves CME, expressed by ratio of red (aggregate) to green (monomer) fluorescence JC-1. FCCP (12 μM) were used as positive control. For both parameters, results are presented as mean $\pm$ SD of at least three independent experiments, each one performed in triplicate. **, *** Significantly different from the matched control cells, with $p \leq 0.01$ and $p \leq 0.001$, respectively.

In general, the mitochondrial toxicity is also reflected by changes in $\Delta\Psi_m$, thereby the effects of the *G. senegalensis* CME on $\Delta\Psi_m$ of insect cells were assessed after 30 min of exposure, by using JC-1 probe (**Figure 24B**). During assays, the metabolism of cells was

supported by 5 mM of glucose, and control cells exhibit a strong $\Delta\Psi_m$ with a JC-1 aggregate/monomer value close to 8. Additionally, the cell treatment with (carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone) FCCP (12 μ M), a well-known uncoupler of the mitochondrial oxidative phosphorylation, decay the JC-1 aggregate/monomer ratio to values close to 1, indicating a full mitochondrial membrane depolarization. *G. senegalensis* CME promotes a concentration-dependent decrease of $\Delta\Psi_m$ of insect cells, and a complete mitochondria depolarization is detected at 25 μ g/mL extract (**Figure 24B**). These data suggest that in addition to complex II inhibition, *G. senegalensis* CME also increases the mitochondrial inner-membrane permeability to protons, working as a uncoupler. Combining results of **Figures 22, 23, and 24**, it can be suggested that *G. senegalensis* CME has compounds with ability to cross the cell membrane and to reach the mitochondria, where they promote mitochondrial membrane depolarization and inhibition of complex II. Thus, *G. senegalensis* CME exhibits insecticidal activity working as mitochondrial toxin, that decreases insect cell's ability to generate ATP by oxidative phosphorylation and increases ROS generation. The consequent mitochondrial dysfunction and oxidative stress can activate the pathological cascades that trigger cell death by apoptosis (254,261,262). Considering the chemical composition data and the above-mentioned antifungal activity, several lipophilic compounds, including guieranone A and chlorophyll-related compounds, should be key players for the insecticidal potential of *G. senegalensis* CME.

1.3.3. The insecticidal potential of *G. senegalensis* CME is not related with the inhibition of acetylcholinesterase activity

AChE is an enzyme that regulates in insects the acetylcholine-dependent connectivity by hydrolyzing acetylcholine and hence terminating the impulse transmission of cholinergic synapses (263). Consequently, AChE has been used as a target in insecticide development throughout the years. Molecules capable of inhibiting this enzyme raise the levels of acetylcholine in the neuromuscular junction, leading to increased activation of acetylcholine receptors found on post-synaptic membranes resulting in enhanced muscle activation, contraction, and strength. Most insecticides that target AChE belong to the classes of organophosphates and carbamates and have been used for several decades (264). Thus, the capacity of *G. senegalensis* CME to inhibit AChE enzyme was also explored.

Figure 25 displays the activity of AChE in the absence and presence of increasing concentrations of *G. senegalensis* CME, using acetylthiocholine at low (0.625 mM) and high (20 mM) substrate concentrations. Data show that the CME does not inhibit this enzyme in the range of concentrations tested. Therefore, the insecticidal potential of *G. senegalensis*

CME cannot be correlated with AChE inhibition. It is considered an advantage since many pathogenic insects have developed resistance for AChE inhibitors (265–269).

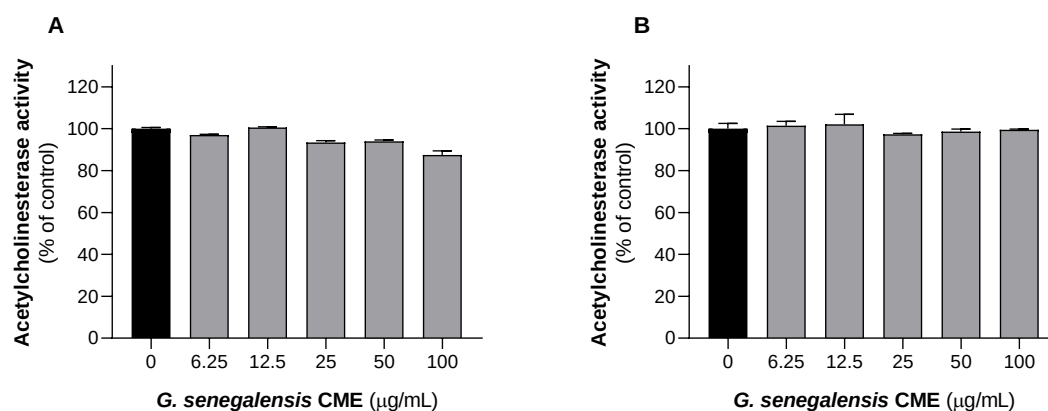


Figure 25. Effects of increasing concentrations of *G. senegalensis* CME on the activity of acetylcholinesterase assessed in the presence of low (**A** - 0.625 mM) and high (**B** - 20 mM) acetylthiocholine concentrations, respectively. Results are expressed as percentage of control and presented as mean $\pm$ SD of three independent assays, each one performed in triplicate.

1.4. Toxicological profile in human neuronal and skin cells

1.4.1. Effects on cell viability and membrane integrity

A toxicological screening of *G. senegalensis* CME and its fractions on neuronal and keratinocytes human cell models was performed to assess their safety profile to determine the selectivity degree for fungal and insect cells. The obtained results of *G. senegalensis* CME on human neuronal (SH-SY5Y) and keratinocytes (HaCaT) cells, by using MTT reduction and LDH release assays, are displayed in **Figure 26**.

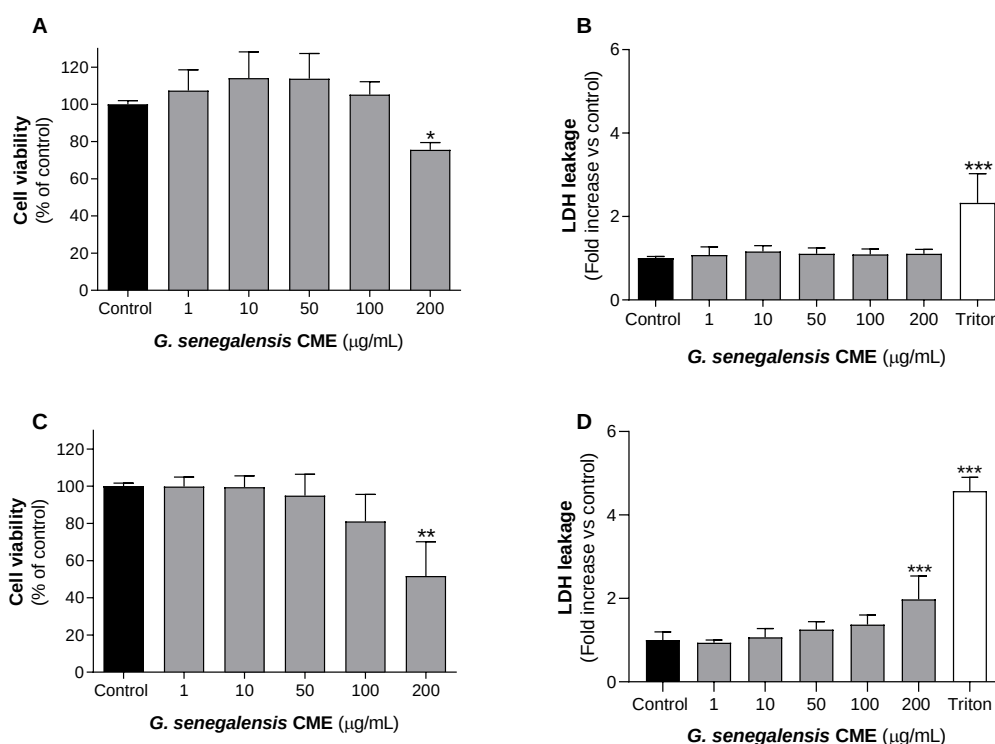


Figure 26. Effect of increasing concentrations of *G. senegalensis* leaves CME on the viability (**A**, **C**) and membrane integrity (**B**, **D**) of HaCaT (**A**, **B**) and SH-SY5Y (**C**, **D**), evaluated respectively by the MTT reduction and the LDH leakage assays, after a 48 h incubation period. Results are expressed as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of three independent assays, each one performed in triplicate. *, **, *** Significantly different from the control cells, with $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

G. senegalensis CME solely affects the cell viability of SH-SY5Y and of HaCaT cells at 200 µg/mL, the highest tested concentration. Furthermore, its cytotoxicity involves rupture of the plasma membrane for SH-SY5Y cells but not for HaCaT cells (**Figure 26B** and **D**). Thus, the mechanisms underlying the *G. senegalensis* CME toxicity for human cells are dependent of the human cell-type. Combining the overall results of **Figure 26** with those

previously described for ML-DmBG2-c2 cells (**Figure 20**), it can be asserted that *G. senegalensis* CME exhibits an interesting degree of selective toxicity for insect cells ($EC_{50} = 47.9 \mu\text{g/mL}$) in comparison with neuronal and keratinocytes human cells ($EC_{50} > 200 \mu\text{g/mL}$). The results obtained with fungal species (**Figure 16**) support the idea that *G. senegalensis* CME presents a degree of selectivity for pathogenic yeasts substantially higher than that detected for insect cells.

Figure 27 show the effects of increasing concentrations of *G. senegalensis* fractions on human cells, assessed by the MTT reduction assay. Data show that *G. senegalensis* MF do not affect the cell viability of both human cells until 100 $\mu\text{g/mL}$, the highest tested concentration. In opposition, *G. senegalensis* EAF promotes toxicity in both cell lines, exhibiting cytotoxicity for SH-SY5Y cells at concentrations equal to or higher than 50 $\mu\text{g/mL}$ and for HaCaT cells only at 100 $\mu\text{g/mL}$, corroborating the higher sensitivity of neuronal human cells for toxic agents. Despite this, the selectivity of *G. senegalensis* EAF for yeast strains, MIC value of 8 $\mu\text{g/mL}$ for the pathogenic *C. neoformans* yeast, remains evident.

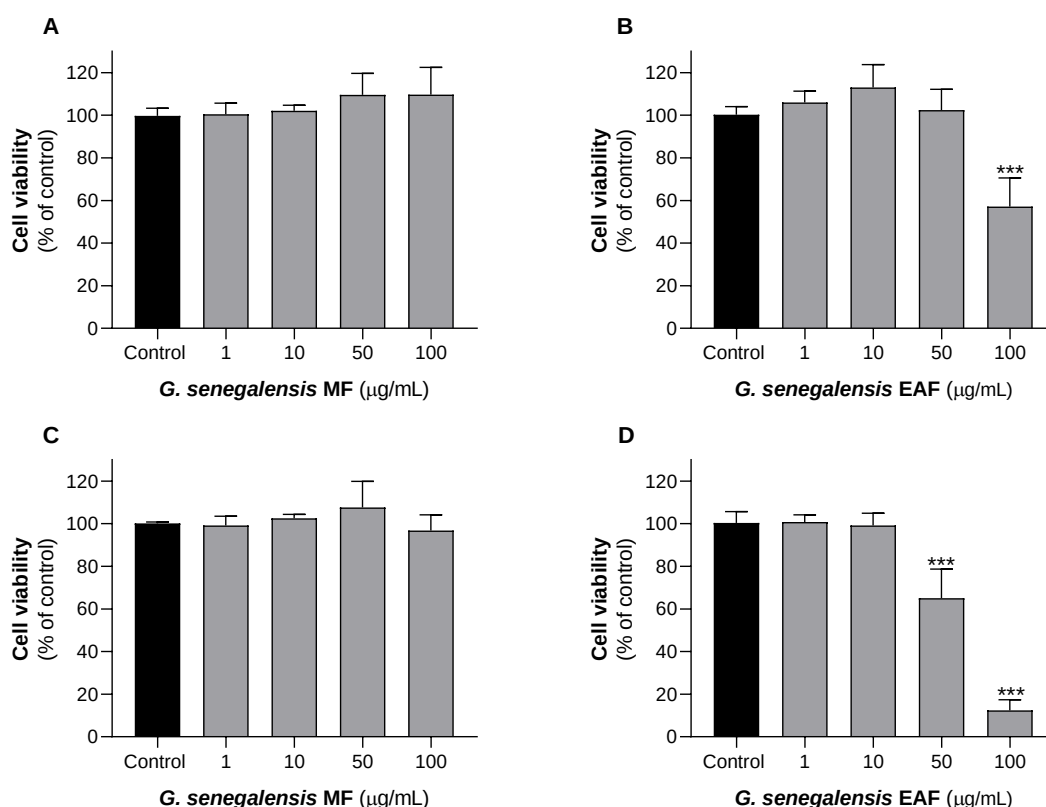


Figure 27. Effect of increasing concentrations of *G. senegalensis* MF (**A, C**) and EAF (**B, D**) on HaCaT (**A, B**) and SH-SY5Y (**C, D**) cells, evaluated by the MTT reduction, after a 48 h incubation period. Results are expressed as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of, at least, three independent assays, each one performed in triplicate. *** Significantly different from the control cells, with $p \leq 0.001$.

1.4.2. Positive impact of *G. senegalensis* CME on ROS generation by human cell lines and protection against the harmful effects of UV radiation

Figure 28 show the effects of increasing concentrations of *G. senegalensis* CME on the ROS generation of HaCaT and SH-SY5Y cells, normalized by cell density using MTT results as a measure. As shown in **Figure 28**, *G. senegalensis* CME promotes a concentration-dependent decrease in ROS generation in both human cell lines. It is important to highlight that this antioxidant activity in human cells runs in opposition to the effects on insect cells, where a pro-oxidant activity was detected. Antioxidant activity and competence to protect human cells against the toxicity induced by oxidant toxic agents were previously reported for extracts obtained from different parts of *G. senegalensis* (197,251,270). The positive impact on human cells ROS production was assigned to the total phenolic composition of *G. senegalensis* extracts and/or to the presence of flavonol 3-O-glycosylated derivatives (197,251,270).

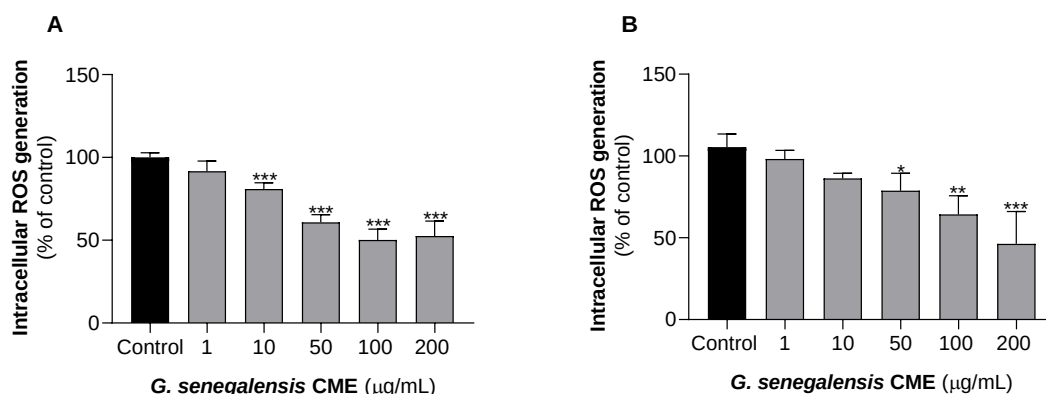


Figure 28. Effect of increasing concentrations of *G. senegalensis* leaves CME on the cellular ROS generation of HaCaT (A) and SH-SY5Y (B) cells assessed by using DCFH-DA probe. Results are expressed as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of four independent assays, each one performed in triplicate. *, **, *** Significantly different from the control cells, with $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

The photosafety profile of an extract is a prerequisite for their use in development of topical formulations with antifungal activity (271). Thus, the effects of cellular exposure to UVA radiation for 15 min on viability of HaCaT cells non-treated and treated with increasing concentrations of *G. senegalensis* CME were assessed. Under the assay conditions, the UVA radiation has minor effects on cellular viability of both untreated and treated HaCaT cells, as shown in **Figure 29A**.

To assess the effects of UVA radiation on cellular ROS generation and the putative protective effects of the extract, it was performed a new series of assays. Thus, cells were pre-incubated with 50 µg/mL of *G. senegalensis* CME for 0.5 h and then exposed to UVA radiation for 15 min. The results of **Figure 29B** show that keratinocytes exposure to UVA radiation trigger a significant increase of ROS generation, which is inverted by cell pre-treatment with *G. senegalensis* CME. In fact, cells pre-treatment with *G. senegalensis* CME promotes a significant decrease of ROS production, either in the presence or in the absence of UVA radiation (**Figure 29B**).

Furthermore, ROS generation by cells pre-treated with *G. senegalensis* CME and exposed to UVA radiation is even lower than the production of ROS by HaCaT cells under control condition (i.e. in the absence of both radiation and CME pre-treatment). Several works have demonstrated that flavonoids protect cells against UV radiation, decreasing the ROS generation (272–274). Thus, the positive outcomes on HaCaT cells under UV radiation might be related with the presence of flavonoids in the *G. senegalensis* CME.

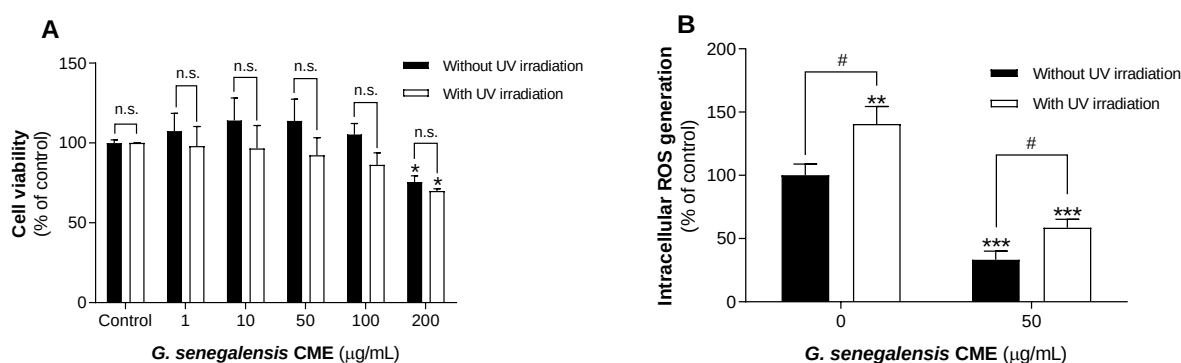


Figure 29. (A) Effects of the pre-incubation of HaCaT cells with *G. senegalensis* CME for 48 h on the harmful effects of exposure to UV radiation (365 nm) for 15 min, assessed by cell viability (MTT reduction assay). (B) Effects of the pre-treatment of 50 µg/mL of *G. senegalensis* CME for 0.5 h before the exposure to UV radiation (365 nm) for 15 min, assessed by intracellular ROS generation (DCFH-DA fluorescence assay). *, **, *** Significantly different from control (untreated cells) with $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. #, ## Significantly different from the cells exposed to UV radiation, with $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. n.s., without significant difference.

