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METABÓLITOS BIOATIVOS DE FUNGOS ISOLADOS DA *Bertholletia excelsa* Bonpl. E SUA AÇÃO SOBRE MELANOGÊNESE EM MODELO *Zebrafish* (Hamilton, 1822).

ADRIANA MACIEL FERREIRA

MACAPÁ-AP
Maio - 2023
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“Precisamos ter consciência de que muitas mulheres morreram para que pudéssemos ficar vivas, termos liberdade de escolher e fazer o que quisermos”

Elza Soares

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FERREIRA, ADRIANA MACIEL. METABÓLITOS BIOATIVOS DE FUNGOS ISOLADOS DA *Bertholletia excelsa* Bonpl. E SUA AÇÃO SOBRE MELANOGÊNESE EM MODELO *Zebrafish* (Hamilton, 1822). 2023. 59f. Tese (Doutorado em Biodiversidade e Biotecnologia - Rede BIONORTE) - Universidade Federal do Amapá, Macapá, 2023.

RESUMO

A obtenção de novos compostos bioativos com diferentes mecanismos de ação, com menores efeitos indesejáveis, maior segurança na utilização e maior eficácia é uma necessidade constante na saúde. *Bertholletia excelsa* Bonpl., conhecida como castanha da Amazônica, é um dos produtos de exportação mais importantes da floresta Amazônica, sendo o Brasil responsável por 75% do mercado desse produto. A bioprospecção é uma das ferramentas utilizadas para a obtenção de produtos naturais com ação sobre a melanogênese. Uma das fontes promissoras utilizadas nesse tipo de investigação são os metabólitos secundários produzidos por microrganismos endofíticos obtidos a partir de espécies vegetais. Deste modo, este estudo teve como objetivo a obtenção de produtos bioativos produzidos por fungos endofíticos isolados das amêndoas de *Bertholletia excelsa* Bonpl. Atuando sobre a melanogênese. Para tanto, foi utilizado modelos *in vitro e in vivo,* com o objetivo de identificar substâncias com atividades biológicas *in vitro* metabolizadas pelos fungos que atuem sobre a melanogênese em Zebrafish além identificar o mecanismo de ação dos compostos identificados no extrato sobre estudos in docking.

Palavras-chave: Metabolitos secundários*;* Fungos endofíticos; inibidor da tirosinase; Castanha da Amazônia.

FERREIRA, ADRIANA MACIEL. BIOACTIVE METABOLITES OF FUNGI ISOLATED FROM *Bertholletia excelsa* Bonpl. AND ITS ACTION ON MELANOGENESIS IN MODEL Zebrafish (Hamilton, 1822). 2023. 59f. Tese (Doutorado em Biodiversidade e Biotecnologia- Rede BIONORTE) - Universidade Federal do Amapá, Macapá, 2023.

ABSTRACT

Obtaining new bioactive compounds with different action controls, with smaller effects, greater safety in use and greater power is a constant need in health. *Bertholletia excelsa* Bonpl., known as Amazonia nut, is one of the most important export products from the Amazon rainforest, with Brazil accounting for 75% of the market for this product. Bioprospecting is one of the tools used to obtain natural products with action on melanogenesis. One of the promising sources used in this type of investigation is the secondary metabolites produced by endophytic microorganisms obtained from plant species. Thus, this study aimed to obtain bioactive products produced by endophytic fungi isolated from almonds of *Bertholletia excelsa* Bonpl. Acting on melanogenesis. For that, in vitro and *in vivo* models were used, with the objective of identifying substances with in vitro biological activities metabolized by fungi that act on melanogenesis in Zebrafish.

Keywords: Secondary metabolites; Endophytic fungi; Tyrosinase inhibitors; Amazon nuts.

Sumário

INTRODUÇÃO

Bertholletia excelsa é a única espécie do gênero *Bertholletia*. Conhecida como "castanha do Brasil", "castanha do Pará" ou "castanha da Amazônia", suas sementes são um recurso alimentar muito apreciado pelas populações amazônicas, e o comércio das sementes ao nível internacional também já é bastante expressivo. É encontrada em florestas não inundadas da região amazônica da Bolívia, Brasil, Colômbia, Guiana, Guiana Francesa, Peru, Suriname, Venezuela, Trindade e Tobago [1].

A castanheira do Brasil (*B. excelsa*) é uma árvore amazônica que pode alcançar mais de 50 metros de altura, uma espécie arbórea pertencente à família Lecythidaceae, nativa da Amazônia. Representa a única espécie existente no gênero *Bertholletia* [2]. A castanha do brasil ou castanha-do-pará (*Bertholletia excelsa*), uma das riquezas da floresta Amazônica, representa importante componente na pauta de exportação da região (Fig. 1). Sua exploração desempenha papel fundamental na organização socioeconômica de grandes áreas extrativistas da floresta Amazônica [3].

Figura 1. Mapa de distribuição geográfica da *Bertholletia excelsa* Bonpl. Ocorrência confirmadas: Verde - Norte (Acre, Amazonas, Amapá, Pará, Rondônia e Roraima); Amarelo – Centro-Oeste (Mato Grosso). Fonte: floradobrasil.jbrj.gov.br/.

A planta produz ouriços que amadurecem e caem no chão da floresta, suas amêndoas são um dos principais produtos extrativista exportados pela região Norte, trazendo divisas para a economia dos estados da Amazônia brasileira, sendo encontrada ainda na

Bolívia, Colômbia, Guianas, Peru e Venezuela [4-5-6]. A amêndoa extraída da castanheira é bastante apreciada pelos consumidores devido ao seu sabor, assim como pelas características nutricionais. Rica em fibra alimentar, carboidratos, proteínas, lipídios essenciais (ômega 3 e 6), tocoferóis, compostos fenólicos, carotenoides, selênio e a vitamina E [7]. O óleo extraído das amêndoas é rico em ácidos graxos insaturados também apresenta relevância nutricional. Estas substâncias presentes nas amêndoas da castanha do brasil permitem atividade antioxidante, atuando sobre os radicais livres e prevenindo o envelhecimento celular precoce ao passo que podem agir contra doenças como doenças inflamatórias, câncer e arteriosclerose [7].

Os alimentos possuem uma microbiota natural variável concentrada principalmente na parte superficial, constituída por fungos e bactérias que possuem a função de deteriorá-lo, assim como exercer eventualmente atividade patogênica para o homem [8]. Na castanha do Brasil, os fungos filamentosos saprófitas são os que mais participam deste processo, podendo ser encontrados no ar, na água e no solo, sendo este último lugar, o reservatório primário de muitos fungos [8].

Entre os danos causados pela deterioração fúngica estão: a diminuição do poder germinativo, alterações no sabor e aroma, descoloração e mudanças químicas e nutricionais [9]. Vários estudos realizados sobre a microbiota da Castanha do Brasil mostraram que as espécies comumente isoladas são *Aspergillus flavus* Link; *Aspergillus nomius* Kurtzm an; *Aspergillus parasiticus* Speare; *Aspergillus niger* Tiegh; *Aspergillus tamarii* Kita; *Aspergillus pulverulentus* (McAlpine) Thom; *Aspergillus flavo* - furcatus Bat. e H. Maia, *Penicillium* glabrum (Wehmer) Westling; *Penicillium citrinum* Thom; *Rhizopus* spp. e *Fusarium oxysporum* Schltdl [10-11-12-13].

Estudos da microbiota em amêndoas da castanha do Brasil, demostra uma maior ocorrência dos gêneros *Aspergillus*, *Penicillium* e *Fusarium*, considerando-os nativos da microflora da castanha -do-Brasil [4].

Os fungos endofíticos são aquele que passam parte de seu ciclo de vida dentro de tecidos saudáveis de um hospedeiro, sem causar-lhe prejuízo [14-15-16]. Diversos estudos relatam que a interação com um endofítico pode proporcionar diversos benefícios ao

hospedeiro, tais como adaptação ao estresse ambiental, sinalização celular, produção de hormônios e antibiose [14-17]. Porém um ponto em comum entre todas as interações endófito-hospedeiro é o fornecimento de nutrientes e proteção contra o estresse ambiental externo e contra a competição microbiana para o micro-organismo endofítico [17].

Os micro-organismos endofíticos receberam uma maior atenção a partir da descoberta da produção de paclitaxel (Taxol®) pelo fungo endofítico *Taxomyces andreanae*. Este endófito foi isolado da planta *Taxus brevifolia*, sendo esta planta o primeiro organismo no qual o paclitaxel foi encontrado [14]. O paclitaxel é um agente antimicrotúbulo com atividade antitumoral, utilizado no tratamento de diversos tipos de câncer, como câncer de mama, ovário, pulmão entre outros [14-18].

Metabólitos secundários são sintetizados quando o crescimento microbiano está na fase estacionária, são frequentemente bioativos e de baixa massa molecular. Apresentam grande importância à humanidade, devido às atividades antibióticas e de importância farmacêutica, bem como atividades imunossupressoras e tóxicas. Estes não são normalmente derivados do substrato utilizado para o crescimento celular, sendo sintetizados a partir de um metabólito primário [19].

Em geral, esses metabólitos parecem ser formados quando grandes quantidades de precursores de metabólitos primários, tais como aminoácidos, acetato, piruvato e outros, são acumulados [20]. Apresentam algumas características como: distribuição taxonômica restrita - nem todas as linhagens de uma mesma espécie são capazes de produzir determinado metabólito; não são essenciais para o crescimento e reprodução do organismo; condições de cultivo, especialmente a composição do meio, controlam a formação destes metabólitos; são produzidos como um grupo de estruturas intimamente relacionadas; podem ser produzidos e são codificados por conjuntos de genes dispensáveis [19-21].

O isolamento, caracterização e identificação da bioatividade dos metabólitos dos fungos obtidos da *B. excelsa* podem conduzir à novas moléculas com potencial terapêutico, pois o estudo da prospecção química a partir de microrganismos em geral, levou a descoberta de vários compostos, os quais se destacam antibióticos, imunossupressores, agentes redutores de colesterol, entre outros [22]. Nos estudos das substâncias, um balanço entre a

atividade biológica versus a toxicidade é um parâmetro fundamental para verificar sua aplicabilidade [23]. Portanto, a busca de novos metabólicos de tais microrganismos é essencial, pois seus produtos naturais podem ser de grande relevância para o desenvolvimento biotecnológico de novos fármacos e na justificativa da manutenção da biodiversidade. Estudos recentes demostraram a eficiência dos metabolitos de fungos frente a melanogênese e no controle da dislipidemia. Uma vez que a descoberta de novas substâncias com atividade biológica pode levar ao desenvolvimento de novas tecnologias nacionais destinadas à área clínica de tratamentos dermatológicos e à comercialização de produtos cosméticos destinados à venda para a população que é acometida por alguma alteração na melanogênese, além de auxiliar na descoberta de novos compostos ou fármacos oriundo de produtos naturais com ação sobre carcinomas.

OBJETIVOS

1.1 GERAL

Isolar metabólitos secundários de fungos endofíticos de *Bertholletia excelsa* Bonpl. e avaliar as ações sobre melanogênese em modelo zebrafish (Hamilton, 1822)

1.2 ESPECÍFICOS

- Isolar, Cultivar e identificar os fungos extraídos da espécie vegetal *B. excelsa*;
- Selecionar os fungos produtores de metabólitos secundários ativos;
- Extração dos metabólitos secundários e analisar o perfil químico por HPTLC e Ressonância Magnética Nuclear – RMN de ^{1}H.
- Avaliar os efeitos dos extratos de fungo sobre a melanogênese em modelo *Zebrafish* (Hamilton, 1822);

molecules

MDPI

Review

Anti-Melanogenic Potential of Natural and Synthetic Substances: Application in Zebrafish Model

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Abstract: Melanogenesis is a biosynthetic pathway for the formation of the pigment melanin in human skin. A key enzyme in the process of pigmentation through melanin is tyrosinase, which catalyzes the first and only limiting step in melanogenesis. Since the discovery of its methanogenic properties, tyrosinase has been the focus of research related to the anti-melanogenesis. In addition to developing more effective and commercially safe inhibitors, more studies are required to better understand the mechanisms involved in the skin depigmentation process. However, in vivo assays are necessary to develop and validate new drugs or molecules for this purpose, and to accomplish this, zebrafish has been identified as a model organism for in vivo application. In addition, such model would allow tracking and studying the depigmenting activity of many bioactive compounds, important to genetics, medicinal chemistry and even the cosmetic industry. Studies have shown the similarity between human and zebrafish genomes, encouraging their use as a model to understand the mechanism of action of a tested compound. Interestingly, zebrafish skin shares many similarities with human skin, suggesting that this model organism is suitable for studying melanogenesis inhibitors. Accordingly, several bioactive compounds reported herein for this model are compared in terms of their molecular structure and possible mode of action in zebrafish embryos. In particular, this article described the main metabolites of *Trichoderma* fungi, in addition to substances from natural and synthetic sources.

Keywords: melanogenesis inhibitors; tyrosinase; melanin; danio rerio

1. Introduction

Estimates indicate that approximately 15% of the world's populations invest in skin whitening [1] with melanogenesis as one of the main reasons. Melanogenesis is a complex process with different physiological stages. Any imbalance in this process can cause different types of pigmentation deficiency,

classified as hypopigmentation or hyperpigmentation, and may occur with or without changes in the number of melanocytes. The serious pathological consequence of such physiological imbalance is cancer derived from melanocytes, or melanomas, which are among the most aggressive, metastatic, and lethal forms of skin cancer [2].

In addition to physiological imbalance, other factors can inhibit melanin production. These include pharmaceutical or cosmetic additives, which cause adverse side effects, such as skin irritation, cytotoxicity, and carcinogenicity. In addition, because of the low stability of some formulations and low penetration into the skin, their multiple use must be limited [3]. Moreover, studies reported that many have been linked to neurodegenerative diseases, including Parkinson's, Alzheimer's, and Huntington's diseases [4–7].

Melanogenesis occurs in melanocytes, which are found in the basal layer of the epidermis, with tyrosinase as the unifying biochemical characteristic of melanogenesis in plants and animals. Tyrosinase (EC 1.14.18.1), a copper oxidase, is a type 3 copper containing metalloenzyme widely distributed in bacteria, fungi, insects, plants, and animals, including humans, to produce melanin pigments [8]. Initially, tyrosinase is synthesized on the surface of the rough endoplasmic reticulum. It is considered a key and limiting enzyme for the in vivo synthesis of melanin. Melanin plays a key role in several biological functions, including the pigmentation process of mammalian dermis. As a component of primary immune response, it is a triggering agent of the wound healing system in plants and fungi [9].

The effect of tyrosinase is observed in various living organisms. In plants, it is observed in degradation processes. In fungi, tyrosinase acts in the differentiation of reproductive organs during spore formation [10–12]. In humans, tyrosinase, which regulates melanin, is responsible for the coloration of the skin, eyes, and hair, with high diversity among human populations [13–15].

The discovery of new molecules from natural products and fungal extracts which have anti-melanogenesis activity is ongoing. Since these molecules would be expected to minimize the side effects of pigmentation treatment, they represent a potent, low-cost and effective alternative [16–19].

Fungal metabolites have stood out as substantial melanogenesis inhibitors owing to their pharmacological potential [20]. Thus, fungi of different genera, which demonstrate anti-melanogenic activity with antibiotic action, and growth regulators in vegetables and fruits, among others, have attracted the interest of researchers who are pursuing the discovery and isolation of new compounds in the agricultural, food and pharmaceutical industries [21].

However, according to the World Health Organization (WHO), researchers must follow the NEQ (Needs Evaluation Questionnaire) validation process when carrying out pharmacological tests in traditional in vivo and in vitro systems in order to increase investment in research and innovation, mainly in underdeveloped countries.

Several in vivo models have been used extensively to investigate anti-tyrosinase mechanisms [22]; however, some are limited from a practical point of view and others from a physio/pathological point of view. Consequently, researchers have resorted to emerging models, such as zebrafish (*Danio rerio*). This model has the advantages of small size, ease of handling and maintenance, and rapid reproduction rate, as well as the high efficiency of drug penetration through skin and gills [23,24]. Moreover, zebrafish have a fully characterized genome with

functional domains of many key proteins nearly identical to their human homologues [25,26].

In addition, the use of the zebrafish model has enabled the development of new approaches, the refinement of techniques, and the insertion of quantitative and qualitative parameters into the screening of bioactive compounds based on phenotypes. In particular, zebrafish analysis has been linked to the presence or absence of melanin since the pigmentation process can be observed on the surface of the zebrafish embryo without complicated experimental procedures [27–30].

Therefore, this work aimed to review the most recent scientific information available on melanogenesis inhibitors of natural (plant or fungal) and synthetic origin using zebrafish as an experimental model. An important part of the review involves clarifying how the zebrafish depigmenting sy stem works and whether it resembles that of humans. The inclusion criteria for this study were original articles exclusive to the genus and species studied with full text available in portuguese, english or other languages. Exclusion criteria included abstracts, online sites without scientific sources, incomplete text, and unrelated or repeated articles, according to the methodology previously described [31].

The descriptive words used in our search were (a) *Trichoderma* spp. and their secondary metabolites; correlated to the potential, (b) anti-melanogenic agent, (c) Tyrosine, (d) natural and synthetic products in the zebrafish model.

2. Melanin and Tyrosinase Mechanism of Action

Melanin is synthesized by melanocytes, which are directly related to neighboring keratinocytes. It is an amorphous substance formed by the polymerization of phenolic and indole compounds. Specific to the skin, melanin protects the epidermis against harmful stimuli, such as UV-radiation, through melanogenesis, the process regulating autocrine or paracrine factors, including α-melanocyte-stimulating hormone and endothelin. Together with this intricate system, keratinocytes and skin cells, such as fibroblasts and immune cells, are regulators of the behavior of melanocytes, which, in turn, are produced by paracrine factors.

This series of reactions makes the polymerized material available spontaneously as melanin. The formation of melanin is dependent on the catalysis of L-tyrosine in L-DOPA, but not intermediate dopachrome, also called (TRP2), though both are direct products of the tyrosinase cycle (Figure 1).

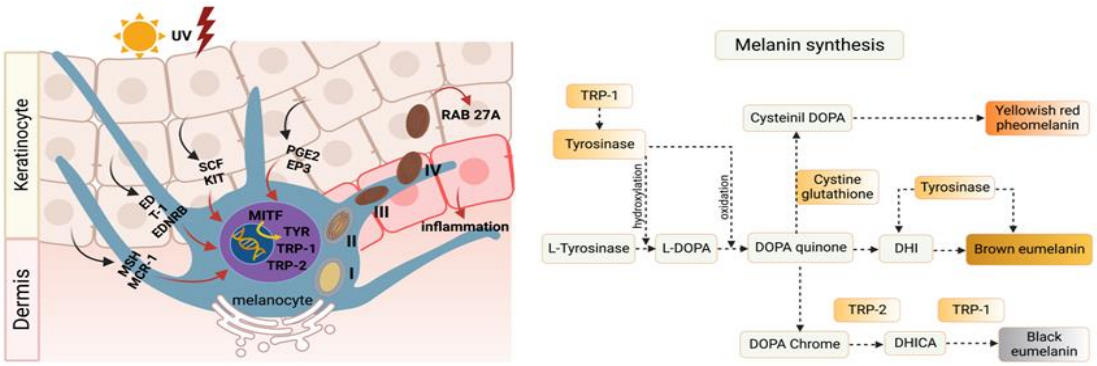


Figure 1. Synthetic pathway of melanin. Melanin synthesis begins with the catalysis of substrates L-phenylalanine and L-tyrosine to produce L-DOPA via phenylalanine hydroxylase (PAH), tyrosinase and, partially, tyrosinase hydroxylase 1 (TH-1). Pathways are then divided into eumelanogenesis or

pheomelanogenesis. The other melanogenic enzymes are TRP-2 (DCT) and TRP-1 for eumelanogenesis.