1.5. Main conclusions

- i) *G. senegalensis* leaves CME is a complex mixture of compounds containing sugars, phospholipids, Liebermann–Burchard reactive compounds, guieranone A, porphyrin-containing compounds, and a great diversity of phenolic compounds, including three flavonol 3-O-glycosylated derivatives identified for the first time in this plant species;
- ii) The solid-phase fractionation procedure used for separation of the compounds of *G. senegalensis* CME in different fractions allows to establish a relationship between chemical composition and bioactivity. The fractionation method enabled the removal of water-soluble compounds, and the separation of the remaining compounds based on their polarity degree into two distinct fractions. The first fraction, EAF, is enriched in lipophilic compounds including guieranone A and chlorophyll-related compounds. The second fraction, MF, is predominantly composed of phenolic compounds;
- iii) *G. senegalensis* CME exhibits a weak antifungal activity against pathogenic filamentous fungi strains, a bioactivity significantly enhanced by its EAF. These data indicate that the lipophilic compounds, mainly guieranone A and chlorophyll-related compounds, are responsible for the antifungal activity;
- iv) The EAF of the *G. senegalensis* CME demonstrates strong antifungal activity against pathogenic yeast strains, such as *C. neoformans* and *C. krusei*, with MIC values comparable to commercially available fungicide penconazole and antifungal fluconazole;
- v) The EAF inhibits yeast mitochondrial respiratory complexes I and II, as well as the tyrosinase enzyme, at concentrations in which it has *in vivo* effects. Thus, the antifungal activity of *G. senegalensis* EAF against yeasts is attributed to specific mitochondrial toxicity and disruption of tyrosinase-dependent biochemical pathways;
- vi) *In vitro* assays with *D. melanogaster* cells unveil that *G. senegalensis* CME exhibits toxicity towards neuronal insect cells, activating mechanisms that do not involve cell death through necrosis;
- vii) The insecticidal potential of *G. senegalensis* CME is improved by EAF, but *G. senegalensis* MF also revealed toxicity for insect cells. These data indicate that both *G. senegalensis* CME fractions, EAF and MF, contain compounds that confer insecticidal potential to *G. senegalensis* CME;

- viii) The toxicity of *G. senegalensis* CME on insect cells is linked to elevated ROS production and mitochondrial damage, characterized by a specific inhibition of mitochondrial respiratory complex II and depolarization of the mitochondrial inner membrane potential;
- ix) Compared to human neuronal and keratinocyte cells, *G. senegalensis* CME exhibits selective toxicity towards insect cells, while its EAF exhibits an improved degree of selectivity towards pathogenic yeasts;
- x) *G. senegalensis* CME has a positive impact on ROS generation by human cell lines, providing protection against UVA radiation for skin cells;
- xi) *G. senegalensis* leaves are a renewable resource to obtain mixtures of bioactive compounds with high potential to support the development of innovative antifungal and insecticidal formulations to address the resistance challenge.

2. *Ficus polita* leaves: from the chemical characterization to the antifungal and insecticide potential

2.1. Chemical characterization

2.1.1. Chemical composition of *F. polita* CME, MF and EAF

The methanol extractable compounds of *F. polita* leaves allows to obtain a CME with an extraction yield of $11.36 \pm 2.41\%$. This extract was submitted to a clean-up solid phase fractionation procedure on a Hypersep C18 non-end-capped column to discard high hydrophilic compounds with water and to collect the lipophilic components with ethyl acetate (fraction yield: $24.21 \pm 1.82\%$) and the remaining compounds with methanol (fraction yield: $12.98 \pm 5.59\%$) (**Table 11**). A chemical analysis, supported by conventional methodologies, was performed to assess the total levels of Folin-Ciocalteu reactive phenolic compounds, total sugars, phospholipids, Liebermann–Burchard reactive compounds (e.g., steroids and triterpenes), as well as chlorophyll-related compounds with subsequent determination of the EF for each type of compounds in the two fractions, as indicated in **Table 11**.

Table 11. Extraction yields of *F. polita* leaves with methanol (CME) and of its solid-phase fractionation obtained with ethyl acetate (EAF) and methanol (MF). There is also a general characterization in terms of total levels of Folin–Ciocalteu reactive phenolic compounds, sugars, phospholipids, Liebermann–Burchard reactive compounds, and chlorophylls with the EF indicated for each compound.

(g compound equivalents per Kg dry extract)	CME	EAF	EF	MF	EF
Extraction/fraction yield (%)	11.36 ± 2.41	24.21 ± 1.82	n.a.	12.98 ± 5.59	n.a.
Folin-Ciocalteu reactive phenolic compounds	12.12 ± 2.97	9.01 ± 2.90	0.74	15.78 ± 7.51	1.30
Total Sugars	54.84 ± 4.69	30.20 ± 3.02	0.55	47.91 ± 3.43	0.87
Phospholipids	29.01 ± 0.98	49.85 ± 6.08	1.72	62.90 ± 7.93	2.17
Liebermann–Burchard reactive compounds (steroids and/or triterpenes)	56.11 ± 17.37	178.41 ± 17.96	3.18	41.94 ± 10.36	0.75
Chlorophylls	12.43 ± 1.75	20.60 ± 2.56	1.66	3.29 ± 0.06	0.26

The concentration of Folin-Ciocalteu reactive phenolic compounds in *F. polita* CME is 12.12 ± 2.97 g/Kg dry extract, expressed as gallic acid equivalents. These compounds are mainly obtained in *F. polita* MF, as revealed by a concentration of 15.78 ± 7.51 g/Kg dry extract with an EF of 1.30. Despite this, a significant amount of Folin-Ciocalteu reactive phenolic compounds is eluted by ethyl acetate, since their concentration in *F. polita* EAF is 9.01 ± 2.90 g/Kg dry extract, EF of 0.74. Regarding total sugars content in *F. polita* CME, MF and EAF, obtained by reaction with phenol in the presence of sulfuric acid (229), *F. polita* CME exhibits a concentration of 54.84 ± 4.69 g/Kg dry extract, the EAF of 30.20 ± 3.02 g/Kg dry extract and MF of 47.91 ± 3.43 g/Kg dry extract. These data indicate that there is no sugar enrichment in the fractions obtained from *F. polita* CME, suggesting that the free sugars are removed by water in the clean-up procedure, while the sugars attached to organic compounds (e.g., phenolics, lipids) are obtained in EAF and MF (**Table 11**).

The total phospholipid content in *F. polita* CME, EAF and MF is, respectively, 29.01 ± 0.98 , 49.85 ± 6.08 and 62.90 ± 7.90 g/Kg dry extract, expressed in C16:0/18:1 PC equivalents. Thus, *F. polita* CME is a complex mixture rich in phospholipids, which are distributed by both EAF (49.85 ± 6.08 g/Kg dry extract, EF = 1.72) and MF (62.90 ± 7.93 g/Kg dry extract, EF = 2.17) fractions. Liebermann–Burchard reactive compounds in *F. polita* leaves CME (56.11 ± 17.37 g/Kg dry extract, expressed as stigmasterol equivalents) are mainly retained in EAF (178.41 ± 17.96 g/Kg dry extract, EF = 3.18) even though their concentration in MF (41.94 ± 10.36 g/Kg dry extract, EF = 0.75) is also relevant. Chlorophylls in *F. polita* CME (12.43 ± 1.75 g/Kg dry extract, expressed as chlorophyll a equivalents) were also detected in both fractions, mainly in EAF (20.60 ± 2.56 g/Kg dry extract EF = 1.66), as shown in **Table 11**. These data indicate that the compounds with less polar degree like chlorophylls and Liebermann–Burchard reactive compounds are mainly concentrated in *F. polita* EAF, while phospholipids and Folin-Ciocalteu reactive phenolic compounds are preferentially concentrated in MF.

2.1.2. Characterization of the phenolic profile of *F. polita* CME and its fractions, MF and EAF

HPLC-DAD-ESI/MSⁿ analysis of the CME obtained from the leaves of *F. polita* allowed to identify and quantify several phenolic compounds, as shown in UV chromatogram recorded at 335 nm (**Figure 30A**) with peak identifications present in **Table 12**. Combining the MS/MS analysis with UV-Vis absorption features, it was possible to identify seven cinnamoyl derivatives (**1-7**), three flavonoids (**8-10**) and a coumarin derivative (**11**) (**Figure 30** and **Table 12**).

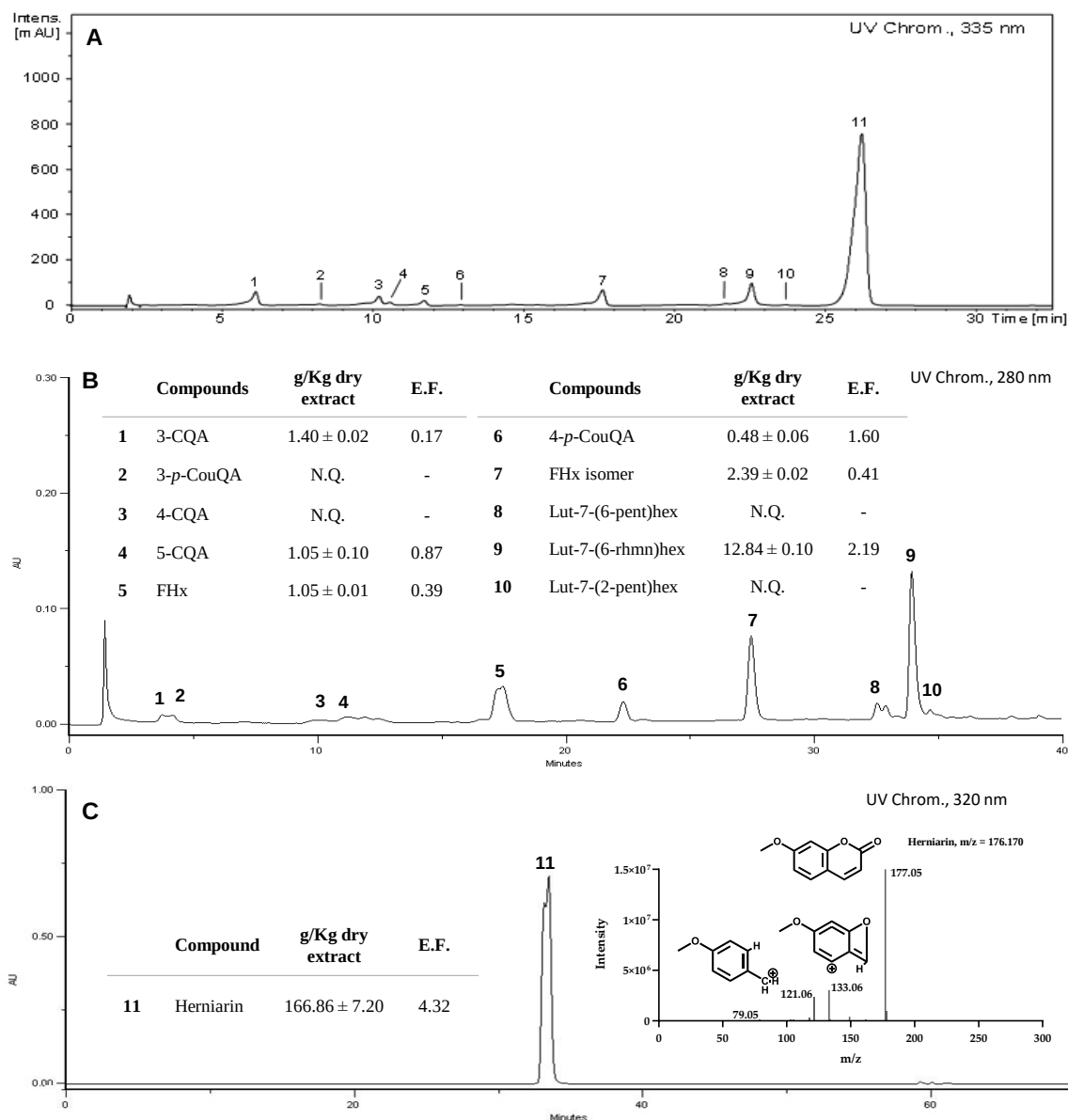


Figure 30. HPLC-UV profile of *F. polita* leaves CME (A), MF (B) and EAF (C). Compounds were analyzed by reverse phase HPLC-DAD-ESI-MSⁿ using a C18 column with spectra acquisition in continuous scan mode from 200 to 600 nm, and the chromatograms recorded at 335, 280 and 320 nm. Compounds identification as indicated in the insert of chromatogram B, in Table 12 and text. N.Q. Not quantified.

Table 12. Identification and quantification (dry weight basis) of each phenolic compound detected in the *F. polita* leaves CME by HPLC-DAD-ESI-MSⁿ: Rt, UV absorption features, molecular formula, MS features in negative or positive (for herniarin) ion modes with indication of main MS² detected fragments.

Compounds ²		Rt (min)	UV (nm)	[M-H] ⁻ , <i>m/z</i>	MS ² fragments, <i>m/z</i> (%)	g/Kg dry extract
cinnamoyl quinic acids						
1	3-CQA	6.1	296sh, 324	353	191(100), 179(35)	8.02 ± 0.20
2	3- <i>p</i> -CouQA	8.3	288sh, 310	337	163(100)	0.54 ± 0.04
3	4-CQA	10.2	295sh, 324	353	190(40), 179(80), 173(100)	5.04 ± 0.05
4	5-CQA	10.6	295sh, 323	353	191(100), 179(3), 173(5)	1.21 ± 0.09
6	4- <i>p</i> -CouQA	13.0	285sh, 312	337	173(100)	0.30 ± 0.08
					Σ	15.11 ± 0.14
feruloyl Hexosides						
5	FHx	11.7	290sh, 320	355	193(100), 149(20)	2.66 ± 0.01
7	FHx isomer	17.6	290sh, 322	355	193(100), 149(15)	5.90 ± 0.11
					Σ	8.56 ± 0.06
flavone derivatives						
8	Lut-7-(6-pent)hex	21.7	255, 266sh, 345	579	285(100)	0.18 ± 0.004
9	Lut-7-(6-rhnm)hex	22.6	255, 266sh, 345	593	285(100)	5.85 ± 0.12
10	Lut-7-(2-pent)hex	23.7	255, 266sh, 345	579	447(50), 429(3), 285(100)	0.40 ± 0.01
					Σ	6.43 ± 0.07

Table 12. Identification and quantification (dry weight basis) of each phenolic compound detected in the *F. polita* leaves CME by HPLC-DAD-ESI-MSⁿ: Rt, UV absorption features, molecular formula, MS features in negative or positive (for herniarin) ion modes with indication of main MS² detected fragments (continued).

Compounds ²		Rt (min)	UV (nm)	[M-H] ⁻ , <i>m/z</i>	MS ² fragments, <i>m/z</i> (%)	g/Kg dry extract
coumarin derivatives						
				[M+H] ⁺ , <i>m/z</i>		
11	Herniarin	26.2	294sh, 324	177	149.06(2), 133.06 (15), 121.06(10), 79.05 (1)	38.61 ± 0.42
Σ						68.71 ± 0.30

Concerning cinnamoyl derivatives, compounds **1**, **3** and **4** present the same deprotonated molecular ion at *m/z* 353 amu (characteristic of caffeoylquinic acid) and can be differentiated by their MS fragmentation patterns and relative abundance of the resulting ions (**Table 12**). According to Clifford and colleagues (275), they can be labelled as 3-O-caffeoylquinic acid (**1**), 4-O-caffeoylquinic acid (**3**) and 5-O-caffeoylquinic acid (**4**), and they match with the standards. Considering UV spectra, deprotonated molecular ions, and MS fragmentation pattern of compounds **2** and **6**, they are isomers, identified as 3- and 4-*p*-coumaroylquinic acid, respectively (**Table 12**). Compounds **5** and **7** are also isomers that exhibit a deprotonated molecular ion at *m/z* 355 amu and UV spectra identical to compounds **2** and **6**. In their MS fragmentations, the loss of the fragment of 162 amu (radicalhexosyl) producing a base peak at *m/z* 193 amu [ferulic acid-H]⁻ is detected. The ion at *m/z* 149 amu resulting from the decarboxylation of ferulic acid [ferulic acid-H-44]⁻ is also present. Therefore, these compounds can be identified as two ferulic acid hexoside isomers.

Compounds **8-10** (**8** and **10** in trace amounts) have UV-Vis spectral features of flavonoids with two substituents in the ring B (255, 266 sh, 345 nm). Their deprotonated molecular ions ([M-H]⁻) at *m/z* 579, 593 and 579 amu, respectively, are characteristic of flavonoid diglycosides. Furthermore, MS fragmentations reveal a base peak at *m/z* 285 amu

that corresponds to the deprotonated molecular ion of luteolin (5,7,3',4'-tetrahydroxyflavone) (**Table 12**). Concerning compounds **8** and **9**, the absence of ions resulting from the fragmentation of the interglycosidic linkage suggests that compound **8** is luteolin-7-O-pentosyl(1→6)hexoside, and the compound **9** is compatible with luteolin-7-O-rhamnosyl(1→6) hexoside, probably luteolin-7-O-rutinoside. Compound **10** was labelled as luteolin-7-O-pentosyl (1→2)hexoside, considering its fragmentation pattern with ions at m/z 447 ($[(MH) - 132]^-$) and 429 ($[(MH) - 150]^-$), indicating an interglycosidic linkage different from 1→6, probably 1→2. At the end of the chromatogram, it is detected compound **11** that matches with the standard herniarin (7-methoxycoumarin). It has UV-Vis spectrum with absorption bands with maximum at 294 nm and 324 nm, and a protonated molecular ion at m/z 177 amu ($[M+H]^+$) and MS² fragmentation pattern displayed in **Figure 30C**.

The quantification of eleven phenolic compounds detected in *F. polita* leaves CME, performed by HPLC-DAD analysis, is indicated in **Table 12**. Suitable external standards were used to obtain the calibration curves and determine the limits of detection and quantification (**Table 4**). *F. polita* CME is dominated by the coumarin herniarin (compound **11**), denoting ca. 56% of the total phenolic content. Additionally, it is also rich in cinnamoyl derivatives (compounds **1-7**) representing ca. 34% of the total phenolic content. The smallest part refers to flavonoids, specifically luteolin derivatives (compounds **8-10**), which account only for ca. 9% of the total polyphenols. It is also important to highlight that the main phenolic compound in *F. polita* CME, herniarin, has a structure that does not allow it to react with the FCR.

Ten phenolic compounds identified and quantified in *F. polita* CME were also detected in MF, seven cinnamoyl derivatives (**1, 2, 3, 4, 5, 6, 7**) and three flavonoids (**8, 9, 10**), as shown in chromatogram recorded at 280 nm displayed in **Figure 30B**. Regarding their quantification in *F. polita* MF, compounds **2, 3, 8** and **10** were not quantified since they are detected in trace amounts. Cinnamoyl derivatives **1, 4, 5**, and **7** exhibit concentrations lower than in CME, while the cinnamoyl derivative, compound **6**, and the flavonoid, compound **9**, are concentrated in this fraction. In fact, luteolin-7-(6-rhm)hex (**9**) with a concentration of 12.84 ± 0.10 g/Kg dry extract, is the major compound, representing ca. 67% of the total phenolics in *F. polita* MF (**Figure 30B**, insert).

From eleven phenolic compounds identified and quantified in *F. polita* CME, only herniarin (**11**) was detected in EAF, as shown in the UV chromatogram recorded at 320 nm (**Figure 30C**). Concerning its quantification, herniarin (**11**) exhibits a concentration of 166.86 ± 7.20 g/Kg dry extract, indicating that *F. polita* leaves, in general, and that EAF, in particular, are excellent sources to obtain this bioactive coumarin. Despite the higher concentrations

of coumarin, the absence of both cinnamoyl derivatives and flavonoids can explain the lowest levels Folin-Ciocalteu reactive phenolic compounds in *F. polita* EAF.

Previous studies report the occurrence of terpenes, cerebrosides, phytosterols and *E*-3,5,4'-trihydroxy-stilbene-3,5-*O*- β -*D*-diglucopyranoside in *F. polita* collected in Nigeria and Camaroon (201,204,205). However, it is here reported, for the first time, a detailed composition of phenolics in *F. polita* leaves. It is important to refer that cinnamoyl derivatives, specially caffeic acid isomers (e.g., 3-, 4- and 5-*O*-caffeoylquinic acids) were considered phenolic fingerprints of the genus *Ficus* (276–279). On the other hand, luteolin derivatives and herniarin were also reported in several species of the genus *Ficus* (280–283).

2.1.3. Characterization of the chlorophyll profile of *F. polita* EAF

Compounds with higher lipophilicity were analyzed by HPLC-DAD-ESI/MSⁿ with a C30 YMC column, using *tert*-butyl methyl ether and methanol as mobile phase. A typical UV-Vis chromatogram recorded at 420 nm is shown in **Figure 31**. Combining UV-Vis spectrum features with MS data, six compounds were identified: chlorophyll *b* (1), chlorophyll *a* (3) and four compounds related to chlorophyll *a* (2, 4, 5, 6), as indicated in **Table 13**.