Melanin is an amorphous polymer, negatively charged, but derived from the auto-oxidative polycondensation of several quinone groups with hydrophobic properties [32]. Therefore, the pathway of melanogenesis (Figure 1) can be conveniently divided into two phases: proximal, which consists of the enzymatic oxidation of a monophenol (tyrosine) and/or o-diphenol (L-DOPA), to its corresponding *O*-quinone and distal, which is represented by chemical and enzymatic reactions occurring after the formation of dopachrome to direct the synthesis of eumelanins, which are either derived from DHICA (5,6-dihydroxyindole-2-carboxylic acid; brown) or from DHI (5,6-dihydroxyindole; black) [33–37].

Therefore, synthesis of eumelanin is directly linked to the process of melanin pigments responsible for retaining the ability to deactivate free radicals, peroxides and absorb heavy metals and toxic electrophilic metabolites, thus exhibiting strong antioxidant activity in addition to absorbing light in a wide spectrum range including UV [3,38]. By deregulating this system, hyperpigmentation can occur. This is equivalent to tyrosinase hyperactivity, which is normally associated with pathological disorders, such as spots, melasma and the appearance of melanomas [39]. Therefore, it is fundamentally important to regulate tyrosinase productions so that balance in the melanogenesis process is maintained and pathogenicity is avoided [40,41].

3. Inhibitors of Melanogenesis by Fungi of the Genus Trichoderma

The literature presents several potential tyrosinase inhibitors, both from natural and synthetic sources. However, studies that investigate the molecular and functional characterization of this enzyme are rare, mainly those specific to anti-melanogenic activity originating from organisms, such as heterotrophs. In this sense, fungi stand out for their potential, with greater occurrence in the genus *Trichoderma* (Hypocreales, Ascomycota), having more than 300 species with high adaptive capacity, favoring their presence in different natural environments under different climatic conditions [42–44].

Species of the genus *Trichoderma*, order Hypocreales, have greater phytogeographic occurrences in the soil of regions with a humid tropical climate. Such conditions produce a class of Hyphomycetes characterized as filamentous and cosmopolitan fungi with diverse biotechnological applications [43,45,46]. Given these characteristics, various species of *Trichoderma* use a wide variety of compounds as a source of carbon and nitrogen, a typical characteristic of fungi from saprophytic soil [47].

Drawing on their chemodiversity, tyrosinase inhibitors biosynthesized by fungi are derived from isoflavones and pyrones, along with terpenes, steroids, and alkaloids, which can reversibly or irreversibly inactivate the enzyme [48].

In particular, some studies report *Trichoderma reesei* and *Trichoderma harzianum* as significant producers of extracellular tyrosinase, previously characterized, isolated and purified by precipitation with ammonium sulfate (85%). Purified tyrosinase exhibited a final specific activity of 69.39 and 65.11 U/mg of protein, values which double the purification of 21.09 and 14.93 for *T. reesei* and *T. harzianum*, respectively [49,50].

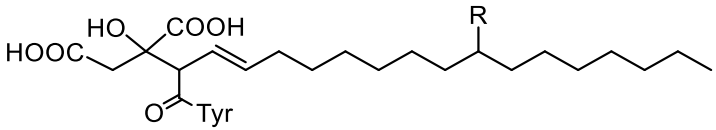
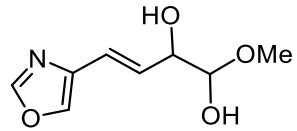
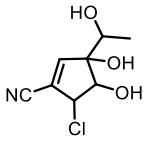
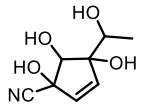
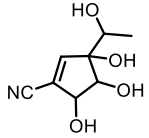
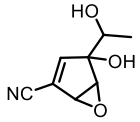
Other studies described the activity of fungal extracts of *Trichoderma atroviride*, *Trichoderma gamsii*, *Trichoderma guizhouense* and *Trichoderma songyi*. These extracts were reported to have tyrosinase inhibitory capacity associated with the elimination of reactive quinone products. Furthermore, a trichoviridine cyclopentyl isocyanide, MR566A and MR566B, isolated from *T. reesei*, showed moderate cytotoxicity against the human melanoma cell line A375-S2 [51].

Studies show that antioxidant activity is associated with the ability to inhibit tyrosinase. However, fungal extracts of *Trichoderma atroviride*, *Trichoderma gamsii*, *Trichoderma guizhouense* and *Trichoderma songyi*, of marine origin, which showed a considerable ability to inhibit tyrosinase (IC 50 < 100 μg/mL), demonstrated low radical scavenging activity (<50%). This suggests the presence of other mechanisms that inhibit tyrosinase, such as competitive inhibitors, including copper chelators that inhibit this metal coenzyme, or suicide inhibitors that inactivate tyrosinase by altering tertiary and quaternary structures of the enzyme [52].

Viridiofungins, broad spectrum antifungal agents, are derived from the secondary metabolite of *Trichoderma viride*. They act as inhibitors of tyrosinase and farnesyl transferase and the farnesylation of the oncogenic Ras protein, indicating their potential to treat cancer [53]. Furthermore, an oxazole derivative called melanoxazal, which is isolated from the fermentation broth of *Trichoderma strain* ATF-451, showed strong inhibitory activity against mushroom tyrosinase [54].

A strain of *T. harzianum*, an isomer designated as MR304A, was isolated and identified as an isocyanide compound, demonstrating inhibition of melanin formation in *Streptomyces bikiniensis* and B16A melanoma cells [55]. Still related to *T. harzianum* isolated from soils, the authors verified the inhibition of melanin synthesis by two new tyrosine inhibitors. MR566A, along with a new oxazole compound, MR93B, exhibited activity similar to that of MR93A. in addition to isocyanide compounds, identified as derivatives of alkyl citrate (Table 1), [56,57] a group of isocyanide compounds acting in the inhibition of tyrosinase activity [20].

Table 1. Secondary metabolites found in fungi of the genus *Trichoderma* with anti-melanogenic effect. Structures of tyrosinase inhibitors from *Trichoderma* spp.

Fungus	Molecules and Their Derivatives	References
Trichoderma viride	 Viridiofungins and derivatives (R = -OH; -H; -C=O)	Reino et al. [53]
Trichoderma spp.	 Melanoxazal	Takahashi et al. [54]
Trichoderma harzianum	 MR566A 1-(3-chloro-1,2-dihydroxy-4-isocyano-4-cyclopenten-1-yl)ethanol  MR566B 1-(1,2,3-trihydroxy-3-isocyano-4-cyclopenten-1-yl)ethanol  1-(1,4,5-Trihydroxy-3isocyano-cyclopenten-2-enyl)ethanol  2-Hydroxy-4-isocyano-a-methyl-6-oxabicyclo[3.1.0]hex-3-ene-3-methanol	Lee et al. [55]

The strain *Trichoderma viride* H1-7 from a marine environment presented a tyrosinase inhibitory factor through the Homothallin II structure (Table 1). It was preliminarily isolated and studied as an antibiotic from *Trichoderma koningii* and *T. harzianum* [58]. These fungi are excellent producers of extracellular enzymes, and seven new molecules were isolated from the metabolites of these species, demonstrating anti-melanogenic activity by binding to the copper active site [48].

In addition to experimental research involving endophytic fungi with enzymatic activity against tyrosinase, the supernatant of the metabolite of *Trichoderma atroviride* has found an industrial use in the manufacture of functional whitening cosmetics, but it is also a potential inhibitor of tyrosinase [59].

4. Anti-Melanogenic Activity in Zebrafish Embryo

Assays involving the mechanisms of action of tyrosinase have become increasingly important for two reasons: (a) the elucidation of inhibitory pathways in melanin pigment synthesis; and (b) the growing demand for anti-melanogenic agents capable of reducing or inhibiting the unwanted side effects of current treatments [60]. Consequently, R&D efforts have turned to experimental models, such as zebrafish, able to facilitate the in vivo screening of anti-melanogenic agents. Such models are low in cost, but high in fertilization rate and genetic homogeneity, relative to mammalian models, thus enabling screening for tyrosinase-reactive drugs and cosmetics [60].

Compared to the zebrafish model, traditional models have both physiological and economic disadvantages. Therefore, experimental robustness and safety in zebrafish, as a phenotype-based screening model for melanogenic inhibitors or stimulators, have advanced considerably in recent years [61,62].

Because it is a model with biological similarity to more complex organisms, its genome shares more than 70% genes with humans [63,64]. This small teleost has three types of pigment cells: iridophores (containing reflective lines, blue), xanthophores (yellow) and melanophores, factors that have favored its use (Figure 2). The existence of homogeneity in the genetic characteristics of genes related to melanogenesis in zebrafish, such as TYR that gives instructions for making tyrosinase, which resemble mammalian genes, is decisive for the selection of this experimental model in anti-melanogenic studies [65,66].

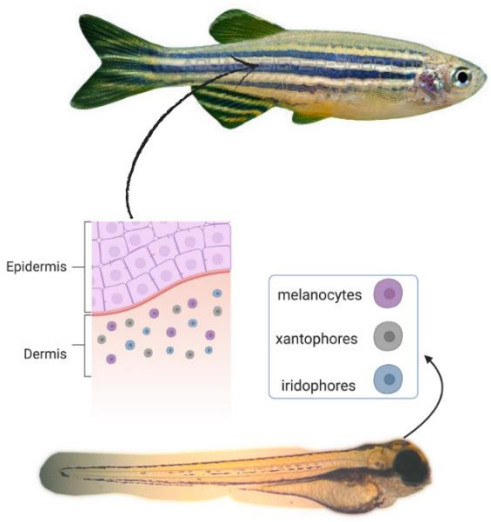


Figure 2. Zebrafish pigment cells include xanthophores (xp), iridophores (ip) and melanophores (mp). The main external barrier is the epidermis, which consists of two layers of cells connected by tight junctions. Certain substances can pass through the epidermis mesenchymal space by diffusion or by active transport (adapted from Irene et al. [67]).

The zebrafish model can be used to understand phylogeny mechanisms and TYR patterns expressed in melanophores relative to time. Specifically, the formation of pigmentation in zebrafish begins directly in the epithelium and pigment of the developing retina with subsequent transcription of TYR within 16.5 h post fertilization (hpf). Melanin in melanophores can be detected in the dorsolateral skin and retina at approximately 24 hpf. Because melanin is synthesized in melanophores in the early stages of zebrafish embryonic development, microscope-assisted observation is possible [30,61,66].

On the other hand, tyrosinase inhibitors derived from secondary metabolites of bacteria and fungi are known to produce anti-melanogenic compounds. Currently, many of these molecules have been identified (Figure 3) and tested for their anti-melanogenic activity in zebrafish [68,69].

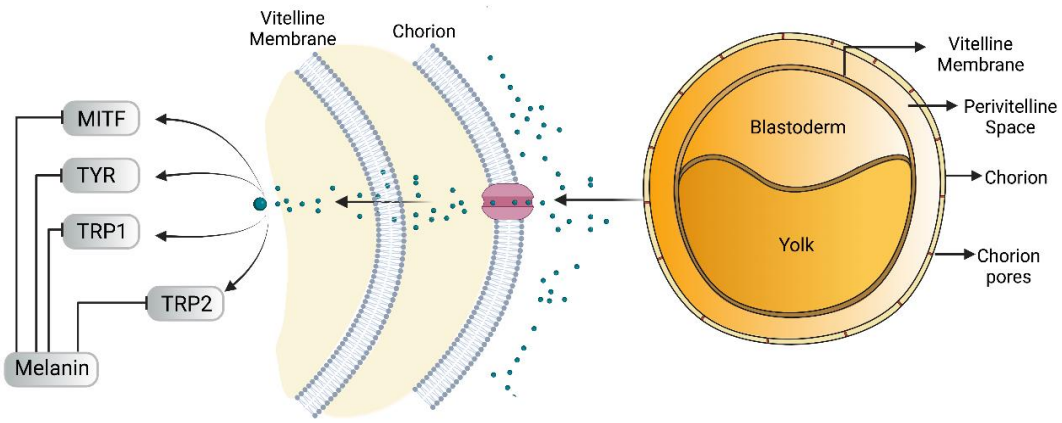


Figure 3. Schematic diagram showing the possible mechanism of action of depigmenting agents on zebrafish embryo. The embryonic chorion is composed of a nanoporous outer membrane 500–700 μM in diameter. The chorion is composed of a three-layer structure (extraembryonic mesoderm) with four main polypeptides. Small or hydrophobic molecules can diffuse across the lipid bilayer (adapted from Bonsignorio et al. [70]; Jon et al. [71]).

Among the most commercially used tyrosinase inhibitors, kojic acid and its derivatives are derived from secondary metabolites produced by fungi of the genera *Aspergillus* and *Penicillium* [35,72]. These metabolites are used in the cosmetics industry for skin whitening, as well as a food additive to prevent enzymatic browning in the food oxidation process. However, they are also used as a standard in research involving melanogenesis inhibitory activity in the zebrafish model [3,37]. Kojic acid is hydrophilic and acts as a Cu^{2+} chelating agent at the active site of tyrosinase to suppress the tautomerization of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid [68,73].

Ethanolic extract of *Laetiporus sulphureus* (LSE) and *Agaricus silvaticus* (ASE), edible mushrooms, underwent biochemical mapping for their anti-melanogenic effect and were found to effectively inhibit melanogenesis in a dose-dependent

manner (400–500 μg/mL). However, the exploited extracts at the depigmenting dose did not show adverse effects on the melanocytes of zebrafish embryos [74].

To validate the *in vivo* anti-melanogenesis activity of *Antrodia cinnamomea* ethanol extracts, a study was based on the zebrafish phenotype. In experiments, the AC_Et50_Hex extract fraction exhibited depigmenting activity similar to that of kojic acid (56.1% vs. 52.3%), but with lower dosage (50 ppm vs. 1400 ppm), in addition to demonstrating less toxicity to embryos [75].

Studies which evaluated the ability of modified Shiitake extract (A37) and wild Shiitake extract (WE) demonstrated that A37 conferred less pigmentation in zebrafish embryos and inhibited the growth of melanoma cells better than WE. The difference in cell cycle profile suggests that the greater anticancer effect of the A37 extract results from changes in the metabolite produced as a result of mutation such that A37 is also capable of inhibiting GSK3β phosphorylation. Both extracts contain 14 compounds in common [76].

Azelaic acid [(1, 7-heptanedicarboxylic acid) Table 1], produced by *Pityrosporum ovale*, a strain found naturally in wheat, rye and barley [77,78], is often used for the treatment of acne, rosacea, skin pigmentation and freckles. The compound can bind to amino and carboxyl groups and prevent the interaction of tyrosine in the active site of tyrosinase, acting as a competitive inhibitor [73,79,80].

Interestingly, azelaic acid has demonstrated thioredoxin reductase inhibition in cultured human keratinocytes, melanocytes, melanoma cells, murine melanoma cells and purified enzymes from *Escherichia coli*, rat liver and human melanoma. [77,81] This may explain the antiproliferative and cytotoxic effect, the synthesis of deoxyribonucleotides. Moreover, azelaic acid, when combined with taurine, an antioxidant compound, inhibits tyrosinase by activating the ERK pathway [82,83].

5. Natural Products Used as Melanogenesis Inhibitors in Zebrafish

Melanogenic inhibitors 1-phenyl-2-thiourea, arbutin, kojic acid, 2-mercaptobenzothiazole and synthesized compounds (haginin, YT16i) [61] were used in zebrafish embryos, and the inhibitory effects on pigmentation were indicated. However, compound YT16i showed major abnormalities in terms of morphological deformities and cardiac function, along with high toxicity at higher concentrations (Table 2). [66]

Table 2. Compounds and metabolites derived from plant species used as melanogenesis inhibitors in zebrafish embryos relative to concentration and toxicity.

Entry	Bioactive Compound/Structure	Mechanism	Toxicity/Concentration	Reference
1	Fisetin	Blocks tyrosinase-induced tyrosine oxidation	Did not show (25 μM, 50 μM, 75 μM, and 100 μM)	Ilandarage et al. [61]
2	KDZ-001	TYR active site	Did not show (10 μM)	Kyu-Seok et al. [60]
3	1-phenyl-2-thiourea	Unknown	Did not show	Ilandarage et al. [52]

4	2-mercaptobenzothiazole	Unknown	Did not show	Ilandarage et al. [61] 2020; Tae-Young et al. [66]
5	Haginin	Unknown	Did not show	Tae-Young et al. [66]
6	YT16i	Unknown	Showed toxicity (1 mM)	Tae-Young et al. [66]
7	triclocarban (3,4,4′-trichlorocarbanilide)	Unknown	Showed toxicity (50 μg/L).	Giulia et al. [62]
8	Adenosine	Inhibits melanogenesis by down-regulating tyrosinase	Did not show (400 μM)	Mi Yoon et al. [84]
9	*Ecklonia cava* seaweed extract	Unknown	Slight toxicity (400 μM)	Kang et al. [85]
10	*Sargassum siliquastrum* seaweed extract	Unknown	Did not show (400 μM)	Kang et al. [85]
11	*Ganoderma formosanum* mycelium extract	Blocks tyrosinase-induced tyrosine oxidation	Did not show (400 ppm)	Kai et al. [86]

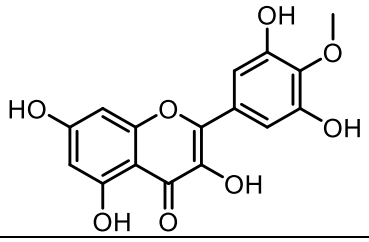
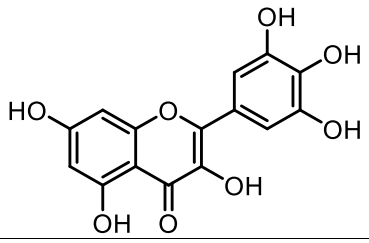
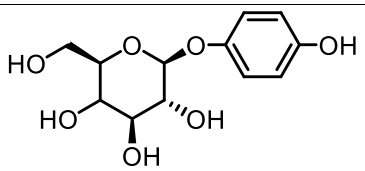
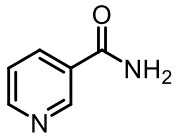
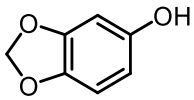
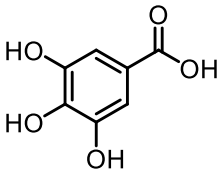
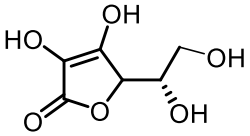
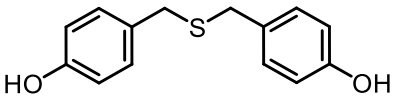
Triclocarban (3,4,4′-trichlorocarbanilide) has TYR inhibition activity and is present in soaps, shampoos, cosmetic detergents, and toothpastes [62]. At a concentration of 50 μg/L, zebrafish embryos exposed to triclocarban showed signs of toxicity, such as mortality and a significant index of teratogenicity [62]. Based on this characteristic, several studies have already shown that the exposure of developing embryos to chemicals considered pollutants will cause the dysregulation of thyroid hormones, resulting in craniofacial and ocular pathologies [87,88].