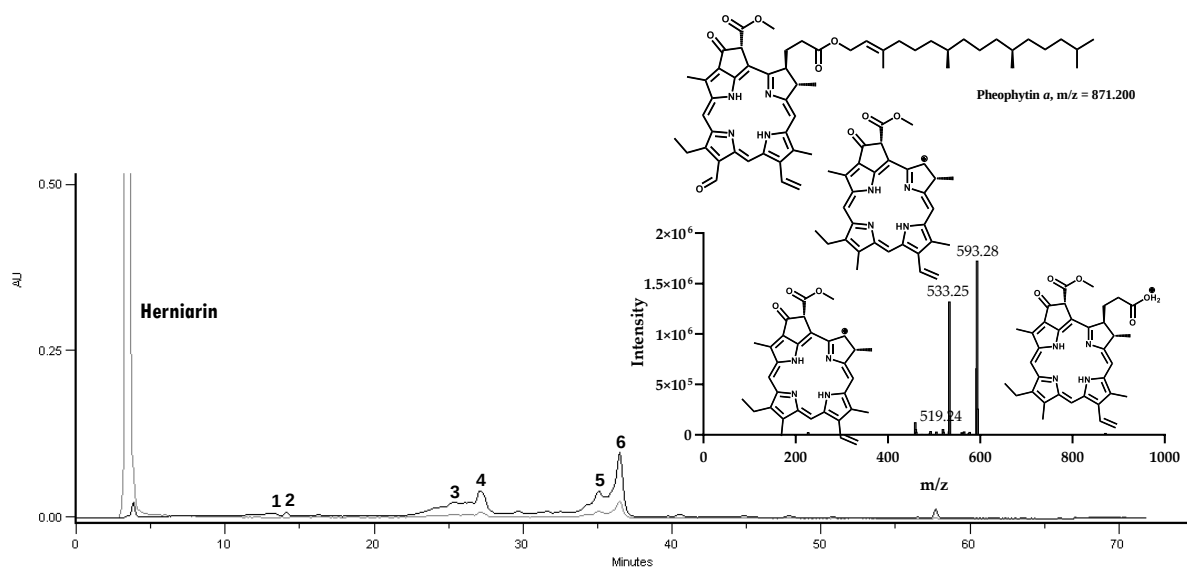


Figure 31. HPLC-UV profile of *F. polita* leaves EAF. Compounds were analyzed by reverse phase HPLC-DAD-ESI-MSⁿ using a C30 column with spectra acquisition in continuous scan mode from 200 to 600 nm, and the chromatograms recorded at 320 and 420 nm. Compounds identification as indicated in **Table 13** and text.

Table 13. Identification and quantification (dry weight basis) of each chlorophyll detected in *F. polita* leaves EAF by HPLC-ESI-MSⁿ: Rt, UV absorption features, molecular formula, MS features in positive ion mode with indication of main MS² detected fragments.

	Compounds	Rt (min)	UV (nm)	Formula (M)	[M+H] ⁺ (m/z)	MS ² [M+H] ⁺ , m/z (%)	g/Kg dry extract
1	Chl <i>b</i>	14.25	444, 664	C ₅₅ H ₇₀ MgN ₄ O ₆	907.55	553.1992(30), 227.1603(100)	0.18 ± 0.08
2	HO-Chl <i>a</i>	16.03	409, 665	C ₅₅ H ₇₃ MgN ₄ O ₆	909.53	555.25(100), 227.16(15)	0.12 ± 0.07
3	Chl <i>a</i>	25.60	407, 665	C ₅₅ H ₇₄ N ₄ O ₉	893.70	539.2418(100) 227.1598(30)	6.41 ± 1.1
4	HO-Lact- pheophytin <i>a</i>	27.40	406, 667	C ₅₆ H ₇₄ N ₄ O ₉	903.65	625.2648(20) 607.2546(20) 565.2444 (80)	3.23 ± 1.51
5	HO-Pheophytin <i>a</i>	35.82	407, 508, 665	C ₅₅ H ₇₅ N ₄ O ₆	887.57	609.27	6.29 ± 2.11
6	Pheophytin <i>a</i>	36.64	373, 405, 666	C ₅₅ H ₇₄ N ₄ O ₅	871.57	593.28(100), 533.25(80), 459.22(10)	5.06 ± 1.28
Σ							21.29 ± 1.79

Compounds **1** and **3** present [M+H]⁺ ions at *m/z* 907.55 and 893.70 and a MS fragmentation pattern with a loss of the phytyl group (-278 amu) and a propionic acid (-74 amu), detected by the ions at *m/z* 553.1992 (C₃₂H₂₅MgN₄O₄) and 539.2418 (C₃₂H₂₇MgN₄O₃), respectively. Thus, the compounds were identified as chlorophyll *b* (**1**) and as chlorophyll *a* (**3**). Compound **6**, with [M+H]⁺ ion at 871.57, was identified as pheophytin *a*, a chlorophyll *a* derivative resulting from the replacement of the central magnesium by two protons with the MS fragmentation pattern displayed in the insert of the **Figure 31**. The other chlorophyll *a*-related compounds arise from oxidation processes in *F. polita* leaves. Compounds **2** and **5**, with [M+H]⁺ at *m/z* 909.53 and 887.65, are, respectively HO-chlorophyll *a* and HO-pheophytin *a*, resulting from the substitution of the hydrogen atom at C13² by a hydroxyl group. Further oxidation implies the rearrangement of the isocyclic ring into a lactone group, leading to the 15¹-hydroxy-lactone derivatives. Thus, compound **4**, with [M+H]⁺ at *m/z* 903.65 and a MS fragmentation with ions at *m/z* 625.2648, 607.2546 and 565.2444, was identified as HO-lactone-pheophytin *a*. Thus, chlorophyll *a* (**3**) and its derivatives without magnesium (**5** and **6**) are the most representative of the chlorophyll-related compounds in *F. polita* CME (**Table 13**).

2.1.4. Antioxidant activity of CME, MF and EAF

The antioxidant activity of *F. polita* CME, MF and EAF was characterized by assessing their capacity to scavenge $O_2^{\cdot-}$ and $\cdot NO$ as well as to inhibit the activity of xanthine oxidase. Xanthine oxidase plays a key role on catabolism of purine nucleic acids, catalyzing the oxidation of hypoxanthine to xanthine, and of xanthine to uric acid with production of $O_2^{\cdot-}$, being considered the main cytosolic source of this free radical (284).

The scavenging activity of increasing concentrations of *F. polita* CME, MF or EAF against $\cdot NO$, generated by photolytic decomposition of SNP, and $O_2^{\cdot-}$, generated by NADH/phenazine methosulfate system (NADH/PMS), are displayed in **Figure 32A**, and **B**, respectively. Regarding $\cdot NO$, *F. polita* CME and MF exhibit similar activity, almost reaching 50% of scavenging at 200 $\mu g/mL$, while EAF only reaches ca. 25% at this concentration. The scavenging activity against $O_2^{\cdot-}$ is also very limited for *F. polita* CME and its fractions (MF and EAF), but the two fractions have similar activity, and higher than CME. Considering effects on xanthine oxidase enzyme, *F. polita* CME, MF and EAF present ability to inhibit the enzyme, with IC_{50} being ca. 383, 453 and 276 $\mu g/mL$, respectively, as shown in **Figure 32C**.

Thus, *F. polita* CME and its fractions scavenge free radicals with physiological relevance and inhibit xanthine oxidase enzyme, bioactivities usually assigned to phenolic composition of plant extracts (285). Combining **Figure 32** results with the chemical composition data, it can be asserted that the scavenging activity against $O_2^{\cdot-}$ and $\cdot NO$ and the inhibition of xanthine oxidase cannot be explained by the levels of phenolic compounds in *F. polita* CME and in its fractions.

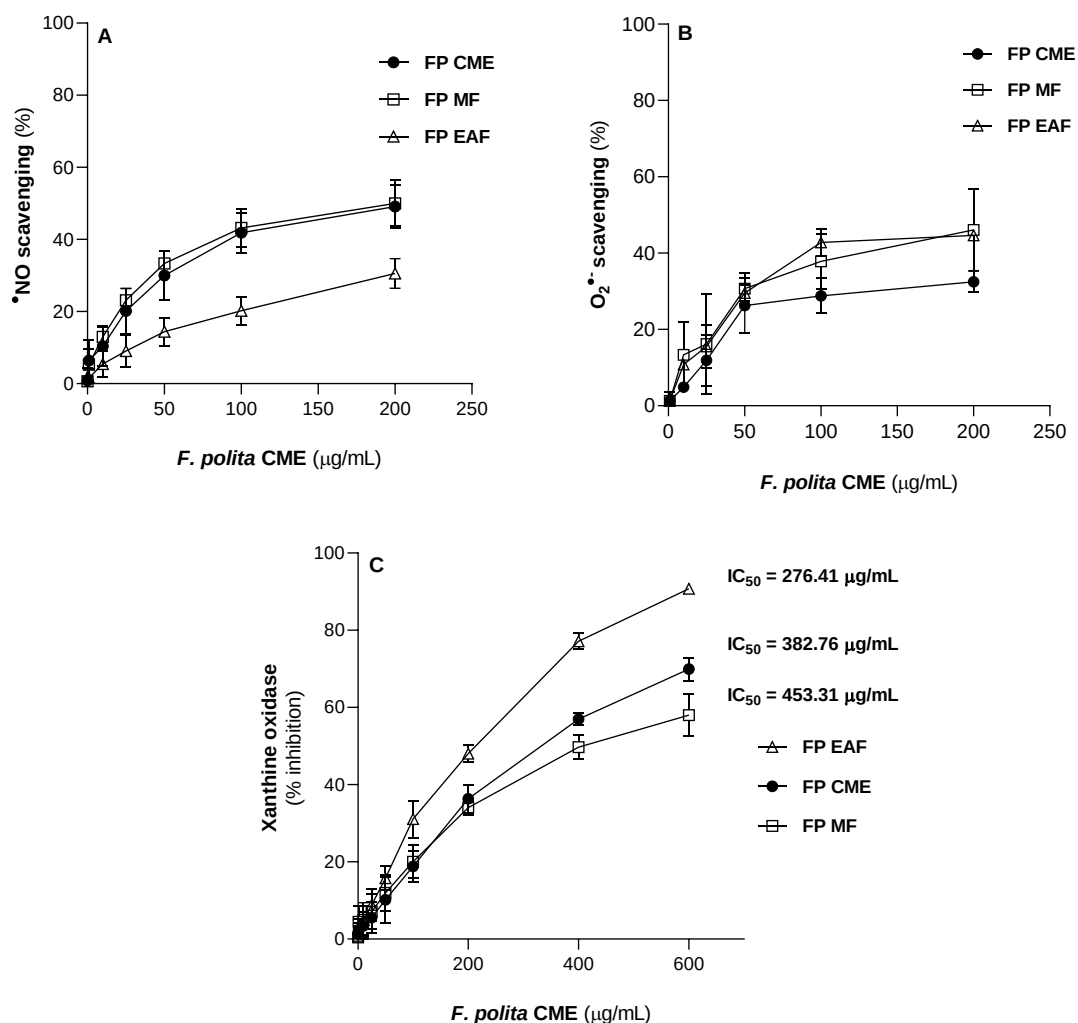


Figure 32. Effect of increasing concentrations of *F. polita* leaves CME and of its fractions, MF and EAF, on the scavenging of the •NO (A) and of O₂•⁻ (B) and on the activity of xanthine oxidase (C) assessed in the presence of xanthine (enzyme substrate). Results are expressed as percentage of control and presented as mean ± SD of, at least, three independent assays, each one performed in triplicate.

2.2. Antifungal activity

2.2.1. Fungal susceptibility to *F. polita* CME, MF and EAF

F. polita CME and its fractions, MF and EAF, were screened against filamentous fungi species, *F. oxysporum*, *A. fumigatus* and *C. gloeosporioides*, assessing the MIC and MFC. Results are shown in **Table 14**. Although CME and MF did not reveal antifungal activity up to a concentration of 2000 μg/mL, *F. polita* EAF inhibits the growth of the three pathogenic

filamentous fungi. This fraction exhibits the highest activity against *A. fumigatus*, with a MIC of 1000 µg/mL.

Table 14. Antifungal activity of the *F. polita* CME and its fractions, EAF and MF, against three species of filamentous fungi, assessed by MIC and MFC.

Filamentous fungi	MIC ¹ (µg/mL)			MFC (µg/mL)
	CME	EAF	MF	EAF
<i>Aspergillus fumigatus</i>	> 2000	1000	> 2000	> 1000
<i>Fusarium oxysporum</i>	> 2000	1500	> 2000	> 1500
<i>Colletotrichum gloeosporioides</i>	> 2000	1500	> 2000	≥ 1500

¹Concentration that inhibited at least 90% of growth

The antifungal activity was extended to three pathogenic yeasts (*C. neoformans*, *C. albicans* and *C. krusei*) and to a non-pathogenic strain (*S. cerevisiae*), used as model organism for mechanistic assays. **Table 15** displays the obtained results, indicating the MIC and MFC values of *F. polita* EAF and for its major compound (herniarin), as well as for fluconazole - a reference antifungal used in clinical practice.

Table 15. Antifungal activity of *F. polita* EAF, herniarin and fluconazole against four yeast strains, assessed by MIC and MFC.

Yeast	MIC ¹ (µg/mL)			MFC (µg/mL)		
	EAF	Herniarin	Fluconazole	EAF	Herniarin	Fluconazole
<i>Saccharomyces cerevisiae</i>	1000	1000	8	>1500	>1000	16
<i>Candida albicans</i>	1500	1000	2	>1500	>1000	>128
<i>Candida krusei</i>	1500	1000	32	>1500	>1000	>128
<i>Cryptococcus neoformans</i>	1000	1000	8	≥1500	>1000	>32

¹Concentration that inhibited at least 90% of growth

At 1000 µg/mL, *F. polita* EAF presents a fungistatic activity against *S. cerevisiae* and *C. neoformans*, inhibiting their fungal growth in 90%. However, to cause the same effect in the two tested *Candida* strains, it is necessary to increase the concentration to 1500 µg/mL. Herniarin was tested to establish a relationship between bioactivity and chemical composition. Results show that herniarin presents an equal effect against the four pathogenic fungi strains tested, with MIC values of 1000 µg/mL. However, compared to *F. polita* EAF, herniarin displays better activity against *C. albicans* and *C. krusei*, since EAF presents MIC values of 1500 µg/mL. Thus, the antifungal activity of *F. polita* EAF may be related to the presence of its major compound, but it does not depend solely on its presence.

Cultures of *S. cerevisiae* and *C. neoformans* were grown in SDB liquid medium supplemented with 100 µg/mL of *F. polita* CME or its fractions aiming to clarify the relationship between the antifungal activity and chemical composition (**Figure 33A, B**). The time-dependent growth of yeast strains, at 30 °C, was measured by changes in optical density at 610 nm. Data are displayed in **Figure 33**. In the control condition, the growth of *S. cerevisiae* and *C. neoformans* can be described by an exponential function with specific growth rates of 0.2335 and 0.0266 h⁻¹, respectively. *F. polita* CME and its fractions have minor effects on the specific growth rate of *S. cerevisiae*, but they promote a decrease of specific growth rate of *C. neoformans*, which is dependent of the chemical composition. Thus, inhibitory effects on the growth of *C. neoformans* are detected with decreases in the specific growth rate of 18%, 26% and 36%, respectively for CME, MF and EAF.

Figure 33C, D show the effects of increasing concentrations of *F. polita* EAF (0-200 µg/mL), the most active fraction, on the growth of *S. cerevisiae* (**Figure 33C**) or of *C. neoformans* (**Figure 33D**). Results show a decrease in the specific growth rate with the increase of the concentration of *F. polita* EAF, which allows to state that its inhibitory effects towards both yeast strains are concentration dependent.

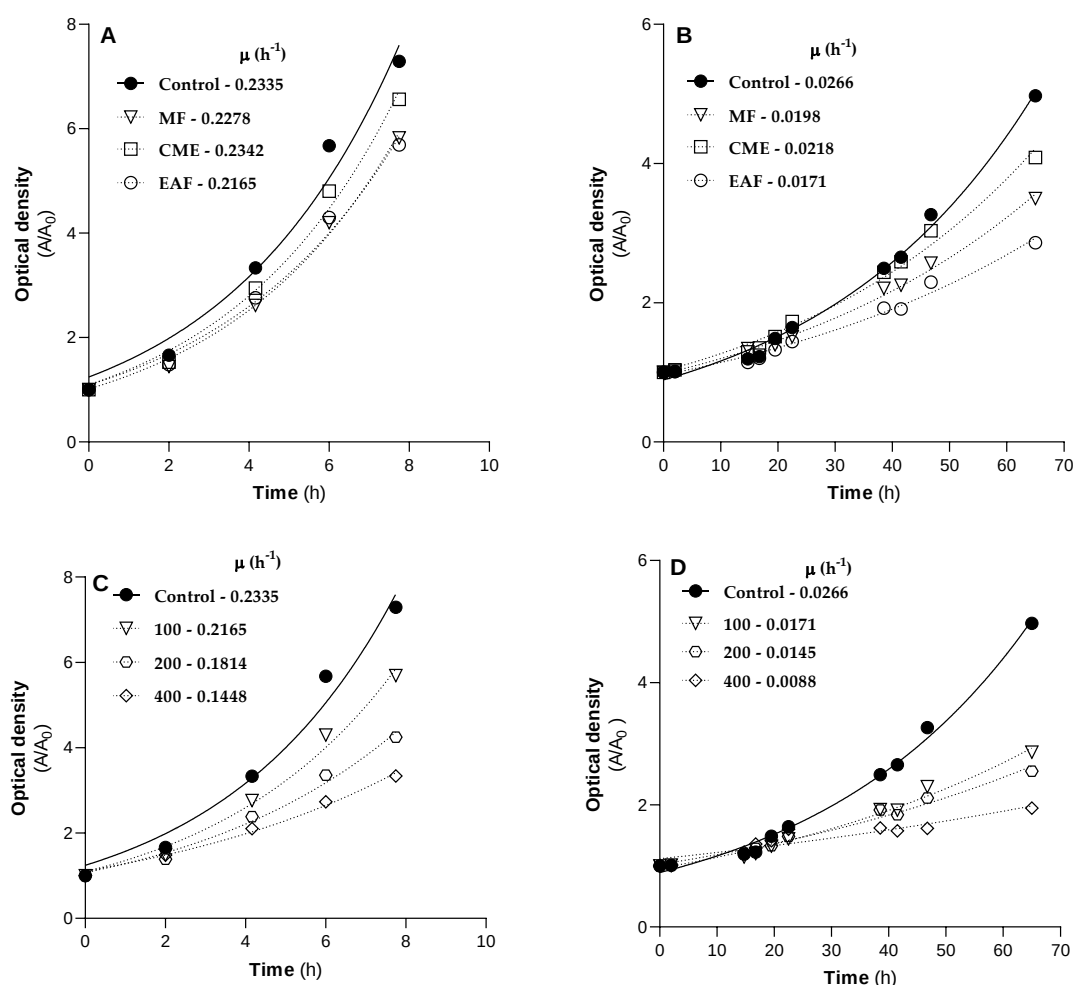


Figure 33. Time-dependent growth of *S. cerevisiae* (A) and *C. neoformans* (B) cultures in SAB medium, at 30 °C, in the absence and presence of 100 $\mu\text{g/mL}$ of *F. polita* leaves CME, MF or EAF. Effects of increasing concentrations of EAF (0 - 400 $\mu\text{g/mL}$) on *S. cerevisiae* (C) and *C. neoformans* (D) growth. The cell number was measured by the optical density at 610 nm. The results shown are representative of three independent experiments using three started yeast cultures.

Given that herniarin is not detected in *F. polita* MF and this fraction exhibits an antifungal activity against *C. neoformans* higher than CME and lower than EAF, the antifungal activity of *F. polita* leaves CME may result from several compounds with different polarity degrees. Additionally, EAF enhances the antifungal activity of *F. polita* CME, emphasizing the benefits of the fractionation procedure. Thus, in addition to herniarin other classes of compounds, such as phospholipids (enriched in both MF and EAF) and steroids/triterpenes (enriched in EAF), should contribute to the detected antifungal activity. However, additive, and synergistic effects, as well as potentiation and antagonism cannot be ruled out in such a complex matrix.

The antifungal activity of the extracts from *F. polita* leaves is reported here for the first time. However, antimicrobial activity against bacteria was previously reported for extracts from *F. polita* leaves (200), and preceding works have also reported that methanol, ethanol:water and ethanol extracts obtained from the *F. polita* roots exhibited antifungal activity against *C. albicans* (201,202,207). The described anti-*Candida* activity was attributed to the presence of terpene compounds without any indication about the mechanism of action (201). In the present work, *F. polita* EAF exhibits an improved antifungal activity and the highest content in Liebermann–Burchard reactive compounds, which includes steroids and triterpenes.