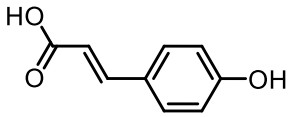
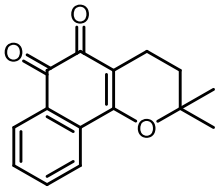
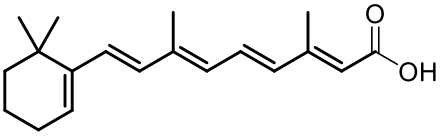
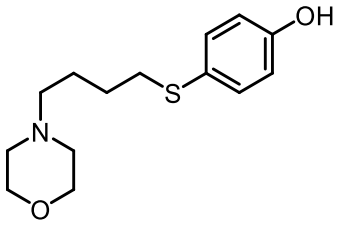
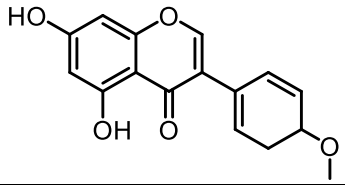
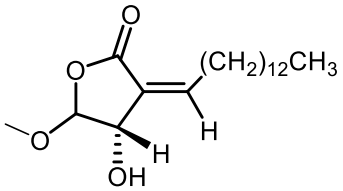
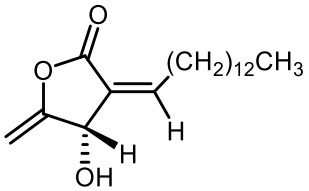
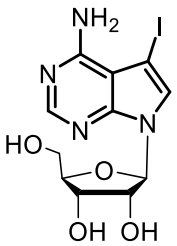
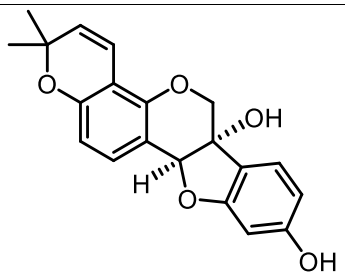
Omeprazole reduces pigment area density in zebrafish embryos by 63% at a concentration of 60 μM. Furthermore, intracellular TYR activity was decreased by 48%, compared to untreated zebrafish embryo, after treatment with omeprazole [89].

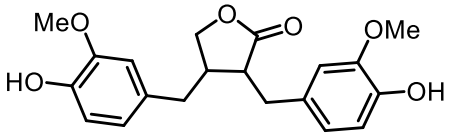
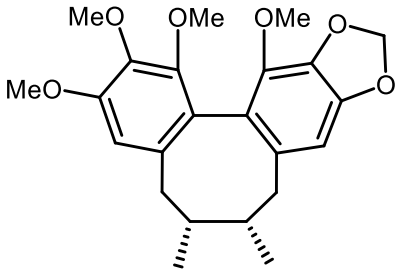
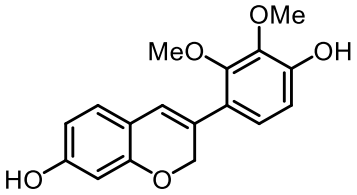
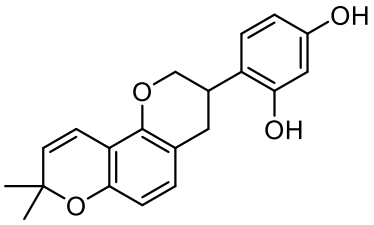
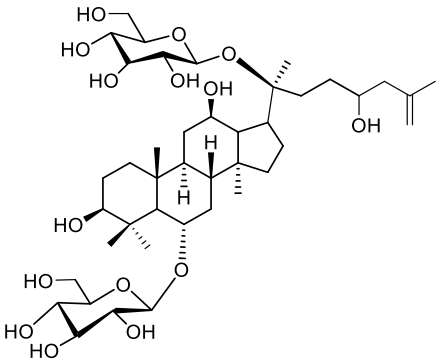
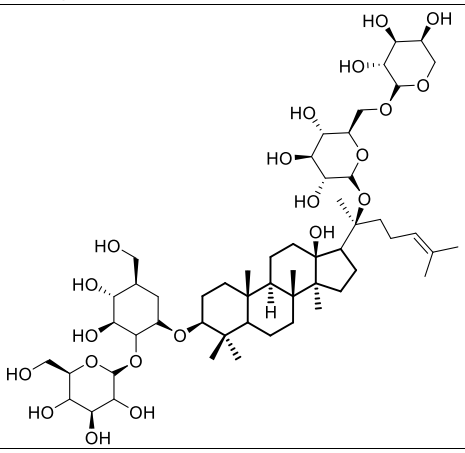
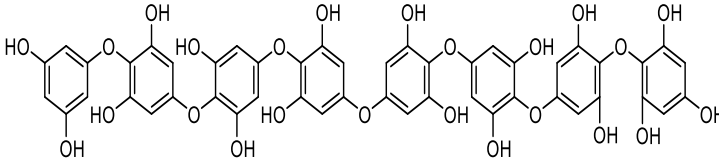
Several studies on plant and fungal extracts used zebrafish as an in vivo experimental model to investigate tyrosinase inhibition or their activity as depigmenting agents. Among these studies, extracts from *Ecklonia cava* and *Sargassum siliquastrum* seaweeds showed slight toxicity. *Phlorofucofuroeckol-A* (PFF-A) isolated from a seaweed species, *Ecklonia cava*, demonstrated an attenuating effect against tyrosinase in the B16F10 cells of zebrafish embryos. When evaluating the safety and efficacy of PFF-A for anti-melanogenic effects, the study tested low doses of PFF-A (1.5–15 nM) [90]. This suggests that low doses of *E. cava* derived PFF-A can suppress embryonic pigmentation and melanogenesis. This indicates the possibility of using PFF-A as an anti-melanogenic agent [90].

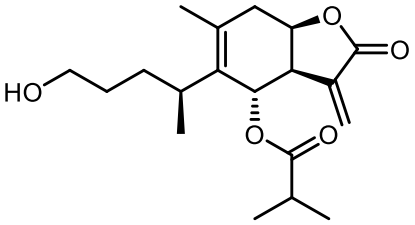
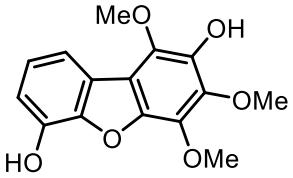
Studies with extracts of the marine *Pseudomonas anoectochilus* and *P. narcissus* showed a potentiated effect in inhibiting zebrafish embryo TYR [85,91], and the natural compound derived from oleic acid, produced in the small intestine as oleoylethanolamide, reduced TYR by about 49.5% at a concentration of 150 μM in zebrafish embryos [92]. In contrast, sesamol, a bioactive lignan from *Sesamum indicum*, inhibited melanin biosynthesis in a concentration-dependent manner in zebrafish embryo. The absence of pigmentation can be explained by reduced TYR activity and gene expression related to melanogenesis [93] (Table 3).

Table 3. Compounds and metabolites derived from plant species used as melanogenesis inhibitors in zebrafish embryos.

Entry	Name	Chemical Structure	Reference
1.	Mearsetin		Huang et al. [22]
2.	Myricetin		Huang et al. [22]
3.	Arbutin		Ilandarage et al. [61]
4.	Niacinamide		Hako-Zaki et al. [81]
5.	Sesamol		Baek et al. [93]
6.	Gallic acid		Kumar et al. [94]
7.	Ascorbic acid		Kumar et al. [94]
8.	Bis(4-hydroxybenzyl)sulfide		Wang et al. [95]

9.	Coumaric acid		Kim et al. [96]
10.	β-Lapachone		Kim et al. [97]
11.	Tretinoin		Huang et al. [98]
12.	2-Morpholinobutyl-4-thiophenol		Huang et al. [98]
13.	Biochanin A		Lin et al. [99]
14.	Subamolide A		Hiu et al. [100]; Wang et al. [101]
15.	Linderanolide B		Hiu et al. [100]; Wang et al. [101]
16.	5-Iodotubersidin		Kim et al. [102]
17.	Glyceollin I		Shin et al. [103]

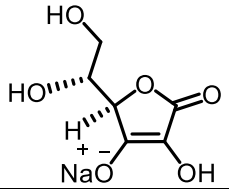
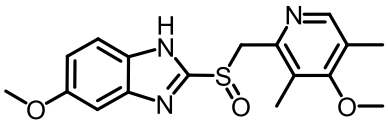
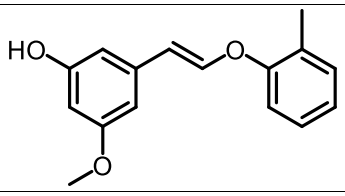
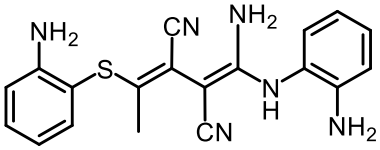
18.	Arctigenin		Park et al. [104]
19.	Gomisin N		Chae et al. [105]
20.	Haginin A		Kim et al. [106]
21.	Glabridin		Chen et al. [107]
22.	Floralginsenoside A		Lee et al. [108]
23.	Ginsenoside Rb2		Lee et al. [109].
24.	Octaphlorethol A		Kin et al. [110]

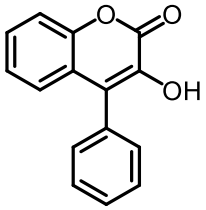
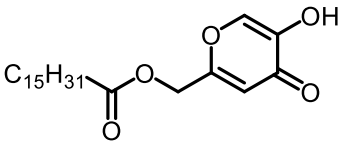
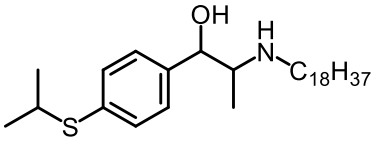
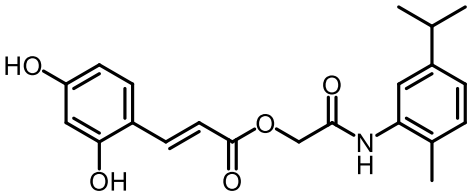
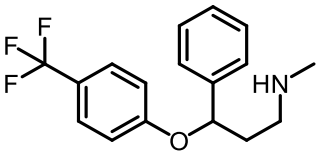
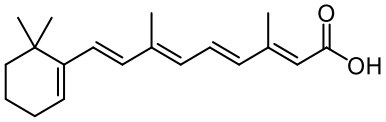
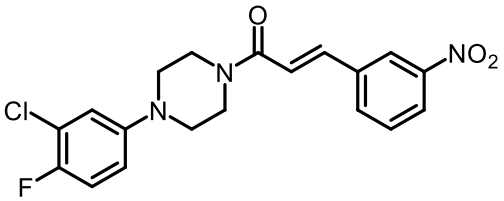
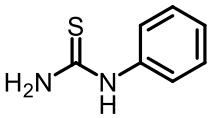
25.	6-O-isobutyrylbritannilactone		Dae et al. [111]
26.	2,3,7,8-tetrachlorodibenzo-p-dioxin		Henry et al. [112]

6. Synthetic Compounds Used as Melanogenesis Inhibitors in Zebrafish

Low molecular weight synthetic compounds between 100 and 300 g/mol can, when subjected to anti-melanogenic activity in zebrafish, be divided into several categories and molecular size. [113] For example, phenylthiorea, [86,96,114] sodium erythorbate, [34] 2-methylphenyl-*E*-(3-hydroxy-5-methoxy)-styryl ether, [98] 4-phenyl hydroxycoumarins, [115] kojic acid palmitate [116] and MEK-I (Table 4) inhibited melanophores in zebrafish embryos. Since these compounds vary by molecular size, stability, hydrophilicity and hydrophobicity, they can permeate the membrane and accommodate bioavailability in the embryo depigmentation process [98].