2.2.2. *F. polita* EAF exhibits antifungal activity related with mitochondrial dysfunction

The effects of increasing concentrations of *F. polita* EAF on mitochondria-dependent oxygen consumption by *S. cerevisiae* were assessed after 5 min exposed, by using a Clark type electrode. Data in **Figure 34A, B** show that *F. polita* EAF significantly decreases the yeast mitochondrial respiration for concentrations equal to or higher than 150 µg/mL. The yeast mitochondria metabolism is supported by glucose and/or glycerol in culture medium, and the use of cyanide (as well as antimycin A) ensures that the recorded effects are related to mitochondrial respiration, as shown in **Figure 34B**. Data of **Figure 34A** also show that the effects of *F. polita* EAF on yeast mitochondria occurs at concentrations below the MIC values and the absence of mitochondrial effects of the herniarin at 100 µg/mL, discarded this compound from the yeast mitochondrial toxicity exhibited by *F. polita* EAF. Thus, these *in vivo* assays indicate that *F. polita* EAF works as mitochondrial toxic for yeasts.

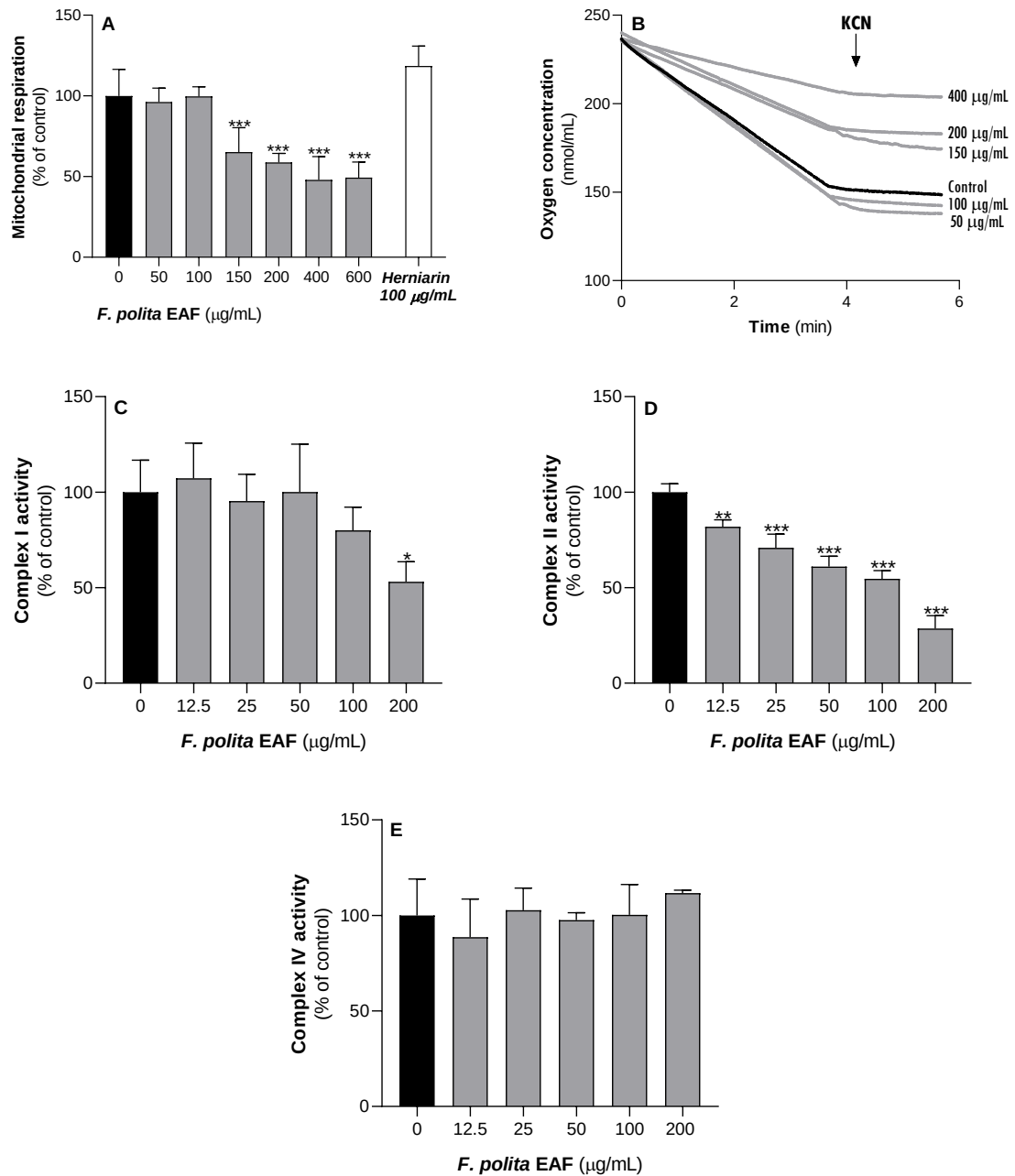


Figure 34. Effect of increasing concentrations of *F. polita* EAF and herniarin (100 μg/mL) on the mitochondrial respiration of *S. cerevisiae* yeasts, at 30 °C, in the respective media (A). Typical assay of the oxygen consumption promoted by 2.2 million yeasts exposed to *F. polita* EAF recorded by a Clark-type electrode in 1 mL of culture medium (B). *In vitro* effects of *F. polita* EAF on the activity of complexes I, II and IV of mitochondria isolated from *S. cerevisiae* cultures grown in control condition, i.e., in the absence of EAF (C, D, E). Results are expressed as percentage of control (cells treated with vehicle) and presented as mean ± SD of three independent assays. *, **, *** Significantly different from control condition, with $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

To characterize *F. polita* EAF mitochondrial toxicity, mitochondria were isolated from yeasts grown under control conditions, and used to assess direct effects on the activity of complexes I, II and IV. The obtained results are displayed in **Figure 34**. As shown in **Figure 34D**, *F. polita* EAF is a strong inhibitor of complex II, with IC_{50} of ca. 85 $\mu\text{g/mL}$. Additionally, *F. polita* EAF promotes significant complex I inhibition at the concentration of 200 $\mu\text{g/mL}$, without detectable effects at the level of complex IV. Therefore, *F. polita* EAF mitochondrial toxicity may emerge from specific inhibition of complex II. By targeting complex II, *F. polita* EAF affects not only electron flux on the mitochondrial redox chain but also the carbon flux in citric acid cycle, both with negative impact on the yeast's capacity to generate ATP by oxidative phosphorylation (286).

2.2.3. Antifungal activity of *F. polita* is not related to tyrosinase inhibition

The effects of *F. polita* CME were also assessed on the activity of tyrosinase, a key enzyme in melanin biosynthesis that plays a crucial role in several fungal pathways. **Figure 35** shows the activity of mushroom tyrosinase in the absence or presence of increasing concentrations of *F. polita* CME, using L-DOPA at 90 μM , as enzyme substrate.

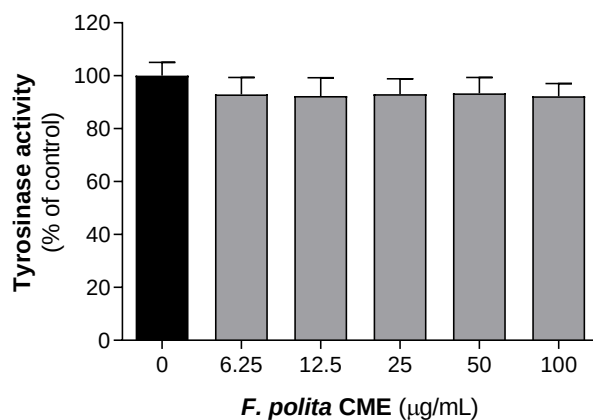


Figure 35. Effect of increasing concentrations of *F. polita* CME on the activity of pure mushroom tyrosinase assessed in the presence of 90 μM of L-DOPA (enzyme substrate). Results are expressed as percentage of control and presented as mean $\pm$ SD of three independent assays, each one performed in triplicate.

Under the assay conditions, *F. polita* CME does not inhibit the pure mushroom tyrosinase, indicating that the antifungal activity is not associated with this key enzyme. However, inhibition of tyrosinase by *Ficus carica* L. leaves extracts was previously reported, a bioactivity attributed to the presence of higher levels of phenolic compounds (287). Thus, the low levels of polyphenols in *F. polita* CME can explain the absence of tyrosinase inhibition.

2.3. Insecticidal activity

2.3.1. Effects of *F. polita* CME and its fractions, MF and EAF, on ML-DmBG2-c2

The insecticidal potential of *F. polita* CME and its fractions was characterized considering effects on neuronal *D. melanogaster* neuronal (ML-DmBG2-c2) cells. Thus, ML-DmBG2-c2 cells were exposed to increasing concentrations (0 - 100 µg/mL) of *F. polita* CME, EAF or MF for 48 h before assessing cell viability effects through the MTT reduction assay. As shown in **Figure 36**, *F. polita* CME only affects the cell viability of ML-DmBG2-c2 cells for concentrations equal to or greater than 100 µg/mL. However, a significant enhancement of the insecticidal potential of *F. polita* leaves is detected for its EAF, as evidenced by the results displayed in **Figure 36C**. On the other hand, *F. polita* MF exhibits lower toxicity against neuronal insect cells than CME, with toxicity detected only at the highest tested concentration (**Figure 36B**). These findings suggest that the toxicity for insect cells is mainly associated to the compounds enriched in the EAF.

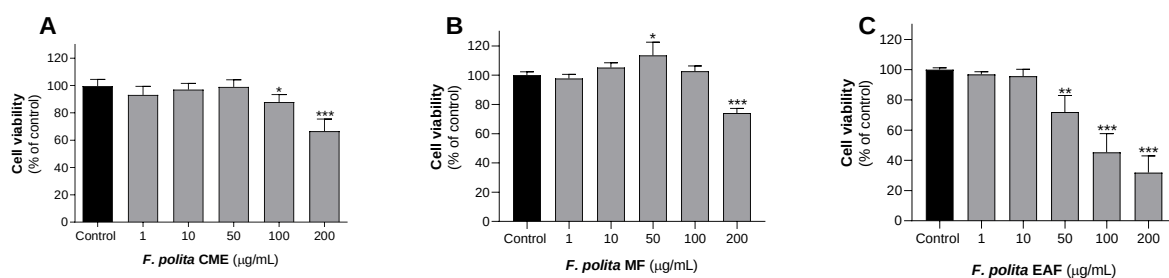


Figure 36. Effect of increasing concentrations of *F. polita* leaves CME and its fractions, MF and EAF, on the viability of ML-DmBG2-c2 (**A**, **B**, **C**), evaluated by the MTT reduction, after a 48 h incubation period. Results are expressed as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of three independent assays, each one performed in triplicate. *, **, *** Significantly different from the control cells, with $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

The screening on neuronal insect cell does not allow to foresee insecticide potential for *F. polita* CME and its fractions. Furthermore, *F. polita* CME does not inhibit AChE enzyme until concentration of 100 µg/mL, as shown in **Figure 37**. Thus, the putative mechanisms of cytotoxicity were not addressed by further assays.

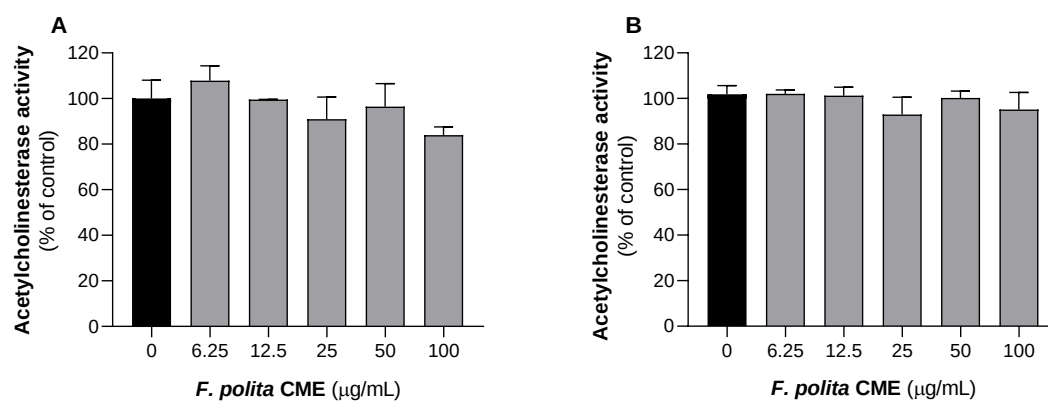


Figure 37. Effects of increasing concentrations of *F. polita* CME on the activity of AChE assessed in the presence of 0.625 (A) and 20 mM (B) acetylthiocholine, respectively. Results are expressed as percentage of control and presented as mean $\pm$ SD of three independent assays, each one performed in triplicate.

2.4. Toxicological profile on human neuronal and skin cells

The antifungal potential of an extract depends on its ability to exhibit antifungal activity within a range of concentrations without toxicity to non-target species, including humans. Therefore, the cytotoxicity of *F. polita* CME and its fractions was also evaluated on human keratinocytes (HaCaT) and neuronal cells (SH-SY5Y). HaCaT and SH-SY5Y cells were exposed to increasing concentrations of *F. polita* CME, EAF or MF for 48 h, and the effects on cell viability were assessed by MTT reduction assay (**Figure 38**). In the range of tested concentrations (up to 200 µg/mL), CME and MF do not affect the HaCaT cell viability, suggesting no cytotoxicity for skin cells. However, *F. polita* EAF affects the viability of these human skin cells for a concentration equal to 200 µg/mL. Data of **Figure 38** also show that neuronal cells are more sensitive, since at 200 µg/mL both MF and EAF significantly decrease the cell viability of SH-SY5Y cells. In fact, toxicity to human cells of *F. polita* CME and its fractions occurs in the same range of concentrations as that detected for insect cells.

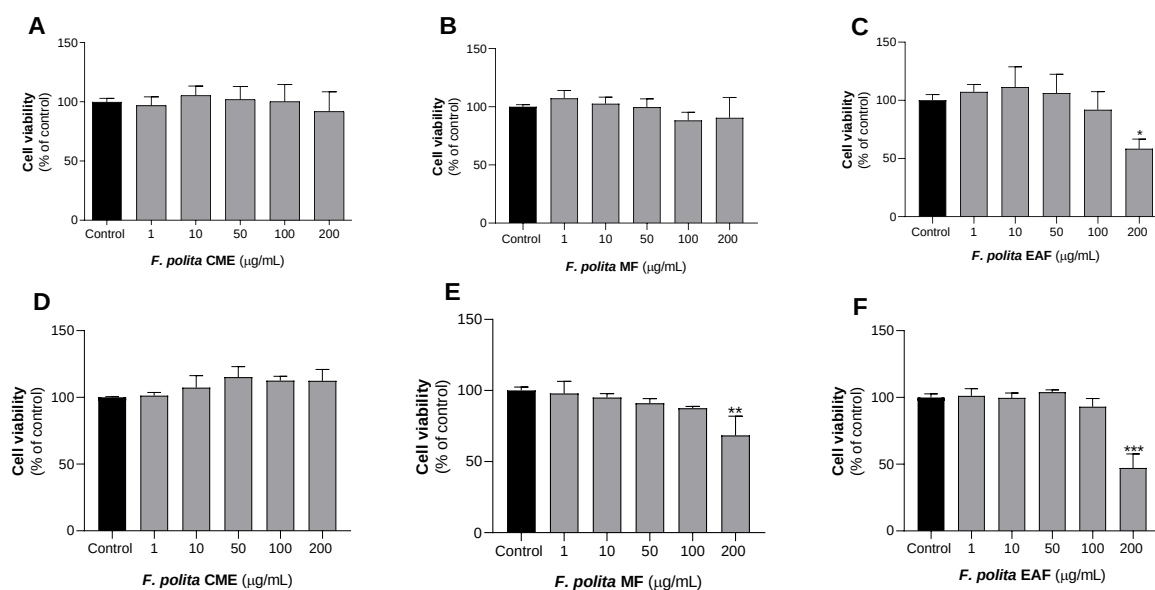


Figure 38. Effect of increasing concentrations of *F. polita* leaves CME and of its fractions, MF and EAF, on the viability of HaCaT (**A, B, C**) and SH-SY-5Y (**D, E, F**) cells evaluated by the MTT reduction assay after a 48 h incubation period. Results are expressed as percentage of control (cells treated with vehicle) and presented as mean $\pm$ SD of at least three independent assays, respectively, each one performed in triplicate. * $p \leq 0.05$ ** $p \leq 0.01$, *** $p \leq 0.001$.

Considering the fungistatic activity of *F. polita* EAF, the MIC values for pathogenic yeast strains are higher than the toxic concentrations for human cells, suggesting no selectivity. By using bioenergetic metabolism assays on millions of yeasts, *F. polita* EAF toxicity for yeasts is already evident for 150 µg/mL, an order of magnitude lower than the MIC value. Thus, bioenergetic metabolism assays exhibit higher sensitivity than MIC, and *F. polita* EAF may exhibit antifungal activity for concentrations that do not affect the human cells.

2.5. Main conclusions

- i) *F. polita* CME is a complex mixture of compounds containing sugars, phospholipids, Liebermann–Burchard reactive compounds, porphyrin-containing molecules and phenolic compounds, with emphasis on herniarin;
- ii) The fractionation procedure of *F. polita* CME allows to obtain an EAF dominated by Liebermann–Burchard reactive compounds, chlorophyll-related compounds and herniarin and a MF mostly composed by phospholipids and phenolic compounds, many of which identified in *F. polita* leaves for the first time;
- iii) *F. polita* EAF enhances the *F. polita* CME antioxidant activity, revealed by scavenging ROS and by inhibition of xanthine oxidase;
- iv) *F. polita* EAF reveals antifungal activity against pathogenic filamentous fungi and yeasts with MIC values between 1000 and 1500 µg/mL;
- v) The antifungal activity of *F. polita* EAF emerge from effects on bioenergetic metabolism of yeasts, resulting from mitochondrial respiratory complex II inhibition;
- vi) Despite the *F. polita* CME and its fractions do not show insecticidal potential, *F. polita* EAF mitochondrial toxicity for yeasts endow it with potential to be considered for development of antifungal formulations.

CHAPTER IV: Integrated Discussion, Conclusion Remarks and Future Perspectives

The increased worldwide incidence of pathogenic fungal strains and insects with resistance to the available antifungals and insecticides are a substantial threat to global public health (1,2,288). The limited range of the synthetic molecules with approval for pest management in agriculture and clinic, associated with their low selectivity and high environmental persistence negatively impact on crop production, sustainability of food supply chains, food security and clinical practice (3,4). To address these challenges, the focus of the research has shifted towards antifungals and insecticides of natural origin.

Sub-Saharan Africa faces even greater challenges due to a lack of adequate food supply and a healthcare system heavily reliant on medicinal plants. This exacerbates the already pressing global problem, highlighting the urgent need for innovative solutions to combat these environmental and public health concerns. However, this region also embraces a crucial resource to address these issues, the traditional medicine that, working on a plant biodiversity with approximately 45,000 species, selected many plant species with antifungal properties and insecticidal activity (9,14). Leveraging the ethnopharmacological relevance of *G. senegalensis* and *F. polita* in Guinea-Bissau, a research strategy was implemented to develop antifungals and insecticides with multi-target activity on pathogenic fungal strains and/or insect cells.

The investigation started with the preparation of a CME from the powdered leaves of *G. senegalensis* or *F. polita*, using a maceration process to preserve thermolabile and volatile compounds (289). A C18 solid-phase column was used for CME compounds fractionation according to their degree of polarity (290). Water-soluble compounds were discarded, while the lipophilic compounds were collected in an EAF, and the remaining compounds in a MF. *G. senegalensis* and *F. polita* CME and fractions were then analyzed for their chemical composition and tested for bioactivity against pathogenic fungi and insect cells.

A work plan with two steps was used for chemical characterization. In the initial step, classical chemical methods were used to determine in crude extracts and fractions the levels of Folin-Ciocalteu reactive phenolic compounds, total sugars, phospholipids, Liebermann–Burchard reactive compounds (e.g., steroids, triterpenes) and chlorophylls. Subsequently, advanced analytical techniques such as HPLC-MS/MS and HPLC-DAD were employed to identify and quantify the phenolic compounds and chlorophyll-related compounds present in the extracts and fractions. For both plant species, the first step of chemical characterization revealed the CME as a complex mixture of compounds from different classes, underscoring the effectiveness of fractionation in simplifying the chemical composition of the mixture, while retaining plant species biomarkers. In *G. senegalensis*,

the Liebermann–Burchard reactive compounds, chlorophylls, total sugars and Folin-Ciocalteu reactive phenolic compounds are concentrated in EAF. Conversely, *G. senegalensis* MF exhibits lower concentration of all these classes of compounds than CME, and chlorophylls are not detected. Phospholipids are detected in both fractions but at a lower concentration than in CME. In *F. polita*, EAF concentrates the Liebermann–Burchard reactive compounds, chlorophylls, and phospholipids, while Folin-Ciocalteu reactive phenolic compounds and phospholipids are concentrated in *F. polita* MF.

The second step of chemical characterization details polyphenols and chlorophyll-related compounds, highlighting plant species biomarkers. Comparing the CME of *G. senegalensis* and *F. polita*, it is evident that *G. senegalensis* exhibits greater quantity and molecular diversity of both phenolic compounds and chlorophyll-related compounds. The phenolic profile of *G. senegalensis* CME exhibits 33 phenolic compounds, including 19 flavonol 3-O-glycosylated derivatives, 4 aglycones, and 10 galloyl derivatives, alongside with the naphthyl butenone guieranone A - the main biomarker of this plant species. *G. senegalensis* EAF retains all the amount of guieranone A and a portion of the nine flavonol 3-O-glycosylated derivatives compounds. In contrast, 5 cinnamoyl quinic acids, 2 feruloyl hexosides, 3 flavone derivatives, and herniarin make up the phenolic profile of *F. polita* CME. Herniarin, the most representative phenolic compound, is completely preserved in the EAF while the other phenolics are mainly detected in MF. These data reveal the uniqueness of the phenolic composition of each plant, highlighting *G. senegalensis* for its greater polyphenol richness. Regarding the chlorophyll-related compounds, *G. senegalensis* EAF contains chlorophylls *a* and *b* and nine catabolites resulting from oxidation reactions before and after the loss of the phytol group of both chlorophylls, while in *F. polita* EAF chlorophylls *a* and *b* are detected along with four catabolites of chlorophyll *a*, also identified in *G. senegalensis* EAF. Thus, *G. senegalensis* EAF reveals higher quantity and diversity of porphyrin-containing compounds.

How the unique chemical compositions of *G. senegalensis* and *F. polita* CME and fractions impact on their antifungal and insecticidal properties will be discussed below.

Assays conducted with filamentous fungi *A. fumigatus*, *F. oxysporum* and *C. gloeosporioides* revealed that the CME and MF obtained from *G. senegalensis* or *F. polita* exhibited limited antifungal activity. However, the EAF obtained from these two plant species showed promising activity, with MIC values ranging from 500 to 2000 µg/mL. The effects against pathogenic (e.g., *C. albicans*, *C. krusei* and *C. neoformans*) and non-pathogenic (e.g., *S. cerevisiae*) yeasts further confirm the antifungal activity of the lipophilic fractions, with MICs ranging from 8 to 1500 µg/mL. Significantly, *G. senegalensis* EAF displays the

strongest antifungal properties against pathogenic yeast strains, with MIC values comparable to those obtained with the commercially available fungicide penconazole and the antifungal fluconazole. Additionally, MIC values are always lower than MFC meaning that the antifungal activity results from fungistatic effects. Furthermore, data obtained in the MIC and MFC susceptibility tests were also confirmed by the time-dependent growth effects of extracts and fractions obtained with both plant species on *C. neoformans* and *S. cerevisiae*.

Considering the relationship between antifungal activity and chemical composition, data indicate that the bioactivity of *G. senegalensis* EAF can be attributed to guieranone A and chlorophyll-related compounds, while that of *F. polita* EAF to chlorophyll-related compounds and herniarin. In the case of *F. polita*, the antifungal activity seems to also rely on compounds that are present in MF, since this fraction has a fungistatic activity higher than CME and lower than EAF. However, *G. senegalensis* EAF exhibits an antifungal activity two orders of magnitude greater than *F. polita* EAF, supporting the idea that the guieranone A effectiveness against yeasts is significantly higher than that of herniarin. It is also necessary to note that Liebermann–Burchard reactive compounds, occurring at similar concentrations in EAF of both plants, can also contribute to the antifungal activity. However, to understand the role of these compounds, it would be necessary to obtain a qualitative and quantitative profile of triterpenes and steroids for both plant species. The presence of phospholipids in the fractions that show bioactivity, particularly when mixed with other lipophilic compounds containing sugars, makes it possible to predict the formation of nanostructured structures (e.g. micelles), which could have a determining role in bioactivity, by increasing the bioavailability of active compounds (291). These issues, not addressed in this thesis, will be considered for future work.

The mechanism of action underlying the antifungal activity of *G. senegalensis* EAF and *F. polita* EAF was investigated considering effects on mitochondria of *S. cerevisiae*, used as model organism, and tyrosinase enzyme. Mitochondria have been the focus of recent studies to discover new antifungal drugs. This research aims to take advantage of particular features of the fungal mitochondrial proteins, including those that form the respiratory chain (292–296). Combining *in vivo* with *ex vivo* assays with cultures of *S. cerevisiae*, it is shown that *G. senegalensis* EAF, at 50 µg/mL, promotes a significant decrease of mitochondrial respiratory complexes I and II activities. Using isolated mitochondria, it is proved direct inhibition of mitochondrial complex I, with an IC₅₀ of 37 µg/mL. Regarding *F. polita* EAF, it is demonstrated its capacity to affect the bioenergetic yeast mitochondrial metabolism for concentrations equal to or higher than 150 µg/mL. It

was also shown that the mitochondrial toxicity results from an inhibition of mitochondrial complex II, IC₅₀ of 85 µg/mL.

The development of drug resistance largely depends upon whether the fungicide affects a single site or multiple sites (248). Most commercially available antifungal drugs have a single-site mechanism of action. Drugs that target different sites of action disrupt several metabolic processes within the fungus, decreasing the emergence of drug-resistant strains (297). Thus, to evaluate whether *G. senegalensis* and *F. polita* extracts and fractions are suitable multi-target formulations to face the current challenges associated with fungal diseases, their ability to inhibit the tyrosinase enzyme was assessed. Tyrosinase is a key enzyme in fungal mechanism defense against external factors and in fungal pathways related to spore formation, which makes it a valuable target in the R&D of novel antifungal agents. Data showed that *G. senegalensis* CME has the capacity to significantly inhibit the activity of this enzyme at concentrations equal to or greater than 25 µg/mL, whereas its *F. polita* counterpart is not active, at least up to a concentration of 100 µg/mL. Thus, the anti-tyrosinase activity was further explored for *G. senegalensis* EAF, the fraction with highest antifungal activity and mitochondrial activity. Results show that *G. senegalensis* EAF decreases, in a concentration-dependent manner, the tyrosinase activity, with IC₅₀ values of 49 µg/mL and 18 µg/mL when enzyme works in the presence of low and high substrate concentrations, respectively. It is also shown that kinetic tyrosinase inhibition promoted by the *G. senegalensis* EAF can be described by a mixed model inhibition, with Ki value of 14.40 µg/mL, suggesting the predominance of non-competitive inhibition.

The insecticidal potential of *G. senegalensis* and *F. polita* extracts and fractions was characterized by using neuronal *D. melanogaster* cells. *In vitro* assays with ML-DmBG2-c2 demonstrated that *G. senegalensis* CME is toxic for neuronal insect cells, and that the toxicity does not involve cell death by necrosis. *G. senegalensis* MF and EAF were also tested in these cells. Data show that EAF presents higher cytotoxicity as compared to CME or MF, with the toxic effects detected for concentrations equal to or higher than 0.5 µg/mL. Qualitatively, the toxicity of *F. polita* CME and fractions for neuronal insect cells follow the same pattern, but for *F. polita* EAF the toxic effects are only detected for concentrations equal to or higher than 50 µg/mL.

Considering the superior outcomes achieved with *G. senegalensis*, the mechanisms underlying the toxicity were investigated. The obtained data unveil that the insect cells toxicity of *G. senegalensis* are associated with an enhancement of ROS production, a mitochondrial membrane depolarization and a decrease of complex II activity. Thus, as

described for *G. senegalensis* antifungal activity, the insecticidal activity is also connected with mitochondrial effects.

AChE enzyme is a traditional target of insecticides, including organophosphates and carbamates (263). Despite the well-known problems associated with this type of insecticide, some authors consider that insect-specific cysteine in AChE enzyme is a unexplored target to develop new insecticides (298). Therefore, the effects of *G. senegalensis* CME and *F. polita* CME on the activity of this enzyme was also evaluated. Data indicate that extracts from both plants do not show activity on the enzyme.

The efficacy of extracts displaying antifungal and/or insecticidal properties should be coupled with their safety for non-target species. Thus, the toxicological profile of *G. senegalensis* and *F. polita* CME and their fractions was assessed on cellular models of human neuronal and skin cells. *In vitro* assays unveiled that *G. senegalensis* CME exhibits some degree of selective toxicity to insect cells ($EC_{50} = 47.9 \mu\text{g/mL}$), when compared to human neuronal and keratinocyte cells ($EC_{50} > 200 \mu\text{g/mL}$). The experiments with human cells also highlight that *G. senegalensis* EAF has an improved degree of selectivity towards pathogenic yeasts. In contrast, the toxicity of *F. polita* CME and fractions for human cells occurs in the same range of concentration as that detected for insect cells, indicating non-selectivity.

Considering the hypothesis of using *G. senegalensis* leaves extracts for development of antifungal formulations for topical application, preliminary assays were carried out to evaluate the role of *G. senegalensis* CME on the interaction of ultraviolet radiation with skin cells. Results show that *G. senegalensis* CME has a photosafety profile and that it provides protection against UVA radiation, decreasing ROS generation.

In conclusion, *G. senegalensis* leaves are a renewable resource to obtain mixtures of bioactive compounds with high potential to support the development of innovative antifungal and insecticidal formulations to address the resistance challenge. Despite *F. polita* CME and its fractions do not show insecticidal potential, *F. polita* EAF mitochondrial toxicity for yeasts endow it with some potential to be considered for the development of antifungal formulations.

Following the outlined research strategy, the next phase of this investigation requires the evaluation of the effects of *G. senegalensis* through *in vivo* insect assays, as well as to broaden the toxicological assessment including several human and non-target species cell lines. Regarding *F. polita*, exploring other bioactivities is paramount to harness the significant amount of herniarin present in this plant species.

CHAPTER V: References

1. Fisher MC, Alastruey-Izquierdo A, Berman J, Bicanic T, Bignell EM, Bowyer P, et al. Tackling the emerging threat of antifungal resistance to human health. *Nat Rev Microbiol* 2022 Sep;20(9):557–71.
2. Lee Y, Robbins N, Cowen LE. Molecular mechanisms governing antifungal drug resistance. *npj Antimicrob Resist* 2023 Jul;1(1):5.
3. Dangarembizi R, Wasserman S, Hoving JC. Emerging and re-emerging fungal threats in Africa. *Parasite Immunol* 2023 Feb;45(2).
4. Woods M, McAlister JA, Geddes-McAlister J. A One Health approach to overcoming fungal disease and antifungal resistance. *WIREs Mech Dis* 2023 Jul;15(4).
5. Gaffi Organization. Global Action for Fungal Infections. Available from: <https://gaffi.org/why/2019> [accessed on 9/08/2023].
6. Diniz-Lima I, Fonseca LM, Reis JS, Santos MARC, Costa KM, Santos CAN, et al. The sweet side of fungal infections: Structural glycan diversity and its importance for pathogenic adaptation. *Medicines* 2022 Jun;9(6):37.
7. WHO. WHO fungal priority pathogens list to guide research, development and public health action. Geneva World Health Organization; 2022.
8. Rhodes J, Fisher MC. Global epidemiology of emerging *Candida auris*. *Curr Opin Microbiol* 2019 Dec;52:84–9.
9. Mahomoodally MF. Traditional Medicines in Africa: An Appraisal of Ten Potent African Medicinal Plants. *Evidence-Based Complement Altern Med* 2013 Dec;2013:1–14.
10. WHO global report on traditional and complementary medicine. Geneva World Health Organization; 2019.
11. Africa WHO. The African Health Monitor. Available from: <https://www.afro.who.int/sites/default/files/2017-06/ahm-special-issue-14.pdf> [accessed on 12/09/2023].
12. Deresa EM, Diriba TF. Phytochemicals as alternative fungicides for controlling plant diseases: A comprehensive review of their efficacy, commercial representatives, advantages, challenges for adoption, and possible solutions. *Heliyon* 2023 Mar;9(3):e13810.
13. Suteu D, Rusu L, Zaharia C, Badeanu M, Daraban G. Challenge of utilization vegetal

- extracts as natural plant protection products. *Appl Sci* 2020 Dec;10(24):8913.
14. Linder HP. The evolution of African plant diversity. *Front Ecol Evol* 2014 Jul;2:99711.
 15. Maroyi A. Alternative medicines for HIV/AIDS in resource-poor settings: insight from traditional medicines use in Sub-Saharan Africa. *Trop J Pharm Res* 2014 Oct;13(9):1527.
 16. Cock IE, Van Vuuren SF. A review of the traditional use of southern African medicinal plants for the treatment of fungal skin infections. *J Ethnopharmacol* 2020 Apr;251:112539.
 17. Masevhe NA, McGaw LJ, Eloff JN. The traditional use of plants to manage candidiasis and related infections in Venda, South Africa. *J Ethnopharmacol* 2015 Jun;168:364–72.
 18. Tedila H, Assefa A, Haji F. Candidiasis and opportunistic mycosis in human. *Nov Res Microbiol J* 2019 Feb;3(1):190–203.
 19. Sobel JD. Vulvovaginal candidosis. *Lancet* 2007 Jun;369(9577):1961–71.
 20. Olum R, Baluku JB, Okidi R, Andia-Biraro I, Bongomin F. Prevalence of HIV-associated esophageal candidiasis in sub-Saharan Africa: A systematic review and meta-analysis. *Trop Med Health* 2020 Dec;48(1):82.
 21. Okoye CA, Nweze E, Ibe C. Invasive candidiasis in Africa, what is the current picture? *Pathog Dis* 2022 Jun;80(1):1–17.
 22. About Invasive Candidiasis. Available from: <https://www.cdc.gov/fungal/diseases/candidiasis/invasive/definition.html> [accessed on 16/08/2023].
 23. Aschale Y, Wubetu M, Abebaw A, Yirga T, Minwuyelet A, Toru M. A systematic review on traditional medicinal plants used for the treatment of viral and fungal infections in Ethiopia. *J Exp Pharmacol* 2021 Aug;13:807–15.
 24. Casadevall A. Cryptococcosis. In: *Harrison's Principles of Internal Medicine* 21 st. McGraw Hill Medical; 2022.
 25. Rajasingham R, Smith RM, Park BJ, Jarvis JN, Govender NP, Chiller TM, et al. Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. *Lancet Infect Dis* 2017 Aug;17(8):873–81.
 26. B. E. Omoruyi. Ethnomedicinal survey of medicinal plants used for the management

- of HIV/AIDS infection among local communities of Nkonkobe Municipality, Eastern Cape, South Africa. *J Med Plants Res* 2012 May;6(19):3603–8.
27. Garcia-Oliveira P, Carreira-Casais A, Pereira E, Dias MI, Pereira C, Calhelha RC, et al. From tradition to health: Chemical and bioactive characterization of five traditional plants. *Molecules* 2022 Oct;27(19):6495.
 28. Lassoued MA, Ben Fatma NEH, Haj Romdhane M, Faidi A, Majdoub H, Sfar S. Photoprotective potential of a Tunisian halophyte plant *Carpobrotus edulis* L. *Eur J Integr Med* 2021 Feb;42:101286.
 29. Omoruyi BE, Ighodaro DI, Afolayan AJ, Bradley G. Inhibition of HIV-1 protease by *Carpobrotus edulis* (L.). *Evidence-Based Complement Altern Med* 2020 Jun;2020:1–14.
 30. Rocha MI, Rodrigues MJ, Pereira C, Pereira H, da Silva MM, Neng N da R, et al. Biochemical profile and *in vitro* neuroprotective properties of *Carpobrotus edulis* L., a medicinal and edible halophyte native to the coast of South Africa. *South African J Bot* 2017 Jul;111:222–31.
 31. Martins A, Vasas A, Viveiros M, Molnár J, Hohmann J, Amaral L. Antibacterial properties of compounds isolated from *Carpobrotus edulis*. *Int J Antimicrob Agents* 2011 May;37(5):438–44.
 32. Otang WM, Grierson DS, Ndip RN. Phytochemical studies and antioxidant activity of two South African medicinal plants traditionally used for the management of opportunistic fungal infections in HIV/AIDS patients. *BMC Complement Altern Med* 2012 Dec;12(1):43.
 33. Okoro BC, Dokunmu TM, Okafor E, Sokoya IA, Israel EN, Olusegun DO, et al. The ethnobotanical, bioactive compounds, pharmacological activities and toxicological evaluation of garlic (*Allium sativum*): A review. *Pharmacol Res - Mod Chinese Med* 2023 Sep;8:100273.
 34. Aremu AO, Van Staden J. The genus *Tulbaghia* (Alliaceae)—A review of its ethnobotany, pharmacology, phytochemistry and conservation needs. *J Ethnopharmacol* 2013 Sep;149(2):387–400.
 35. Makhuvele R, Gbashi S, Njobeh PB. GC-HRTOF-MS metabolite profiling and antioxidant activity of methanolic extracts of *Tulbaghia violacea* Harv. *J King Saud Univ - Sci* 2022 Oct;34(7):102278.

36. Eid HH, Metwally GF. Phytochemical and biological study of callus cultures of *Tulbaghia violacea* Harv. cultivated in Egypt. *Nat Prod Res* 2017 Aug;31(15):1717–24.
37. Mbeng W, Grierson DS, Ndip RN. Ethnobotanical survey of medicinal plants used in the management of opportunistic fungal infections in HIV/AIDS patients in the Amathole District of the Eastern Cape Province, South Africa. *J Med Plants Res* 2012 Mar;6(11):2071–80.
38. Ndlovu M, Serem JC, Selepe MA, Opoku AR, Bester MJ, Apostolides Z, et al. Triterpenoids from *Protorhus longifolia* exhibit hypocholesterolemic potential via regulation of cholesterol biosynthesis and stimulation of low-density lipoprotein uptake in HepG2 Cells. *ACS Omega* 2023 Aug;8(34):30906–16.
39. Gado DA, Abdalla MA, Ahmed AS, Madikizela B, Nkadimeng SM, Ehlers MM, et al. *In vitro* antibacterial activity of *Loxostylis alata* extracts and isolated compounds against *Salmonella* species. *BMC Complement Med Ther* 2021 Dec;21(1):121.
40. Anjani G, Ayustaningwarno F, Eviana R. Critical review on the immunomodulatory activities of carrot's β -carotene and other bioactive compounds. *J Funct Foods* 2022 Dec;99:105303.
41. Rasheed H, Shehzad M, Rabail R, Kowalczewski PŁ, Kidoń M, Jeżowski P, et al. Delving into the nutraceutical benefits of purple carrot against metabolic syndrome and cancer: A review. *Appl Sci* 2022 Mar;12(6):3170.
42. Holzapfel CW, Van Wyk BE, De Castro A, Marais W, Herbst M. A chemotaxonomic survey of kaurene derivatives in the genus *Alepidea* (Apiaceae). *Biochem Syst Ecol* 1995 Nov;23(7–8):799–803.
43. Adam GO, Kim SJ, Egbuna C, Oh HG. Ethnopharmacological properties of African medicinal plants for the treatment of neglected tropical diseases. *Res J Pharmacogn* 2022 Oct;9(4):59–72.
44. Mohseni F, Ahmadiani ES, Hekmatdoust A. The effect of Cumin on anthropometric measurements: A systematic review of randomized controlled clinical trials. *Obes Med* 2021 May;23:100341.
45. Thakur A, Moyo P, van der Westhuizen CJ, Yang HO, Maharaj V. A novel cardenolide glycoside isolated from *Xysmalobium undulatum* reduces levels of the Alzheimer's disease-associated β -amyloid peptides A β 42 *in vitro*. *Pharmaceuticals*

2021 Jul;14(8):743.

46. Vermaak I, Enslin GM, Idowu TO, Viljoen AM. *Xysmalobium undulatum* (uzara) – review of an antidiarrhoeal traditional medicine. *J Ethnopharmacol* 2014 Oct;156:135–46.
47. Ndlovu B, Africa C, Klaasen J, Rahiman F. Does South Africa hold the key to the development of alternative treatments for resistant dermatophyte infections? A review. *J Herb Med* 2021 Oct;29:100474.
48. Nakayama M, Koshioka M, Kondo T, Imizu K. Flavone C-glucosides responsible for yellow pigmentation induced by low temperature in bracts of *Zantedeschia aethiopica*. *Nat Prod Commun* 2015 Mar;10(3):1934578X1501000.
49. Greca M Della, Ferrara M, Fiorentino A, Monaco P, Previtera L. Antialgal compounds from *Zantedeschia aethiopica*. *Phytochemistry* 1998 Nov;49(5):1299–304.
50. Ghuman S, Ncube B, Finnie JF, McGaw LJ, Cooposamy RM, Van Staden J. Antimicrobial activity, phenolic content, and cytotoxicity of medicinal plant extracts used for treating dermatological diseases and wound healing in KwaZulu-Natal, South Africa. *Front Pharmacol* 2016 Sep;7(320):211073.
51. Nazeam JA, Gad HA, El-Hefnawy HM, Singab ANB. Chromatographic separation and detection methods of *Aloe arborescens* Miller constituents: A systematic review. *J Chromatogr B* 2017 Jul;1058:57–67.
52. Li T, Liao Z, Chen M, Liu D, Zhang S, Lin L, et al. Simultaneous qualitative and quantitative determination of compounds in *Aloe arborescens* Mill using liquid chromatography with ion trap time-of-flight mass spectrometry and diode array detectors. *Sep Sci PLUS* 2018 Oct;1(10):640–9.
53. Kambizi L, Sultana N, Afolayan AJ. Bioactive compounds isolated from *Aloe ferox*: A plant traditionally used for the treatment of sexually transmitted infections in the Eastern Cape, South Africa. *Pharm Biol* 2005 Jan 7;42(8):636–9.
54. van Vuuren SF. Antimicrobial activity of South African medicinal plants. *J Ethnopharmacol* 2008 Oct;119(3):462–72.
55. Avula B, Bae JY, Wang YH, Wang M, Ali Z, Khan IA. Chemical profiling and characterization of anthraquinones from two *Bulbine* species and dietary supplements using liquid chromatography–high resolution mass spectrometry. *J AOAC Int* 2021 Sep;104(5):1394–407.