Table 4. Synthetic compounds used as melanogenesis inhibitors in zebrafish embryos.

Entry	Name	Chemical structure	Reference
9	Sodium erythorbate		Chen et al. [34]
12	Omeprazole		Baek et al. [89]
7	2-Methylphenyl-E-(3-hydroxy-5-methoxy)-styryl ether		Huang et al. [98]
2	MEK-1		Huang et al. [98]

8	4-phenyl hydroxycoumarins		Veselinovi´c et al. [115]
4	Kojic acid palmitate		Lajis et al. [116]
1	Suloctidil		Li et al. [117]
3	Compound 6		Abbas et al. [118]
5	Fluoxetina		Shang et al. [125]
6	Tretinoína		Shang et al. [119]
11	(*E*)-1-(4-(3-chloro-4-fluorophenyl)piperazin-1-yl)-3-(3-nitrophenyl)prop-2-en-1-one		Shang et al. [119]
10	Phenylthiourea		Kim et al. [96]; Hsu et al. [86]; Thach et al. [120]

Hydrophobicity is an important feature that demonstrates an affinity for permeating cell membranes. For zebrafish, several membrane layers must be taken into account, including chorion, melanocyte cell membrane and melanosome plasma membrane. [70,121].

Chorion is a porous channel measuring 0.5 to 0.7 µm in diameter with a gap at intervals of 1.5 to 2.5 µm. It surrounds the embryo, thus reducing the rate of diffusion of small molecules in the embryo (Figure 3) [103,106]. Most of these compounds show conformity in the benzene ring structure with a varied number of hydroxyl groups (OH) bonded to it. This chemical feature may explain the cellular permeability that leads to TYR inhibition. [70,122,123].

Twenty-seven new cinamides, consisting of cinnamic acid derivatives similar to 1-aryl piperazines, were synthesized and evaluated for potential tyrosinase inhibitory activity. Among them, 3-chloro-4-fluorophenyl moiety at the N-1 of the

piperazine ring was essential for potent tyrosinase inhibitory effect with 3-nitrocinnamoyl and 2-chloro-3-methoxycinnamoyl. In general, all compounds characterized by the presence of 1-(3-chloro-4-fluorophenyl)piperazine demonstrated the ability to inhibit melanogenesis in A375 human melanoma cells and zebrafish embryos. One of the most potent compounds in this series, 19t, significantly reduced embryonic pigmentation at a concentration of 50 μM, but showed 100% mortality in an acute toxicity test [124].

7. Conclusions

Tyrosinase plays a key role in disorders related to depigmentation changes in humans. Thus, TYR inhibitors may be the best option for treatment. Much research has been advancing in the discovery of new inhibitors. A variety of plants and fungi are important producers of bioactive metabolites inhibiting tyrosinase. *Trichoderma* is the most studied genus in terms of tyrosinase inhibition since metabolites of its species are derived from isoflavones and pyrones, along with terpenes, steroids and alkaloids, which can reversibly or irreversibly inactivate the enzyme. In recent years, research has guided important advances in the development of technologies and in the screening of bioactive compounds. Moreover, in vivo tests have intensified the use of the experimental zebrafish model based on phenotypes in which melanin pigments can be observed on the zebrafish surface, allowing the simple observation of the pigmentation process without complicated experimental procedures. For this reason, the zebrafish is gaining increasing viability as an in vivo model to evaluate the depigmenting activity of melanogenic regulatory compounds.

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Article

Trichoderma asperellum extract isolated from Brazil nuts (*Bertholletia excelsa*. BONPL): *in vivo* and *in silico* studies on melanogenesis in zebrafish

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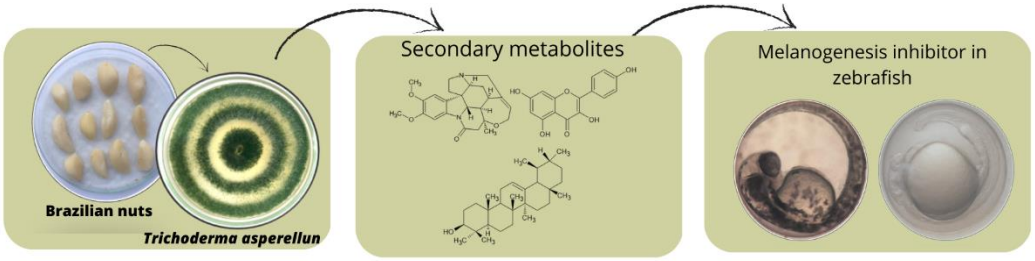
Abstract: Endophytic fungi are those that present part of their life cycle in healthy tissues of different plant hosts in symbiosis without causing harm. At the same time, fungus-plant symbiosis makes it possible for microorganisms to synthesize their own bioactive secondary metabolites while in stationary stage. Such microorganism-derived secondary metabolites contribute. Therefore, this work aimed to. To accomplish this, the endophytic fungus *Trichoderma asperellum* was isolated from *Bertholletia excelsa* (Brazil nut) almonds. The fungus was cultivated and extracted with ethyl acetate, obtaining AM07Ac. Then, using HPTLC (High-performance thin-layer chromatography) and Nuclear Magnetic Resonance (^{1}H NMR), β-amyrin, kaempferol, and brucine were identified as major compounds. Further *in vivo* assays in Zebrafish demonstrated the activity of AM07Ac on melanogenesis by producing a concentration-response inhibitory effect, which, through an *in silico* study, proved to be related to the noted major compounds known to inhibit tyrosinase activity. The inhibition of tyrosinase prevents melanin accumulation in skin. Therefore, these results imply the importance of investigating microorganisms and their pharmacological activities, in particular the endophytic fungus *Trichoderma asperellum* as a generator of active metabolites for melanogenesis modulation.

Keywords: Amazon fungi; Fungus extract; *Trichoderma asperellum*; Melanogenesis; Zebrafish



1. Introduction

Endophytic fungi spend part of their life cycle within healthy tissues of diverse plant hosts in symbiosis without causing any harm. [1-3] At the same time, fungus-plant symbiosis makes it possible for microorganisms to synthesize their own bioactive secondary metabolites with low molecular mass during the stationary phase [4-5] and form in the presence of abundant precursors of primary metabolites, such as amino acids, acetate, pyruvate, and others. [6]

The Amazon holds about 80% of the world's biodiversity. In Brazil, several tree species native to the Amazon region, such as *Bertholletia excelsa* H. B. K., are intrinsic to the food culture of traditional peoples and the economic development of the country. Wood, fruits, oil, and almonds of these species are widely used as raw materials. [7] The oil extracted from almonds is rich in unsaturated fatty acids (oleic, linoleic, and linolenic) with nutritional relevance owing to the content of vitamins and minerals. These substances present in Brazil nut almonds have antioxidant activity, acting on free radicals and preventing premature cell aging, but they can also act against inflammatory diseases, cancer, arteriosclerosis, and others. [8-9] The Amazon rainforest with its hot and humid climate also favors the growth and symbiosis of fungi and other microorganisms. [10]

In particular, these climatic conditions are favorable to the production of bioactive metabolites from endophytic fungi. Compared to higher organisms, these metabolites are renewable, and large-scale production of bioactive metabolites can be carried out using existing technology, such as changing the environment and optimizing cultivation conditions. From the perspective of environmental conservation, only a single, small removal of fungus from the natural environment is required. [11-12]

Recently, enzyme inhibitors have been highlighted as substantial tools with pharmacological potential. [13] As an important example, different genera of fungi produce several bioactive compounds with anti-tyrosinase activity, including antibiotics, enzymes, enzyme inhibitors, and growth promoters. These can be applied in the agricultural, food, and pharmaceutical industries. [14]

During melanogenesis, melanin is produced and stored in melanocytes that contain tyrosinase. Owing to the toxicity of intermediates in this pathway, such as 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and quinones, this reaction is restricted to the interior of specialized organelles in melanocytes, the melanosomes. [15] Excessive melanogenesis can lead to skin darkening and abnormal hyperpigmentation, causing various dermatological problems, such as freckles, melasma, senile lentigines, and even skin cancer. [16] Melanosomes are lysosome-like organelles that contain all the enzymes necessary for the melanogenic process, such as tyrosinase (TYR), dopachrome tautomerase (DCT), and tyrosinase-related proteins 1 and 2 (TYRP-1 and 2). [17] The maturation process of this organelle begins inside melanocytes and ends with the transfer of melanin granules to keratinocytes. [18-19]

In recent decades, several phenolic compounds that inhibit tyrosinase have been studied. [20] These efforts have led to the discovery of inhibitors, such as arbutin, kojic acid, and hydroquinone. [21-22] However, the use of these agents has been limited owing to low stability, insufficient biological activity, side effects, high toxicity, and limited ability to penetrate tissue. [22]

Studies demonstrate that fungi belonging to Aspergillus, Paecilomyces, Agaricus, and Myrothecium produce a range of compounds with inhibitory

activity against tyrosinase. Another genus related to the production of compounds with anti-tyrosinase activity is Trichoderma, likely by combining enzymatic degradation of the cell wall with the production of different secondary metabolites. [23-24]