56. Abdissa N, Heydenreich M, Midiwo JO, Ndakala A, Majer Z, Neumann B, et al. A xanthone and a phenylanthraquinone from the roots of *Bulbine frutescens*, and the revision of six seco-anthraquinones into xanthenes. *Phytochem Lett* 2014 Sep;9(1):67–73.
57. Tambama P, Abegaz B, Mukanganyama S. Antiproliferative activity of the isofuranonaphthoquinone isolated from *Bulbine frutescens* against Jurkat T Cells. *Biomed Res Int* 2014 Jan;2014:1–14.
58. Collen M, Aladejana EB. Review of studies on *Bulbine natalensis* Baker (Asphodelaceae): Ethnobotanical uses, biological and chemical properties. *J Appl Pharm Sci* 2020 Sep;10(9):150–5.
59. Grierson D, Ndip R, Otang W. Antifungal activity of *Arctotis arctotoides* (L.f.) O. Hoffm. and *Gasteria bicolor* Haw. against opportunistic fungi associated with human immunodeficiency virus/acquired immunodeficiency syndrome. *Pharmacogn Mag* 2012;8(30):135.
60. Oyedele OA, Yani V V., Afolayan AJ. Chemical composition of the essential oil from *Arctotis arctotoides* (L.F.) O. Hoffm. (syn. *Vendium arctotoides* Less.). *Flavour Fragr J* 2005 Mar;20(2):232–4.
61. Sultana N, Afolayan AJ. A novel daucosterol derivative and antibacterial activity of compounds from *Arctotis arctotoides*. *Nat Prod Res* 2007 Aug;21(10):889–96.
62. Saleh-e-In MM, Van Staden J. Ethnobotany, phytochemistry and pharmacology of *Arctotis arctotoides* (L.f.) O. Hoffm.: A review. *J Ethnopharmacol* 2018 Jun;220:294–320.
63. Shinyuy LM, Loe GE, Jansen O, Mamede L, Ledoux A, Noukimi SF, et al. Secondary metabolites isolated from *Artemisia afra* and *Artemisia annua* and their anti-malarial, anti-inflammatory and immunomodulating properties — pharmacokinetics and pharmacodynamics: A review. *Metabolites* 2023 Apr;13(5):613.
64. Maciuk A, Mazier D, Duval R. Future antimalarials from *Artemisia*? A rationale for natural product mining against drug-refractory *Plasmodium* stages. *Nat Prod Rep* 2023 Jun;40(6):1130–44.
65. Hulley IM, van Vuuren SF, Sadgrove NJ, van Wyk BE. Antimicrobial activity of *Elytropappus rhinocerotis* (Asteraceae) against micro-organisms associated with foot odour and skin ailments. *J Ethnopharmacol* 2019 Jan;228:92–8.

66. Mshengu BP, Gakuba E, van Heerden FR. Chemical constituents from *Elytropappus rhinocerotis* (L.f.) Less. Biochem Syst Ecol 2017 Dec;75:18–20.
67. Adewinogo SO, Sharma R, Africa CWJ, Marnewick JL, Hussein AA. Chemical study and comparison of the biological activities of the essential oils of *Helichrysum petiolare*, *H. cymosum*, and *H. odoratissimum*. Plants 2022 Oct;11(19):2606.
68. Jadalla B, Moser J, Sharma R, Etsassala N, Egieyeh S, Badmus J, et al. *In vitro* alpha-glucosidase and alpha-amylase inhibitory activities and antioxidant capacity of *Helichrysum cymosum* and *Helichrysum pandurifolium* Schrank Constituents. Separations 2022 Jul;9(8):190.
69. Matanzima Y, Laubscher CP, Mabusela WT, Nchu F. Effects of varying levels of nitrogen on plant growth, anti-*Fusarium oxysporum* activity and 3,5-dicaffeoylquinic acid concentration of extracts from hydroponically cultivated *Helichrysum cymosum* (L.). Med Plants - Int J Phytomedicines Relat Ind 2021;13(1):45–55.
70. Elegbeleye JA, Krishnamoorthy S, Bamidele OP, Adeyanju AA, Adebowale OJ, Agbemavor WSK. Health-promoting foods and food crops of West-Africa origin: The bioactive compounds and immunomodulating potential. J Food Biochem 2022 Nov;46(11).
71. Pollastro F, Minassi A, Fresu LG. Cannabis phenolics and their bioactivities. Curr Med Chem 2018 Apr;25(10):1160–85.
72. Abdelfattah MAO, Dmirieh M, Ben Bakrim W, Mouhtady O, Ghareeb MA, Wink M, et al. Antioxidant and anti-aging effects of *Warburgia salutaris* bark aqueous extract: Evidences from *in silico*, *in vitro* and *in vivo* studies. J Ethnopharmacol 2022 Jun;292:115187.
73. Okello D, Kang Y. Ethnopharmacological potentials of *Warburgia ugandensis* on antimicrobial activities. Chin J Integr Med 2021 Aug;27(8):633–40.
74. Ogunlakin AD, Onifade TR, Ojo OA, Adesanya EO, Berena GA, Ayeni PO, et al. Antidiabetic potential of *Carica papaya* L. and its constituents: From folkloric uses to products development. Bioact Compd Heal Dis 2023 Jun;6(6):126–44.
75. Ndako M, Jigam AA, Kabiru AY, Umar SI, Lawal B. Polar extracts from *Gymnosporia senegalensis* (syn. *Maytenus senegalensis*) root bark, its effects on nociception, edema, and malarial infection. Phytomedicine Plus 2021 Nov;1(4):100113.
76. Zangueu CB, Olounlade AP, Ossokomack M, Djouatsa YNN, Alowanou GG,

- Azebaze AGB, et al. *In vitro* effects of aqueous extract from *Maytenus senegalensis* (Lam.) Exell stem bark on egg hatching, larval migration and adult worms of *Haemonchus contortus*. BMC Vet Res 2018 May;14(1):1–11.
77. Okoye FBC, Agbo MO, Nworu CS, Nwodo NJ, Esimone CO, Osadebe PO, et al. New neolignan glycoside and an unusual benzoyl malic acid derivative from *Maytenus senegalensis* leaves. Nat Prod Res 2015 Jan;29(2):109–15.
 78. Okoye FBC, Debbab A, Wray V, Esimone CO, Osadebe PO, Proksch P. A phenyldilactone, bisnorsesquiterpene, and cytotoxic phenolics from *Maytenus senegalensis* leaves. Tetrahedron Lett 2014 Jul;55(28):3756–60.
 79. Moyo M, Aremu AO. Nutritional, phytochemical and diverse health-promoting qualities of *Cleome gynandra*. Crit Rev Food Sci Nutr 2022 May;62(13):3535–52.
 80. Maina S, Ryu DH, Bakari G, Misinzo G, Nho CW, Kim HY. Variation in phenolic compounds and antioxidant activity of various organs of african cabbage (*Cleome gynandra* L.) accessions at different growth stages. Antioxidants 2021 Dec;10(12):1952.
 81. Omujal F, Okullo J, Bigirimana C, Isubikalu P, Agea J, Malinga M, et al. Physico-chemical, proximate, mineral and bioactive composition of *Garcinia buchananii* baker fruit from Uganda and Rwanda. African J Food, Agric Nutr Dev 2021 Dec;21(105):18932–49.
 82. Stark TD, Salger M, Frank O, Balemba OB, Wakamatsu J, Hofmann T. Antioxidative compounds from *Garcinia buchananii* stem bark. J Nat Prod 2015 Feb;78(2):234–40.
 83. Lyles J, Negrin A, Khan S, He K, Kennelly E. *In vitro* antiplasmodial activity of benzophenones and xanthenes from edible fruits of *Garcinia* species. Planta Med 2014 Jun;80(08/09):676–81.
 84. Mulholland DA, Mwangi EM, Dlova NC, Plant N, Crouch NR, Coombes PH. Non-toxic melanin production inhibitors from *Garcinia livingstonei* (Clusiaceae). J Ethnopharmacol 2013 Sep;149(2):570–5.
 85. Kaikabo AA, Eloff JN. Antibacterial activity of two biflavonoids from *Garcinia livingstonei* leaves against *Mycobacterium smegmatis*. J Ethnopharmacol 2011 Oct;138(1):253–5.
 86. Dirar AI, Devkota HP. Ethnopharmacological uses, phytochemistry and

- pharmacological activities of *Guiera senegalensis* J.F. Gmel. (Combretaceae). J Ethnopharmacol 2021 Mar;267:113433.
87. De Leo M, Braca A, Sanogo R, Cardile V, DeTommasi N, Russo A. Antiproliferative activity of *Pteleopsis suberosa* leaf extract and its flavonoid components in human prostate carcinoma cells. Planta Med 2006 Jun;72(7):604–10.
 88. Leo M De, Tommasi N De, Sanogo R, D'Angelo V, Germanò MP, Bisignano G, et al. Triterpenoid saponins from *Pteleopsis suberosa* stem bark. Phytochemistry 2006 Dec;67(24):2623–9.
 89. Salih EYA, Julkunen-Tiitto R, Luukkanen O, Fyhrqvist P. Anti-*Candida* activity of extracts containing ellagitannins, triterpenes and flavonoids of *Terminalia brownii*, a medicinal plant growing in semi-arid and savannah woodland in Sudan. Pharmaceutics 2022 Nov;14(11).
 90. Karri KC, Berihu HT, Konuku KR. *In vitro* antioxidant activities of ethanolic stem bark extract of *Terminalia brownii*. Drug Invent Today 2020 Mar;14(7):1022–6.
 91. Mongalo NI, McGaw LJ, Segapelo T V., Finnie JF, Van Staden J. Ethnobotany, phytochemistry, toxicology and pharmacological properties of *Terminalia sericea* Burch. ex DC. (Combretaceae) – A review. J Ethnopharmacol 2016 Dec;194:789–802.
 92. Mulaudzi N, Anokwuru CP, Tankeu SY, Combrinck S, Chen W, Vermaak I, et al. Phytochemical profiling and quality control of *Terminalia sericea* Burch. ex DC. using HPTLC metabolomics. Molecules 2021 Jan;26(2):432.
 93. Sobeh M, Mahmoud MF, Hasan RA, Abdelfattah MAO, Osman S, Rashid HO, et al. Chemical composition, antioxidant and hepatoprotective activities of methanol extracts from leaves of *Terminalia bellirica* and *Terminalia sericea* (Combretaceae). PeerJ 2019 Feb;2019(2):e6322.
 94. Abosede Wi O, Jide Afolo A. Aspects of medicinal activities of the stem bark extracts of *Curtisia dentata* (Burm. F.). Int J Pharmacol 2017 Mar;13(3):237–46.
 95. Shai LJ, McGaw LJ, Aderogba MA, Mdee LK, Eloff JN. Four pentacyclic triterpenoids with antifungal and antibacterial activity from *Curtisia dentata* (Burm.f) C.A. Sm. leaves. J Ethnopharmacol 2008 Sep;119(2):238–44.
 96. Rao V, Poonia A. *Citrullus colocynthis* (bitter apple): bioactive compounds, nutritional profile, nutraceutical properties and potential food applications: a review. Food Prod

- Process Nutr 2023 Dec;5(1):1–12.
97. Taye AD, Bizuneh GK, Kasahun AE. Ethnobotanical uses, phytochemistry and biological activity of the genus *Euclea*: A review. Front Pharmacol 2023 Apr;14:1170145.
 98. Maroyi A. Review of ethnomedicinal uses, phytochemistry and pharmacological properties of *Euclea natalensis* A.DC. Molecules 2017 Dec;22(12):2128.
 99. Moremi MP, Kamatou GP, Viljoen AM, Tankeu SY. *Croton gratissimus* - essential oil composition and chemometric analysis of an ethnomedicinally important tree from South Africa. South African J Bot 2021 May;138:141–7.
 100. Magwilu KD, Nguta JM, Mapenay I, Matara D. Phylogeny, phytomedicines, phytochemistry, pharmacological properties, and toxicity of *Croton gratissimus* Burch (Euphorbiaceae). Adv Pharmacol Pharm Sci 2022 May;2022:1–13.
 101. Ogunmefun OT, Ogunojemite OA, Adedokun OA, Olagbemide PT, Kehinde IO, Soliman M. β -Amyrin and benzene-1,2,4-trimethyl from *Euphorbia hirta* L. and *Nauclea latifolia* (Smith) leaves induce dauer diapause via antagonist inhibition of daf-12 receptor. Trop J Nat Prod Res 2023 Jul;7(7).
 102. Muhtar H, Fachriyah E, Kusrini D. Natural deep eutectic solvent-based ultrasonic-assisted extraction of *Euphorbia hirta* L.: Total flavonoid content, total phenolic content, and antioxidant activity. Adv Intell Appl Innov Approach 2023 Jun;2760(1):050010.
 103. Cavalcante NB, Santos AD da C, Almeida JRG da S. The genus *Jatropha* (Euphorbiaceae): A review on secondary chemical metabolites and biological aspects. Chem Biol Interact 2020 Feb;318:108976.
 104. Tiwari M, Panghal A, Mittal V, Gupta R. Bioactive compounds of *Acacia*, health benefits and its utilization in food processing industry: a critical review. Nutr Food Sci 2023 Oct;53(7):1125–46.
 105. Qian H, Wang L, Li Y, Wang B, Li C, Fang L, et al. The traditional uses, phytochemistry and pharmacology of *Abrus precatorius* L.: A comprehensive review. J Ethnopharmacol 2022 Oct;296:115463.
 106. Oyun MB. Chemical characterization of selected tree legumes as indices for their litter quality. J Appl Sci 2006 Aug;6(10):2321–4.
 107. Chen JY, Lian X, Fan YW, Ao ZY, Zhang WJ, Pan YC, et al. Four new stilbenes and

- one new flavonoid with potential antibacterial and anti-SARS-CoV-2 activity from *Cajanus cajan*. J Nat Med 2023 Sep;77(4):858–66.
108. Dade JME, Irie-N'Guessan G, Komlaga G, Say M, Okpekon TA, Boti JB, et al. Pyrrolidine alkaloids and their glycosylated derivatives from the root bark of *Dichrostachys cinerea* (L.) Wight & Arn. (Fabaceae). Phytochem Lett 2016 Jun;16:268–76.
 109. Suresh G, Tiwari AK, Murthy MRK, Kumar DA, Prasad KR, Rao RR, et al. New advanced glycation end-products inhibitors from *Dichrostachys cinerea* Wight & Arn. J Nat Med 2012 Jan;66(1):213–6.
 110. Mbaveng AT, Damen F, Simo Mpetga JD, Awouafack MD, Tane P, Kuete V, et al. Cytotoxicity of crude extract and isolated constituents of the *Dichrostachys cinerea* bark towards multifactorial drug-resistant cancer cells. Evidence-Based Complement Altern Med 2019 Jul;2019:1–11.
 111. Kiki GA à, Pop RM, Sabin O, Bocsan IC, Chedea VS, Socaci SA, et al. Polyphenols from *Dichrostachys cinerea* fruits anti-inflammatory, analgesic, and antioxidant capacity in Freund's adjuvant-induced arthritic rat model. Molecules 2022 Sep;27(17):5445.
 112. Nethathe BB, Ndip RN. Bioactivity of *Hydnora africana* on selected bacterial pathogens: Preliminary phytochemical screening. African J Microbiol Res 2011 Sep;5(18):2820–6.
 113. Wintola O, Afolayan A. Chemical constituents and biological activities of essential oils of *Hydnora africana* Thunb. used to treat associated infections and diseases in South Africa. Appl Sci 2017 Apr;7(5):443.
 114. Mohlakoana M, Moteetee A. Southern African soap plants and screening of selected phytochemicals and quantitative analysis of saponin content. Resources 2021 Sep;10(10):96.
 115. Zulfiqar F, Pandey P, Tripathi SK, Ali Z, Chittiboyina AG, Khan IA. Benzoylcyclopropane derivatives from *Hypoxis hemerocallidea* Corms. Planta Med 2022 Jul;88(08):685–92.
 116. Zulfiqar F, Khan SI, Ali Z, Wang YH, Ross SA, Viljoen AM, et al. Norlignan glucosides from *Hypoxis hemerocallidea* and their potential *in vitro* anti-inflammatory activity via inhibition of iNOS and NF-κB. Phytochemistry 2020 Apr;172:112273.