Among the secondary metabolites of fungi are compounds that can stimulate the production of tyrosinases, such as phenolic compounds, aromatic compounds, and metallic ions. Tyrosinase can oxidize a wide range of phenolic compounds, peptides, and proteins that comprise tyrosyl residues. [25-27] The main step in the oxidation reaction of phenolic compounds by tyrosinase can, however, be impeded by several inhibitory enzymes. [28-30]

Trichoderma asperellum represents a potential source of underexplored bioactive substances. Therefore, both *in vitro* and *in vivo* tests are necessary to validate the biological activities of these substances. To perform such validation, a new model vertebrate has been introduced, Danio rerio, popularly known as Zebrafish (ZF). This is a freshwater fish belonging to the Cyprinidae family (teleost class), measuring between 3 and 4 cm and originating in India. [31] The ZF genome has been fully mapped, and it demonstrated 70% genetic homology with humans. [32] As such, it has become an experimental model for the study of genetic and biological mechanisms of numerous human diseases. [33]

The high fertility rate and rapid development of ZF make it an ideal model to elucidate the molecular basis of several diseases, [34-35] as well as screen for bioactive compounds, such as the presence or absence of melanin, the easily visualized pigment we intend to study in this work. [36]

2. Materials and Methods

2.1. Collection of plant material and isolation of *Trichoderma asperellum* fungi

Bertholletia excelsa almonds (Figure 1) were collected in areas 1 (W 52 ° 18'20.976"; S 0 ° 33'44.44") and 2 (W 51 ° 57'53.338"; S 0 ° 25'21.39"). The endophytic fungus used in this study was isolated from the almond and stored according to the protocol described by Holanda et al. [37].

Sample fragments were mounted on carbon tapes and visualized in a scanning electron microscope (SEM, model HITACHI - TM3030PLUS, Japan) at an accelerated voltage of 20 kV. Our access to SEM was provided by the Research Laboratory of Drugs of the Department of Biological and Health Sciences (DCBS) at the Federal University of Amapá (UNIFAP).

The endophytic fungus *Trichoderma asperellum* was identified conventionally and by molecular methods at the Pluridisciplinary Center for Chemical, Biological, and Agricultural Research (CPQBA) at the State University of Campinas (Unicamp), SP, Brazil.

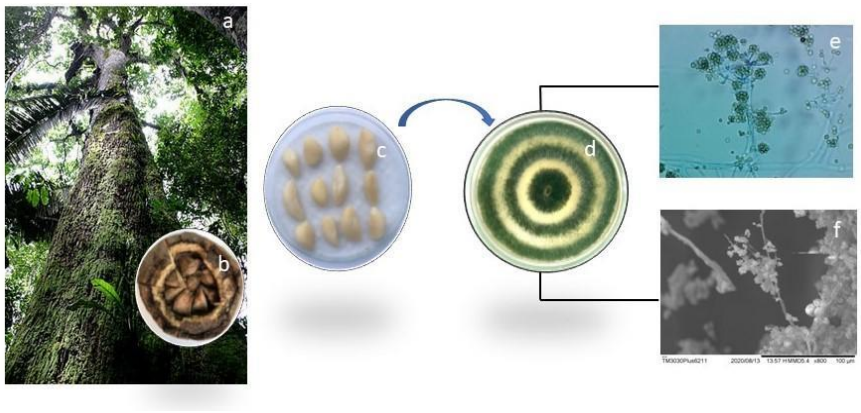


Figure 1. Tree of the species Bertholletia excelsa (a), hedgehog (b), and almonds (c). Morphological identification of the endophytic fungus *Trichoderma asperellum* cultivated in solid medium (d) with SEM at 800 x (f).

2.2. Fungal extract preparation (AM07Ac)

The fungi were cultivated in a 500 mL Erlenmeyer flask containing 200 mL of malt medium (2%) at pH 7.0 under constant stirring in a rotary shaker (Solab, SP, Brazil) for 8 days (28 °C ± 2 °C, 160 rpm). Inoculations were performed on four circular discs (0.5 cm in diameter) of solid culture medium to obtain the extracts. The growth of mycelial mass in a liquid medium was carried out in triplicate. After the cultivation period, fungal growth was stopped with the addition of ethyl acetate, followed by vacuum filtration and partitioning with ethyl acetate (3 x 50 mL). Excess solvent was removed by rotary evaporator (Quimis, model Q344M2) at a temperature of 40 °C. The material was lyophilized (LS 3000, Terroni, Brazil) to obtain the final mass of the dry extract of 0,45g.

2.3. Fungal species identification

2.3.1. Fungal genomic DNA extraction

Genomic DNA from the culture was purified using the phenol DNA extraction protocol described by Aamir et al. [38] The amplification of TEF and Beta-tubulin marker genes was performed by PCR using the extracted genomic DNA as a template. The primers (synthetic oligonucleotides) used for PCR reaction were as follows: 728f/TEFlr for sample CPQBA 2615-22 DRM 02 complementary to the TEF and Bt2a region.

2.3.2. Genetic Sequencing

The amplification product was column purified (GFX PCR DNA and Gel Band Purification Kit, GE Healthcare) and submitted directly to sequencing using an ABI 3500XL Series automatic sequencer (Applied Biosystems). The primers used for sequencing were EF1/EF2. 728f/TEFlr and Bt2a/Bt2b. Both genetic distance analysis and partial sequences of genes obtained from the above-noted primers were assembled into a consensus (single consensus sequence combining the different fragments obtained) and compared with the sequences of organisms represented in GenBank (http://www.ncbi.nlm.nih.gov) and CBS (http://www.westerdijkinstitute.nl/) databases.

The sequences of microorganisms related to the unknown sample were then selected for the construction of the dendrogram. DNA sequences were aligned using the CLUSTAL X program [39] within BioEdit 7.2.6, [40] and genetic distance analyses were conducted using MEGA, version 6.0. [41] The distance matrix was calculated using the Kimura model, [42] and construction of the dendrogram from the genetic distances was carried out using the Neighbor-Joining method [43] with bootstrap values calculated from 1,000 resamplings using the software included in the MEGA 6.0 program.

2.4. Chemical profile of *T. asperellum* fungal extract (AM07Ac)

2.4.1. Characterization of extracts (AM07Ac) by HPTLC and ^{1}H NMR

The extract (AM07Ac) was analyzed by adapting the methodology described by Pinheiro, [44] using the techniques of High-Performance Thin- Layer Chromatography (HPTLC) and Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR).

2.4.1.2. Chemical profiling by HPTLC

Chromatographic analysis was obtained in a robotized HPTLC system composed of application modules (Automatic TLC Sample 4 – ATS4) and a photo documenter (TLC Visualizer - CAMAG (Muttenz, Switzerland)). WinCats 1.4.6 was used to process the chromatographic data.

2.4.1.3. Sample preparation and application

The sample was prepared from the dilution of 10 mg of extract in 1.0 mL of methanol. Aluminum silica gel chromatoplates F-254 60 Å (Silicycle, Quebec, Canada) were used for the chromatographic analyses in the spray-band mode in aliquots of 50 µg/band (5 µL of solution) of extract solutions. In addition, 0,1 µg/band of the standards of (i) β-amyrin terpene (Sigma-Aldrich), kaempferol flavonoid (Sigma-Aldrich), and brucine alkaloid (Sigma-Aldrich) was inoculated. Kaempferol (Sigma-Aldrich) was also used to evaluate antioxidant potential.

2.4.1.4. Chromatographic Procedures

Chromatoplates were eluted in a Camag glass tank (Muttenz, Switzerland) in an isocratic dichloromethane/methanol system (98:2) with a chromatographic path of 70 mm. Chromatoplates were derivatized with selective developer solutions for terpenes and steroids (Vanillin-sulfuric acid 10% - VAS), alkaloids (tartaric acid and potassium iodide - Dragendorff), flavonoids (2-aminoethyl-diphenylborinate and polyethylene glycol 400 - NP/PEG) and for antioxidant compounds (DPPH˙ 0.5%).

2.4.1.5. ^{1}H NMR Spectrum

Samples were prepared using 20 mg of extract solubilized in 600 µL of deuterated methanol (CD_3OD) in a Bruker apparatus, model Ascend™ (Rheinstetten, Germany), operating at 400 MHz. TopSpin 3.6.0 software was used for data control and treatment, FIDs obtained were submitted to a Fourier transform with LB = 0.3 Hz, and pre-saturation sequences with low-power selective irradiation were used to suppress the residual signal of H_2O. The spectra

were treated manually, corrected at the baseline, and calibrated using the solvent's residual signal as an internal reference, CH_3OH – δ_H 3,30. [44]

The abundance of functional groups present in the classes of metabolites of interest was analyzed in the processing of FIDs, grouped, and normalized into specific regions (δ = 0,5 – 1,5 / 1,5 – 3,0 / 3,0 – 4,5 / 4,5 – 6,0 / 6,0 – 9,0 / 9,0 – 10,0).

2.5. Melanogenesis studies in Zebrafish embryos

2.5.1. Experimental Animals

Danio rerio (ZF) of wild AB strain, both sexes, and aged approximately 8 months were purchased from Power Fish (Betta Psicultura – Itaguaí, Rio de Janeiro, Brazil). Specimens were conditioned in aquaria on the Zebrafish Platform of the Research Laboratory of Drugs at UNIFAP by undergoing an adaptation period of 40 days at a controlled temperature (23 ± 2 °C) and 12-hour light/dark cycle. The project was approved by the Ethics Committee for Animal Use (CEUA) of UNIFAP under protocol number 006/2020.