117. Mwinga JL, Asong JA, Amoo SO, Nkadimeng SM, McGaw LJ, Aremu AO, et al. *In vitro* antimicrobial effects of *Hypoxis hemerocallidea* against six pathogens with dermatological relevance and its phytochemical characterization and cytotoxicity evaluation. J Ethnopharmacol 2019 Oct;242:112048.
118. Moyo M, Amoo SO, Aremu AO, Gruz J, Šubrtová M, Doležal K, et al. Plant regeneration and biochemical accumulation of hydroxybenzoic and hydroxycinnamic acid derivatives in *Hypoxis hemerocallidea* organ and callus cultures. Plant Sci 2014 Oct;227:157–64.
119. Arumugam G, Swamy MK, Sinniah UR. *Plectranthus amboinicus* (Lour.) Spreng: botanical, phytochemical, pharmacological and nutritional significance. Molecules 2016 Mar;21(4):369.
120. Kulbat-Warycha K, Oracz J, Żyżelewicz D. Bioactive properties of extracts from *Plectranthus barbatus* (*Coleus forskohlii*) roots received using various extraction methods. Molecules 2022 Dec;27(24):8986.
121. Ishii T, Matayoshi N, Taba K, Kamada T. Two new abietane diterpenoids from *Plectranthus barbatus*. Chem Nat Compd 2022 May;58(3):474–7.
122. Shimira F. *Tetradenia riparia*, an ethnobotanical plant with diverse applications, from antimicrobial to anti-proliferative activity against cancerous cell lines: A systematic review. J Herb Med 2022 Mar;32:100537.
123. Latolla N, Hlangothi B. Three new pentacyclic triterpenes isolated from *Strychnos henningsii* Gilg. and their cytotoxic evaluation. Nat Prod Res 2023 Feb;1–9.
124. Wright CW, Allen D, Cai Y, Chen Z, Phillipson JD, Kirby GC, et al. Selective antiprotozoal activity of some *Strychnos* alkaloids. Phyther Res 1994 May;8(3):149–52.
125. Patil SM, Shirahatti PS, Ramu R. *Azadirachta indica* A. Juss (neem) against diabetes mellitus: a critical review on its phytochemistry, pharmacology, and toxicology. J Pharm Pharmacol 2022 May;74(5):681–710.
126. Gharsallah K, Rezig L, Rajoka MSR, Mehwish HM, Ali MA, Chew SC. *Moringa oleifera*: Processing, phytochemical composition, and industrial applications. South African J Bot 2023 Sep;160:180–93.
127. Marealle AI, Innocent E, Andrae-Marobela K, Qwarse M, Machumi F, Nondo RRSO, et al. Safety evaluation and bioassay-guided isolation of antimycobacterial

- compounds from *Morella salicifolia* root ethanolic extract. J Ethnopharmacol 2022 Oct;296:115501.
128. G. Meniso B, D. Boru A, B. Ganjinaboyina N. Phytochemical investigation and evaluation of antimicrobial activities of stem bark of *Morella salicifolia*. Bull Chem Soc Ethiop 2019 Jul;33(2):293.
 129. Makule E, Schmidt T, Heilmann J, Kraus B. Diarylheptanoid glycosides of *Morella salicifolia* bark. Molecules 2017 Dec;22(12):2266.
 130. Tekalign E, Tadege G, Fisseha N, Nureye D. Suppressive, curative, and prophylactic effects of *Maesa lanceolata* Forssk. against rodent malaria parasite *Plasmodium berghei*. Biomed Res Int 2022 Nov;2022:1–18.
 131. Seumo AS, Nanfack ARD, Ndontsa BL, Bitchagno GTM, Mbouangouere R, Lenta BN, et al. Alkenylbenzoquinones and other compounds from the fruit of *Maesa lanceolata* exhibited potent cytotoxic, antibacterial, and antiradical scavenging activities. Nat Prod Res 2022 Sep;36(17):4373–81.
 132. Surbhi, Kumar A, Singh S, Kumari P, Rasane P. *Eucalyptus*: phytochemical composition, extraction methods and food and medicinal applications. Adv Tradit Med 2023 Jun;23(2):369–80.
 133. Amadike Ugbogu E, Emmanuel O, Ebubechi Uche M, Dike Dike E, Chukwuebuka Okoro B, Ibe C, et al. The ethnobotanical, phytochemistry and pharmacological activities of *Psidium guajava* L. Arab J Chem 2022 May;15(5):103759.
 134. Shettar AK, Sateesh MK, Kaliwal BB, Vedamurthy AB. *In vitro* antidiabetic activities and GC-MS phytochemical analysis of *Ximenia americana* extracts. South African J Bot 2017 Jul;111:202–11.
 135. Eiki N, Manyelo TG, Hassan ZM, Lebelo SL, Sebola NA, Sakong B, et al. Phenolic composition of ten plants species used as ethnoveterinary medicines in Omusati and Kunene regions of Namibia. Sci Rep 2022 Dec;12(1):1–17.
 136. Rivero-Pino F. Oleocanthal – Characterization, production, safety, functionality and *in vivo* evidences. Food Chem 2023 Nov;425:136504.
 137. Elizondo-Luevano JH, Verde-Star J, González-Horta A, Castro-Ríos R, Hernández-García ME, Chávez-Montes A. *In vitro* effect of methanolic extract of *Argemone mexicana* against *Trichomonas vaginalis*. Korean J Parasitol 2020 Apr;58(2):135–45.

138. Mgbeahuruike EE, Holm Y, Vuorela H, Amandikwa C, Fyhrquist P. An ethnobotanical survey and antifungal activity of *Piper guineense* used for the treatment of fungal infections in West-African traditional medicine. *J Ethnopharmacol* 2019 Jan;229:157–66.
139. Ojimelukwe PC. *Piper guineense* - an underutilized aromatic spice with medicinal value. *Adv Tradit Med* 2023 Jun;23(2):381–92.
140. Scott IM, Jensen HR, Philogène BJR, Arnason JT. A review of *Piper* spp. (Piperaceae) phytochemistry, insecticidal activity and mode of action. *Phytochem Rev* 2008 Jan;7(1):65–75.
141. Madikizela B, McGaw LJ. *Pittosporum viridiflorum* Sims (Pittosporaceae): A review on a useful medicinal plant native to South Africa and tropical Africa. *J Ethnopharmacol* 2017 Jun;205:217–30.
142. Wedajo F, Gure A, Meshesha M, Kedir K, Frese M, Sewald N, et al. Cytotoxic compounds from the root bark of *Securidaca longipedunculata*. *Bull Chem Soc Ethiop* 2022 May;36(2):417–22.
143. Mongalo NI, McGaw LJ, Finnie JF, Staden J Van. *Securidaca longipedunculata* Fresen (Polygalaceae): A review of its ethnomedicinal uses, phytochemistry, pharmacological properties and toxicology. *J Ethnopharmacol* 2015 May;165:215–26.
144. Degani L, Riedo C, Gulmini M, Chiantore O. From plant extracts to historical textiles: Characterization of dyestuffs by GC–MS. *Chromatographia* 2014 Nov;77(23–24):1683–96.
145. Witbooi H, Okem A, Makunga NP, Kambizi L. Micropropagation and secondary metabolites in *Agathosma betulina* (Berg.). *South African J Bot* 2017 Jul;111:283–90.
146. Zahr S, Zahr R, El Hajj R, Khalil M. Phytochemistry and biological activities of *Citrus sinensis* and *Citrus limon*: an update. *J Herb Med* 2023 Sep;41:100737.
147. Lima RCL, Gramsbergen SM, Van Staden J, Jäger AK, Kongstad KT, Staerk D. Advancing HPLC-PDA-HRMS-SPE-NMR analysis of coumarins in *Coleonema album* by use of orthogonal reversed-phase C18 and pentafluorophenyl separations. *J Nat Prod* 2017 Apr;80(4):1020–7.
148. Başer KHC, Demirci B, Özek T, Viljoen AM, Victor JE. Composition of the essential

- oils of five *Coleonema* species from South Africa. J Essent Oil Res 2006 Jan;18:26–9.
149. Esterhuizen LL, Meyer R, Dubery IA. Antioxidant activity of metabolites from *Coleonema album* (Rutaceae). Nat Prod Commun 2006 May;1(5):1934578X0600100.
 150. Baskaran P, Moyo M, Van Staden J. *In vitro* plant regeneration, phenolic compound production and pharmacological activities of *Coleonema pulchellum*. South African J Bot 2014 Jan;90:74–9.
 151. Brader G, Bacher M, Hofer O, Greger H. Prenylated phenylpropenes from *Coleonema pulchellum* with antimicrobial activity. Phytochemistry 1997 Jul;45(6):1207–12.
 152. Koduru S, Asekun OT, Grierson DS, Afolayan AJ. Isolation of volatile compounds from *Solanum aculeastrum* (Solanaceae). J Essent Oil Bear Plants 2006 Jan;9(1):65–9.
 153. Koduru S, Grierson DS, Van De Venter M, Afolayan AJ. Anticancer activity of steroid alkaloids isolated from *Solanum aculeastrum*. Pharm Biol 2008 Oct;45(8):613–8.
 154. Kama-Kama F, Omosa LK, Nganga J, Maina N, Osanjo G, Yaouba S, et al. Antimycoplasmal activities of compounds from *Solanum aculeastrum* and *Piliostigma thonningii* against strains from the *Mycoplasma mycoides* Cluster. Front Pharmacol 2017 Dec;8:296432.
 155. Aini FN, Susilo S. Phytochemical profiling of Javanese Ginseng (*Talinum paniculatum*) stem extract using GC-MS analysis and pharmacological potential. Trop J Nat Prod Res 2023 Jul;7(7):3272–8.
 156. Ambily PG, Mathew J, Sudhina M. Analysis of leaf extract of *Zingiber officinale* by a hybrid analytical technique. Curr Trends Biotechnol Pharm 2022 Aug;16(3):316–28.
 157. Medjekal S, Ghadbane M, Boufennara S, Benderradji L, Bodas R, Bousseboua H, et al. Comparing chemical composition and phenolic compounds of some herbals as potential feed additives in ruminant nutrition. In: Environmental Science and Engineering 2nd ed. Springer, Cham; 2021. p. 1389–94.
 158. Heinrich M, Jalil B, Abdel-Tawab M, Echeverria J, Kulić Ž, McGaw LJ, et al. Best Practice in the chemical characterization of extracts used in pharmacological and toxicological research—The ConPhyMP—Guidelines12. Front Pharmacol 2022 Sep

- 13;13.
159. Mutinda ES, Mkala EM, Nanjala C, Waswa EN, Odago WO, Kimutai F, et al. Traditional medicinal uses, pharmacology, phytochemistry, and distribution of the genus *Fagaropsis* (Rutaceae). J Ethnopharmacol 2022 Feb;284:114781.
 160. Gião MS, Pereira CI, Fonseca SC, Pintado ME, Malcata FX. Effect of particle size upon the extent of extraction of antioxidant power from the plants *Agrimonia eupatoria*, *Salvia* sp. and *Satureja montana*. Food Chem 2009 Dec;117(3):412–6.
 161. Zhang QW, Lin LG, Ye WC. Techniques for extraction and isolation of natural products: a comprehensive review. Chin Med 2018 Dec;13(1):20.
 162. Handa SS, Khanuja SPS, Longo G, Rakesh DD. Extraction technologies for medicinal and aromatic plants. Trieste: International Centre for Science and High Technology; 2008.
 163. Doell S, Arens N, Mock H. Liquid chromatography and liquid chromatography–mass spectrometry of plants: Techniques and applications. In: Encyclopedia of Analytical Chemistry Wiley; 2019. p. 1–12.
 164. Merck. HPLC and UHPLC Column Selection Guide - The comprehensive Supelco® portfolio of analytical solutions to meet your U/HPLC and LC-MS needs. 2022.
 165. Sander LC, Rimmer CA, Wilson WB. Characterization of triacontyl (C-30) liquid chromatographic columns. J Chromatogr A 2020 Mar;1614:460732.
 166. Eugster PJ, Guilleme D, Rudaz S, Veuthey JL, Carrupt PA, Wolfender JL. Ultra high pressure liquid chromatography for crude plant extract profiling. J AOAC Int 2011 Feb;94(1):51–70.
 167. Wang M, Wang C, Han X. Selection of internal standards for accurate quantification of complex lipid species in biological extracts by electrospray ionization mass spectrometry – What, how and why? Mass Spectrom Rev 2017 Nov;36(6):693.
 168. Cantón E, Espinel-Ingroff A, Pemán J. Trends in antifungal susceptibility testing using CLSI reference and commercial methods. Expert Rev Anti Infect Ther 2009 Feb;7(1):107–19.
 169. Clinical and Laboratory Standards Institute. M38-A2 Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard-Second Edition. 2008.
 170. Clinical and Laboratory Standards Institute. M27-A3 Reference Method for Broth

Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard-Third Edition. 2008.

171. Monteiro MC, De La Cruz M, Cantizani J, Moreno C, Tormo JR, Mellado E, et al. A new approach to drug discovery: high-throughput screening of microbial natural extracts against *Aspergillus fumigatus* using resazurin. *J Biomol Screen* 2012 Apr;17(4):542–9.
172. Beattie SR, Krysan DJ. Antifungal drug screening: thinking outside the box to identify novel antifungal scaffolds. *Curr Opin Microbiol* 2020 Oct;57:1.
173. Vila T, Lopez-Ribot JL. Screening the pathogen box for identification of *Candida albicans* biofilm inhibitors. *Antimicrob Agents Chemother* 2017 Jan;61(1).
174. Meletiadiis J, Meis JFGM, Mouton JW, Donnelly JP, Verweij PE. Comparison of NCCLS and 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) methods of *in vitro* susceptibility testing of filamentous fungi and development of a new simplified method. *J Clin Microbiol* 2000 Aug;38(8):2949–54.
175. Meletiadiis J, Mouton JW, Meis JFGM, Bouman BA, Donnelly JP, Verweij PE. Colorimetric assay for antifungal susceptibility testing of *Aspergillus* species. *J Clin Microbiol* 2001 Sep;39(9):3402–8.
176. Tsukatani T, Suenaga H, Shiga M, Matsumoto K. A rapid microplate method for the proliferation assay of fungi and the antifungal susceptibility testing using the colorimetric microbial viability assay. *Lett Appl Microbiol* 2014 Aug;59(2):184–92.
177. Organização Oeste Africana da Saúde. Farmacopeia de Ervas da África Ocidental. Bobo-Dioulasso (Burkina Faso); 2020.
178. Sparks TC, Lorsbach BA. Agrochemical discovery - building the next generation of insect control agents. In: *Advances in agrochemicals: Ion channels and G Protein-coupled receptors (GPCRs) as targets for pest control* 2017. p. 1–17.
179. Bantz A, Camon J, Froger JA, Goven D, Raymond V. Exposure to sublethal doses of insecticide and their effects on insects at cellular and physiological levels. *Curr Opin Insect Sci* 2018 Dec;30:73–8.
180. Sparks TC, Nauen R. IRAC: Mode of action classification and insecticide resistance management. *Pestic Biochem Physiol* 2015 Jun;121:122–8.
181. Insecticide Resistance Training. Available from: <https://irac-online.org/documents/insecticide-resistance-training-basic-module/> [accessed on

- 14/02/2024].
182. Sparks TC, Wessels FJ, Lorsbach BA, Nugent BM, Watson GB. The new age of insecticide discovery-the crop protection industry and the impact of natural products. *Pestic Biochem Physiol* 2019 Nov;161:12–22.
 183. Swale DR. Perspectives on new strategies for the identification and development of insecticide targets. *Pestic Biochem Physiol* 2019 Nov;161:23–32.
 184. Updated WHO guidance for controlling vector-borne diseases through indoor residual spraying. Available from: <https://www.who.int/news/item/15-02-2024-updated-who-guidance-for-controlling-vector-borne-diseases-through-indoor-residual-spraying> [accessed on 26/02/2024].
 185. Vector-borne diseases. Available from: <https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases> [accessed on 28/02/2024].
 186. Caminade C, McIntyre KM, Jones AE. Impact of recent and future climate change on vector-borne diseases. *Ann N Y Acad Sci* 2019 Jan;1436(1):157.
 187. World malaria report. Available from: <https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2023> [accessed on 26/02/2024].
 188. Dengue- Global situation. Available from: <https://www.who.int/emergencies/disease-outbreak-news/item/2023-DON498> [accessed on 28/02/2024].
 189. Zika virus. Available from: <https://www.who.int/news-room/fact-sheets/detail/zika-virus> [accessed on 28/02/2024].
 190. He X, Lu L, Huang P, Yu B, Peng L, Zou L, et al. Insect cell-based models: Cell line establishment and application in insecticide screening and toxicology research. *Insects* 2023 Jan;14(2):104.
 191. *Guiera senegalensis*. Available from: <https://greeninstitute.ng/plants/2023/8/29/guiera-senegalensis> [accessed on 05/02/2024].
 192. Aderbauer B, Clausen PH, Kershaw O, Melzig MF. *In vitro* and *in vivo* trypanocidal effect of lipophilic extracts of medicinal plants from Mali and Burkina Faso. *J Ethnopharmacol* 2008 Sep;119(2):225–31.
 193. Bizimana N, Tietjen U, Zessin KH, Diallo D, Djibril C, Melzig MF, et al. Evaluation of medicinal plants from Mali for their *in vitro* and *in vivo* trypanocidal activity. *J Ethnopharmacol* 2006 Feb;103(3):350–6.

194. Fiot J, Sanon S, Azas N, Mahiou V, Jansen O, Angenot L, et al. Phytochemical and pharmacological study of roots and leaves of *Guiera senegalensis* J.F. Gmel (Combretaceae). *J Ethnopharmacol* 2006 Jun;106(2):173–8.
195. Dirar AI, Adhikari-Devkota A, Hassan MM, Wada M, Watanabe T, Devkota HP. Phenolic compounds as potent free radical scavenging and enzyme inhibitory components from the leaves of *Guiera senegalensis*. *Nat Prod Commun* 2019 Jun;14(6).
196. Bosisio E, Mascetti D, Verotta L, Zani F, Mazza P, Talbot M. *Guiera senegalensis* J. F. Gmelin (Combretaceae): Biological activities and chemical investigation. *Phytomedicine* 1997 Feb;3(4):339–48.
197. Sombie PAED, Hilou A, Mounier C, Coulibaly AY, Kiendrebeo M, Millogo JF, et al. Antioxidant and anti-inflammatory activities from galls of *Guiera senegalensis* J.F. Gmel (Combretaceae). *Res J Med Plant* 2011 Apr;5(4):448–61.
198. Adebisi A, Ayo R, Bello I, Habila J. Phytochemical screening and anti-Tb activity of root extracts of *Guiera senegalensis* (J. F. Gmel). *Am J Biosci Bioeng* 2015 Jan;3(6):208–13.
199. Silva O, Gomes ET. Guieranone A, a naphthyl butenone from the leaves of *Guiera senegalensis* with antifungal activity. *J Nat Prod* 2003 Mar;66(3):447–9.
200. Ajayi FT, Omotoso SO, Odejide JO. Evaluation of fodder plants (*Ficus polita*, *Azadirachta indica* and *Vernonia amygdalina*) for their phytochemical and antibacterial properties. *Cogent Food Agric* 2016 Dec;2(1).
201. Kuete V, Kamga J, Sandjo LP, Ngameni B, Poumale HMP, Ambassa P, et al. Antimicrobial activities of the methanol extract, fractions and compounds from *Ficus polita* Vahl. (Moraceae). *BMC Complement Altern Med* 2011 Jan;11(1):1–6.
202. Usman H, Kaigama AU, Ibisagba OO, Fulata A, Ahmed I. Phytoconstituents evaluation and antimicrobial efficacy of the crude flavonoids and saponins rootbark extracts of *Terminalia avicennioides* and *Ficus polita*. *J Herbmmed Pharmacol* 2018 Apr;7(2):106–11.
203. *Ficus polita* Vahl subsp. *polita*. Available from: <https://pza.sanbi.org/ficus-polita-subsp-polita> [accessed on 03/03/2024].
204. Ogunwande IA, Sonibare MA, Thang TD, Dung NX, Soladoye MO, Morohunfolu OO. Comparative analysis of the oils of three *Ficus* species from Nigeria. *J Essent Oil*

- Res 2008 Sep;20(5):386–9.
205. Pergaud SL, Kamga J, Sandjo LP, Poumale HM, Ngameni B, Shiono Y, et al. Politamide, a new constituent from the stem bark of *Ficus polita* Vahl (Moraceae). Arkivoc 2010 Jun;2010(2):323–9.
 206. Etkin NL, Ross PJ. Food as medicine and medicine as food. Soc Sci Med 1982 Jan;16(17):1559–73.
 207. Yahaya MF, Shagal MH, Maitera ON, Dass PM, Yelwa JM, Osemeahon SA. Antimicrobial, antioxidant, cytotoxicity profiles and chemical compositions of ethanolic extracts of *Ficus polita* and *Ficus thonningii* plant. J Res Chem 2021 Sep;2(1).
 208. Singleton VL, Orthofer R, Lamuela-Raventós RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. In: Methods in Enzymology Academic Press Inc.; 1999. p. 152–78.
 209. Nielsen SS. Phenol-Sulfuric Acid Method for Total Carbohydrates. In: Food Analysis Laboratory Manual Boston, MA: Springer; 2010. p. 47–53.
 210. Bartlett EM, Lewis DH. Spectrophotometric determination of phosphate esters in the presence and absence of orthophosphate. Anal Biochem 1970 Jul;36(1):159–67.
 211. Kenny AP. The determination of cholesterol by the Liebermann-Burchard reaction. Biochem J 1952 Dec;52(4):611–9.
 212. Lichtenthaler HK, Buschmann C. Chlorophylls and carotenoids: Measurement and characterization by UV-Vis spectroscopy. Curr Protoc Food Anal Chem 2001 Aug;1(1):F4.3.1-F4.3.8.
 213. Costa AF. Farmacognosia - Volume III. 3rd ed. Fundação Calouste Gulbenkian; 2001.
 214. Ferreres F, Grosso C, Gil-Izquierdo A, Fernandes A, Valentão P, Andrade PB. Comparing the phenolic profile of *Pilocarpus pennatifolius* Lem. by HPLC–DAD–ESI/MSⁿ with respect to authentication and enzyme inhibition potential. Ind Crops Prod 2015 Dec;77:391–401.
 215. Garcia CJ, García-Villalba R, Garrido Y, Gil MI, Tomás-Barberán FA. Untargeted metabolomics approach using UPLC-ESI-QTOF-MS to explore the metabolome of fresh-cut iceberg lettuce. Metabolomics 2016 Aug;12(8):138.
 216. Sousa C, Valentão P, Ferreres F, Seabra RM, Andrade PB. Tronchuda cabbage

- (*Brassica oleracea* L. var. *costata* DC): scavenger of reactive nitrogen species. *J Agric Food Chem* 2008 Jun;56(11):4205–11.
217. Nishikimi M, Appaji Rao N, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochem Biophys Res Commun* 1972 Jan;46(2):849–54.
218. Gnaiger E. Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply. *Respir Physiol* 2001 Nov;128(3):277–97.
219. Monteiro-Cardoso VF, Oliveira MM, Melo T, Domingues MRM, Moreira PI, Ferreira E, et al. Cardiolipin profile changes are associated to the early synaptic mitochondrial dysfunction in Alzheimer's Disease. *J Alzheimer's Dis* 2014 Dec;43(4):1375–92.
220. Hatefi Y, Stiggall DL. Preparation and properties of succinate: Ubiquinone oxidoreductase (complex II). In: *Methods in Enzymology* Academic Press; 1978. p. 21–7.
221. Barrientos A, Fontanesi F, Díaz F. Evaluation of the mitochondrial respiratory chain and oxidative phosphorylation system using polarography and spectrophotometric enzyme assays. *Curr Protoc Hum Genet* 2009 Oct;63(1).
222. Shepherd D, Garland PB. The kinetic properties of citrate synthase from rat liver mitochondria. *Biochem J* 1969 Sep;114(3):597–610.
223. Maisuthisakul P, Gordon MH. Antioxidant and tyrosinase inhibitory activity of mango seed kernel by product. *Food Chem* 2009 Nov;117(2):332–41.
224. Valentão P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, Bastos ML. Antioxidant activity of *Centaurium erythraea* infusion evidenced by its superoxide radical scavenging and xanthine oxidase inhibitory activity. *J Agric Food Chem* 2001 Jul;49(7):3476–9.
225. Videira RA, Andrade PB, Monteiro LS, Valentão P, Ferreira PMT, Pereira DM. Toxicity and structure-activity relationship (SAR) of α,β -dehydroamino acids against human cancer cell lines. *Toxicol Vitro* 2018 Mar;47:26–37.
226. Neves D, Valentão P, Bernardo J, Oliveira MC, Ferreira JMG, Pereira DM, et al. A new insight on elderberry anthocyanins bioactivity: Modulation of mitochondrial redox chain functionality and cell redox state. *J Funct Foods* 2019 May;56:145–55.
227. Worek F, Eyer P, Thiermann H. Determination of acetylcholinesterase activity by the

- Ellman assay: A versatile tool for *in vitro* research on medical countermeasures against organophosphate poisoning. *Drug Test Anal* 2012 Mar;4(3–4):282–91.
228. Dorta E, Lobo MG, Gonzalez M. Reutilization of mango byproducts: Study of the effect of extraction solvent and temperature on their antioxidant properties. *J Food Sci* 2012 Jan;77(1):C80–8.
229. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956 Mar;28(3):350–6.
230. Ancolio C, Azas N, Mahiou V, Ollivier E, Di Giorgio C, Keita A, et al. Antimalarial activity of extracts and alkaloids isolated from six plants used in traditional medicine in Mali and Sao Tome. *Phyther Res* 2002 Nov;16(7):646–9.
231. Azas N, Laurencin N, Delmas F, Di Giorgio C, Gasquet M, Laget M, et al. Synergistic *in vitro* antimalarial activity of plant extracts used as traditional herbal remedies in Mali. *Parasitol Res* 2002 Feb;88(2):165–71.
232. Bouchet N, Lévesque J, Pousset JL. HPLC isolation, identification and quantification of tannins from *Guiera senegalensis*. *Phytochem Anal* 2000 Jan;11(1):52–6.
233. Alam P, Parvez MK, Arbab AH, Al-Dosari MS. Quantitative analysis of rutin, quercetin, naringenin, and gallic acid by validated RP- and NP-HPTLC methods for quality control of anti-HBV active extract of *Guiera senegalensis*. *Pharm Biol* 2017 Jan;55(1):1317–23.
234. Makkar HPS, Becker K. Isolation of tannins from leaves of some trees and shrubs and their properties. *J Agric Food Chem* 1994 Mar;42(3):731–4.
235. Maleš Ž, Medić-Šarić M, Bucar F. Flavonoids of *Guiera senegalensis* J. F. GMEL. - Thin-layer chromatography and numerical methods. *Croat Chem Acta* 1998;71(1):69–79.
236. Ficarra R, Ficarra P, Tommasini S, Carulli M, Melardi S, Di Bella MR, et al. Isolation and characterization of *Guiera senegalensis* J.F.Gmel. active principles. *Boll Chim Farm* 1997 May;136(5):454–9.
237. Lamien CE, Meda A, Mans J, Romito M, Nacoulma OG, Viljoen GJ. Inhibition of fowlpox virus by an aqueous acetone extract from galls of *Guiera senegalensis* J. F. Gmel (Combretaceae). *J Ethnopharmacol* 2005 Jan;96(1–2):249–53.
238. Viera I, Roca M, Perez-Galvez A. Mass Spectrometry of non-allomerized chlorophylls *a* and *b* derivatives from plants. *Curr Org Chem* 2018 May;22(9):842–76.

239. Schelbert S, Aubry S, Burla B, Agne B, Kessler F, Krupinska K, et al. Pheophytin pheophorbide hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in *Arabidopsis*. *Plant Cell* 2009 Apr;21(3):767–85.
240. Uaciquete A, Korsten L, Van der Waals JE. Epidemiology of cashew anthracnose (*Colletotrichum gloeosporioides* Penz.) in Mozambique. *Crop Prot* 2013 Jul;49:66–72.
241. Sanders GM, Korsten L. Comparison of cross inoculation potential of South African avocado and mango isolates of *Colletotrichum gloeosporioides*. *Microbiol Res* 2003 Jan;158(2):143–50.
242. Ishii H, Watanabe H, Yamaoka Y, Schnabel G. Sensitivity to fungicides in isolates of *Colletotrichum gloeosporioides* and *C. acutatum* species complexes and efficacy against anthracnose diseases. *Pestic Biochem Physiol* 2022 Mar;182:105049.
243. Maekawa LE, Lamping R, Marcacci S, Maekawa MY, Nassri MRG, Koga-ito CY. Antimicrobial activity of chlorophyll-based solution on *Candida albicans* and *Enterococcus faecalis*. *RSBO* 2008 Jun;4(2):36–40.
244. Kustov A V., Belykh D V., Smirnova NL, Venediktov EA, Kudayarova T V., Kruchin SO, et al. Synthesis and investigation of water-soluble chlorophyll pigments for antimicrobial photodynamic therapy. *Dye Pigment* 2018 Feb;149:553–9.
245. Silva MG, Mendes D, Oliveira MM, Andrade PB, Videira RA, Peixoto F. Mitochondria as a toxicological target for fungicides. In: *Mitochondrial Intoxication* Elsevier; 2023. p. 493–526.
246. Zhao RZ, Jiang S, Zhang L, Yu Z Bin. Mitochondrial electron transport chain, ROS generation and uncoupling. *Int J Mol Med* 2019 Jul;44(1):3–15.
247. Du S, Hu X, Shao X, Qian X. Novel trifluoromethyl sydnone derivatives: Design, synthesis and fungicidal activity. *Bioorganic Med Chem Lett* 2021 Jul;44:128114.
248. Pesticide Environmental Stewardship. Available from: <https://pesticidestewardship.org/resistance/fungicide-resistance/raised-resistance-risks/> [accessed on 10/02/2024].
249. Gisi U, Sierotzki H, Cook A, McCaffery A. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Manag Sci* 2002 Sep;58(9):859–67.
250. Mounkoro P, Michel T, Benhachemi R, Surpateanu G, Iorga BI, Fisher N, et al. Mitochondrial complex III Qi-site inhibitor resistance mutations found in laboratory

- selected mutants and field isolates. *Pest Manag Sci* 2019 Aug;75(8):2107–14.
251. Muddathir AM, Yamauchi K, Batubara I, Mohieldin EAM, Mitsunaga T. Anti-tyrosinase, total phenolic content and antioxidant activity of selected Sudanese medicinal plants. *South African J Bot* 2017 Mar;109:9–15.
 252. Dirar AI, Alsaadi DHM, Wada M, Mohamed MA, Watanabe T, Devkota HP. Effects of extraction solvents on total phenolic and flavonoid contents and biological activities of extracts from Sudanese medicinal plants. *South African J Bot* 2019 Jan;120:261–7.
 253. Nairn R, Cresswell W, Nairn J. Mushroom tyrosinase: A model system to combine experimental investigation of enzyme-catalyzed reactions, data handling using R, and enzyme-inhibitor structural studies. *Biochem Mol Biol Educ* 2015 Sep;43(5):370–6.
 254. Lee W, An G, Park H, Lim W, Song G. Diflubenzuron leads to apoptotic cell death through ROS generation and mitochondrial dysfunction in bovine mammary epithelial cells. *Pestic Biochem Physiol* 2021 Aug;177:104893.
 255. Chen P, Hu YF, Wang L, Xiao WF, Bao XY, Pan C, et al. Mitochondrial apoptotic pathway is activated by H_2O_2 -mediated oxidative stress in BmN-SWU1 cells from *Bombyx mori* ovary. *PLoS One* 2015 Jul;10(7).
 256. Summers CB, Felton GW. Prooxidant effects of phenolic acids on the generalist herbivore *Helicoverpa zea* (Lepidoptera: Noctuidae): Potential mode of action for phenolic compounds in plant anti-herbivore chemistry. *Insect Biochem Mol Biol* 1994 Oct;24(9):943–53.
 257. Agliassa C, Maffei ME. *Origanum vulgare* terpenoids induce oxidative stress and reduce the feeding activity of *Spodoptera littoralis*. *Int J Mol Sci* 2018 Sep;19(9):2805.
 258. Lopes AIF, Monteiro M, Araújo ARL, Rodrigues ARO, Castanheira EMS, Pereira DM, et al. Cytotoxic plant extracts towards insect cells: Bioactivity and nanoencapsulation studies for application as biopesticides. *Molecules* 2020 Dec;25(24):5855.
 259. Rai Y, Pathak R, Kumari N, Sah DK, Pandey S, Kalra N, et al. Mitochondrial biogenesis and metabolic hyperactivation limits the application of MTT assay in the estimation of radiation induced growth inhibition. *Sci Rep* 2018 Dec;8(1):1–15.
 260. Murphy MP. Mitochondrial dysfunction indirectly elevates ROS production by the endoplasmic reticulum. *Cell Metab* 2013 Aug;18(2):145–6.

261. Zhang J, Wang X, Vikash V, Ye Q, Wu D, Liu Y, et al. ROS and ROS-mediated cellular signaling. *Oxid Med Cell Longev* 2016;2016.
262. Lebieczinska M, Karkucinska-Wieckowska A, Giorgi C, Karczmarewicz E, Pronicka E, Pinton P, et al. Oxidative stress-dependent p66Shc phosphorylation in skin fibroblasts of children with mitochondrial disorders. *Biochim Biophys Acta - Bioenerg* 2010 Jun;1797(6–7):952–60.
263. Pang YP. Insect acetylcholinesterase as a target for effective and environmentally safe insecticides. *Adv In Insect Phys* 2014 Jan;46:435–94.
264. Zolfaghari M, Xiao Y, Mohammad F, Azam S, Yin F, Peng ZK, et al. Resistance mechanism of *Plutella xylostella* (L.) associated with amino acid substitutions in acetylcholinesterase-1: Insights from homology modeling, docking and molecular dynamic simulation. *Insects* 2024 Feb;15(3):144.
265. Sindhu T, Venkatesan T, Prabhu D, Jeyakanthan J, Gracy GR, Jalali SK, et al. Insecticide-resistance mechanism of *Plutella xylostella* (L.) associated with amino acid substitutions in acetylcholinesterase-1: A molecular docking and molecular dynamics investigation. *Comput Biol Chem* 2018 Dec;77:240–50.
266. Malekmohammadi M. Resistance of Colorado potato beetle (*Leptinotarsa decemlineata* Say) to commonly used insecticides in Iran. *J Asia Pac Entomol* 2014 Sep;17(3):213–20.
267. Vontas JG, Hejazi MJ, Hawkes NJ, Cosmidis N, Loukas M, Hemingway J. Resistance-associated point mutations of organophosphate insensitive acetylcholinesterase, in the olive fruit fly *Bactrocera oleae*. *Insect Mol Biol* 2002 Aug;11(4):329–36.
268. Walsh SB, Dolden TA, Moores GD, Kristensen M, Lewis T, Devonshire AL, et al. Identification and characterization of mutations in housefly (*Musca domestica*) acetylcholinesterase involved in insecticide resistance. *Biochem J* 2001 Oct;359(1):175–81.
269. Mutero A, Pralavorio M, Bride JM, Fournier D. Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. *Proc Natl Acad Sci* 1994 Jun;91(13):5922–6.
270. Suematsu N, Hosoda M, Fujimori K. Protective effects of quercetin against hydrogen peroxide-induced apoptosis in human neuronal SH-SY5Y cells. *Neurosci Lett* 2011

- Oct;504(3):223–7.
271. Veloso C, Videira RA, Andrade PB, Cardoso C, Vitorino C. Topical fixed-dose combinations: Current *in vitro* methodologies for pre-clinical development. *Int J Pharm* 2022 Apr;617:121621.
272. Verma A, Zanoletti A, Kareem KY, Adelodun B, Kumar P, Ajibade FO, et al. Skin protection from solar ultraviolet radiation using natural compounds: a review. *Environ Chem Lett* 2024 Feb;22(1):273–95.
273. Santhra Krishnan P, Salian A, Dutta S, Mandal S. A roadmap to UV-protective natural resources: classification, characteristics, and applications. *Mater Chem Front* 2021 Oct;5(21):7696–723.
274. Moukova A, Malina L, Kolarova H, Bajgar R. Hyperoside as a UV photoprotective or photostimulating compound - Evaluation of the effect of UV radiation with selected UV-absorbing organic compounds on skin cells. *Int J Mol Sci* 2023 Jun;24(12):9910.
275. Clifford MN, Johnston KL, Knight S, Kuhnert N. Hierarchical scheme for LC-MSⁿ identification of chlorogenic acids. *J Agric Food Chem* 2003 May;51(10):2900–11.
276. Andrade C, Ferreres F, Gomes NGM, Duangsrissai S, Srisombat N, Vajrodaya S, et al. Phenolic profiling and biological potential of *Ficus curtipes* corner leaves and stem bark: 5-lipoxygenase inhibition and interference with NO levels in LPS-stimulated RAW 264.7 macrophages. *Biomolecules* 2019 Aug;9(9):400.
277. Mouho DG, Oliveira AP, Kodjo CG, Valentão P, Gil-Izquierdo A, Andrade PB, et al. Chemical findings and *in vitro* biological studies to uphold the use of *Ficus exasperata* Vahl leaf and stem bark. *Food Chem Toxicol* 2018 Feb;112:134–44.
278. Oliveira AP, Valentão P, Pereira JA, Silva BM, Tavares F, Andrade PB. *Ficus carica* L.: Metabolic and biological screening. *Food Chem Toxicol* 2009 Nov;47(11):2841–6.
279. Elhawary SS, Younis IY, El Bishbishy MH, Khattab AR. LC–MS/MS-based chemometric analysis of phytochemical diversity in 13 *Ficus* spp. (Moraceae): Correlation to their *in vitro* antimicrobial and *in silico* quorum sensing inhibitory activities. *Ind Crops Prod* 2018 Dec;126:261–71.
280. Fahmy MA, Hassan EE, Ibrahim NE, Hassan EM, Hassan ZM, Omara EA. Protective role of *Ficus carica* extract against hepato-testicular side effects and genotoxicity induced by cisplatin. *Pharmacogn J* 2020 May;12(3):645–56.

281. Mawa S, Jantan I, Husain K. Isolation of terpenoids from the stem of *Ficus aurantiaca* Griff and their effects on reactive oxygen species production and chemotactic activity of neutrophils. *Molecules* 2016 Jan;21(1):9.
282. Wira DW, Mardawati E, Djali M, Balia RL. The characterization of *Ficus lyrata* Warb fruit extract and the effect on toxicity, physicochemical, and microbiology properties of chicken carcass. *Int J Adv Sci Eng Inf Technol* 2020 Feb 25;10(1):362.
283. Zingue S, Michel T, Nde CBM, Njuh AN, Cisilotto J, Ndinteh DT, et al. Estrogen-like and tissue-selective effects of 7-methoxycoumarin from *Ficus umbellata* (Moraceae): An *in vitro* and *in vivo* study. *BMC Complement Altern Med* 2017 Aug 2;17(1).
284. Mohamed Isa S, Ablat A, Mohamad J. The antioxidant and xanthine oxidase inhibitory activity of *Plumeria rubra* flowers. *Molecules* 2018 Feb;23(2):400.
285. Cimanga K, Ying L, De Bruyne T, Apers S, Cos P, Hermans N, et al. Radical scavenging and xanthine oxidase inhibitory activity of phenolic compounds from *Bridelia ferruginea* stem bark. *J Pharm Pharmacol* 2001 Feb;53(5):757–61.
286. Mendes D, Peixoto F, Oliveira MM, Andrade PB, Videira RA. Mitochondria research and neurodegenerative diseases: On the track to understanding the biological world of high complexity. *Mitochondrion* 2022 Jul;65:67–79.
287. Renda G, Barut B, Ceren R, Aydin E. *In vitro* tyrosinase inhibitory, DNA interaction studies, and LC-HRMS analysis of *Ficus carica* leaves. *Turkish J Chem* 2023 Jan;47(2):465–75.
288. Siddiqui JA, Fan R, Naz H, Bamisile BS, Hafeez M, Ghani MI, et al. Insights into insecticide-resistance mechanisms in invasive species: Challenges and control strategies. *Front Physiol* 2022 Jan;13:1112278.
289. Farooq S, Mir SA, Shah MA, Manickavasagan A. Extraction techniques. In: *Plant Extracts: Applications in the Food Industry* Academic Press; 2022. p. 23–37.
290. Abubakar AR, Haque M. Preparation of medicinal plants: basic extraction and fractionation procedures for experimental purposes. *J Pharm Bioallied Sci* 2020 Jan;12(1):1.
291. Burdíkuvá J, Mravec F, Pekař M. The formation of mixed micelles of sugar surfactants and phospholipids and their interactions with hyaluronan. *Colloid Polym Sci* 2016 May;294(5):823–31.
292. Nishikawa H, Fukuda Y, Mitsuyama J, Tashiro M, Tanaka A, Takazono T, et al. *In*

- vitro* and *in vivo* antifungal activities of T-2307, a novel arylamidine, against *Cryptococcus gattii*: an emerging fungal pathogen. J Antimicrob Chemother 2017 Jun;72(6):1709–13.
293. Singh SB, Liu W, Li X, Chen T, Shafiee A, Card D, et al. Antifungal spectrum, *in vivo* efficacy, and structure-activity relationship of ilicicolin H. ACS Med Chem Lett 2012 Oct;3(10):814–7.
294. Oliver JD, Sibley GEM, Beckmann N, Dobb KS, Slater MJ, McEntee L, et al. F901318 represents a novel class of antifungal drug that inhibits dihydroorotate dehydrogenase. Proc Natl Acad Sci U S A 2016 Nov;113(45):12809–14.
295. McLellan CA, Vincent BM, Solis N V., Lancaster AK, Sullivan LB, Hartland CL, et al. Inhibiting mitochondrial phosphate transport as an unexploited antifungal strategy. Nat Chem Biol 2017 Dec;14(2):135–41.
296. Qin Y, Wang J, Lv Q, Han B. Recent progress in research on mitochondrion-targeted antifungal drugs: a review. Antimicrob Agents Chemother 2023 Jun;67(6).
297. Deising HB, Reimann S, Pascholati SF. Mechanisms and significance of fungicide resistance. Brazilian J Microbiol 2008 Apr;39(2):286.
298. Dou D, Park JG, Rana S, Madden BJ, Jiang H, Pang YP. Novel selective and irreversible mosquito acetylcholinesterase inhibitors for controlling malaria and other mosquito-borne diseases. Sci Rep 2013 Jan;3(1):1–11.