2.5.2. Protocol to determine the effect of fungal extract (AM07Ac) on melanin synthesis in Zebrafish.

The reproduction test followed the recommendations of the Organization for Economic Cooperation and Development (OECD, 236) / (OECD, 2013). Eggs were collected from at least three groups to avoid genotypic variants, following the technique described by Yang et al. [45] The eggs were washed with aquarium water, randomized, separated into 7 groups with N = 50 eggs, and treated in triplicate. The groups were treated with *T. asperellum* fungal extract (AM07Ac) at concentrations 4.8, 15, and 30 mg/L and transferred to wells of a 96-well plate containing a final volume of 250 μL. Solutions of PTU (N-phenylthiourea) 25 μM (Sigma-Aldrich, MO, USA) and kojic acid 25 μM (Sigma-Aldrich, Korea) were used as positive controls, while aquarium water and DMSO at 3% were used as negative controls. Microplates with the treated and control groups were maintained at a controlled temperature of 28 ± 2 °C in an incubator (SOLAB - SL, Brazil). Teratogenic alterations, vitality, and absence of pigments were observed through analysis in an optical microscope at 24, 36, 48, 60, 72, 84, and 96 hours post-fertilization (hpf) (OECD, 2013).

2.5.3. Toxicity of melanogenic inhibitors

ZF embryo growth patterns were monitored at intervals of 24, 48, 72, and 96 hpf to determine the potential toxicity of melanogenic inhibitors. In the test, 200 embryos were used for each treatment to evaluate embryonic mortality, morphological malformations, and heartbeat disturbances.

2.5.4. Analysis of pigmentation in zebrafish

ImageJ (Fiji distribution, version 1.52p, National Institutes of Health, Bethesda, MD, USA) was used to analyze the degree of pigmentation in zebrafish embryos. The images were first converted to 8-bit gray images, and the threshold was set to select only the pigmented area. The particle parameter was employed with an appropriate pixel size threshold to remove artifacts, such as eyes and shadows around the yolk sac, from the images. For the Nile Red experiment, the

integrated density of each image was measured as Nile Red fluorescence intensity using ImageJ, following the methodology used in a previous study. [46]

The proportion of melanocytes was determined with ImageJ software (NIH), using equal-sized boxes for the dorsal view of whole embryos. Quantitative values were calculated as a percentage of black proportion per whole image. To assess the significance of differences between the control and experimental group, all statistical data were obtained from one-way ANOVA with Dunnett's post-test using IBM SPSS Statistics Data Editor software (Version 19). The significance level was set at $*p \leq 0.05$ versus the DMSO control group, and the data were represented as the means ± SEM (standard error of the mean). To calculate the percentage of melanocytes in ZF embryos, photographs of the dorsal and integral view of the embryos were first taken, and then the images were used for the quantitative measurement of melanogenesis in each ZF embryo (Fig. 2).[47]

2.6. Optimization and Molecular Docking to identify secondary metabolites

The 3D crystal structure of human tyrosinase (PDB ID: 5M8N) was downloaded from the RCSB Protein Data Bank (https://www.rcsb.org/) with a resolution of 2.60 Å and used in the molecular docking study. [48-49] The secondary metabolites identified in the composition of the AM07Ac extract (β-amyrin, kaempferol, and brucine) were drawn, and their bioactive conformation energies were minimized in ChemSketch software by the Molecular Mechanics method (MM+) with a force field based on CHARMM parameterization. [50-51] The receptor protein was prepared using Discovery Studio software (ACCELRYS, 2008) to remove water molecules and heteroatoms. The positive control ligands used in the molecular docking simulations were mimosine (MMS), PTU, and kojic acid.

Pyrx (version 0.8.30) was used as a graphical interface to couple the receptor protein with the extracted secondary metabolite ligands and positive control groups. The grid center coordinate parameters were defined as −30.7782, −4.4638.034, and −23.0952, according to the positive control MMS. To obtain greater computational precision, exhaustiveness parameter 8 was generated for the receptor, and the conformation with the highest affinity was selected as the final pose to be visualized in the software in Pymol 2.5.0. [52]

2.7. Statistical analysis

Results of the Area Under the Curve (AUC) application were organized as mean ± SD (standard deviation) and presented in graphs. Analysis of variance (One-way ANOVA) was performed, followed by Tukey's test for multiple comparisons. Results that showed differences of $p < 0.05$ were considered statistically significant between the treatment and control groups.

3. Results

3.1. Identification and phylogeny

Fragments of TEF and beta-tubulin genes were successfully amplified and sequenced from the genomic DNA extracted from the sample. The genetic distance analysis (Figure 1) recovered sample CPQBA 2615-22 DRM 02 in a cluster with a resolution of 98% with the strain Type CBS 433.97 for the species *Trichoderma asperellum*.

Thus, the results of the analyses carried out in the databases and the phylogeny suggest the final identification of sample CPQBA 2615-22 DRM 02 as *Trichoderma asperellum* Samuels, Lieckf. & Nirenberg. [53] Partial sequence of the samples is shown in Figure 2.

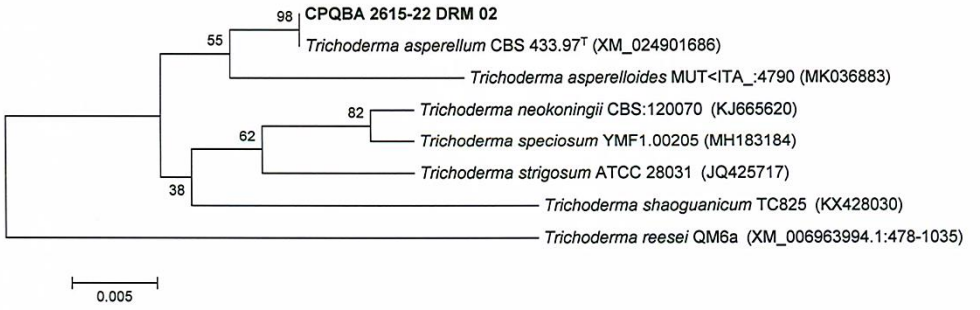


Figure 2. Dendrogram based on genetic distance using the neighbor-joining method to demonstrate the relationship between the partial sequence of the TEF region of the CPQBA 2615-22DRM sample and sequences of related microorganism strains present in the MycoBank (CBS KNAW, actual Westerdijk Fungal Biodiversity Institute) and GenBank databases.

3.2. Chemical profile of the fungal extract of *Trichoderma asperellum* (AM07Ac) by HPTLC and ^{1}H NMR

3.2.1. Characterization by HPTLC

Analysis of the chemical profile by HPTLC allowed evaluation of the chemical complexity of the extract (Figure 3). When a 10% sulfuric acid (VAS) vanillin solution was used, the extract showed a reaction indicative of a diversified composition of terpenes and steroids (Figure 3A). By using the NP/PEG reagent and observing the chromatoplate under 366 nm radiation, formation of green bands typical of flavonoids could be visualized, as also seen with the standard used, kaempferol (Kae), in addition to the characteristic blue color of other phenolic compounds (Figure 3B). A thin orange band also indicated a positive reaction for alkaloids (Figure 3C).

The tests also showed the ability of AM07Ac extract to sequester DPPH radicals (purple) through the formation of yellow bands (DPPH in molecular form). Results indicate the antioxidant capacity of the constituents present in the AM07Ac extract compared to the positive control kaempferol (Kae), as seen in Figure 3D.

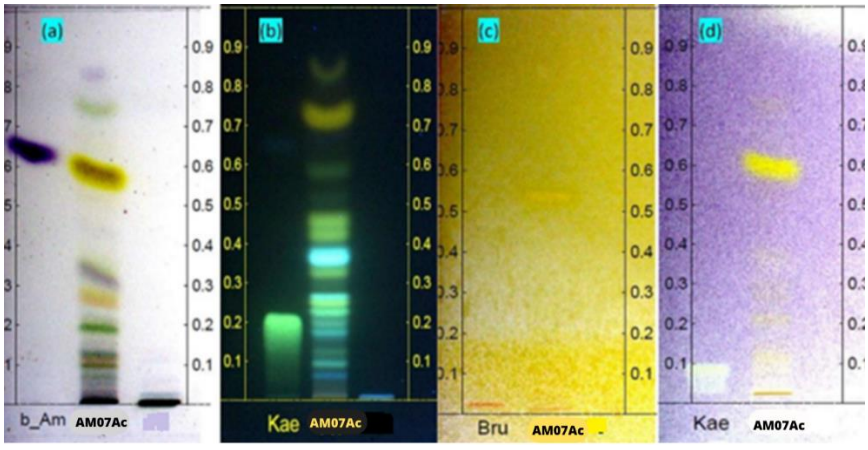


Figure 3. Chemical profile of extracts by HPTLC on chromatoplates derivatized with selective solutions for (a) terpenes and steroids in purple compared to the B-amyrin (b_Am) standard; (b) flavonoids in green and phenolic compounds in blue compared to the kaempferol (Kae) standard; (c) alkaloids in orange compared to the brucine (Bru) standard; (d) and antioxidant compounds in yellow compared to the kaempferol (Kae) standard. Standards were compared with *T. asperellum* crude extract (AM07AC).

3.2.2. Characterization by ^{1}H NMR

The chemical composition of the AM07AC extract was also analyzed through the abundance of functional hydrogen groups and chemical shifts observed in the ^{1}H NMR spectrum. The region of the spectrum, corresponding to olefinic hydrogens δH 4.5 – 6.0 ppm, had the highest signal intensity with 15% of the area, while the region of δH 4.5 – 6.0 ppm, corresponding to signals from hydrogens linked to oxygenated carbons, had a peak intensity equal to 2.06% of the area. The δH 1.5 – 3.0 ppm range of hydrogens bonded to unsaturated carbons had an area equivalent to 0.35%. Signals related to methyl, methylene, and methine group hydrogens at δH 0.5 to 1.5 ppm obtained an area percentage of 0.16%, as shown in Table 1. [44]

Table 1 - Spectroscopic characterization by ^{1}H NMR of extract AM07AC.

Chemical Shift (ppm)	Assignments	Area (%)
0.5 - 1.5	-CH_n; -CH_n	0.16
1.5 - 3.0	CH_n-C=O; CH_n-N; Ar-CH_n; Ar-CH_n-	0.35
3.0 - 4.5	CH_n-C=O; -CH_n-O-; -CH_n-N-	2.06
4.5 - 6.0	Ph-O-CH_n; HC=C- (non-conjugated)	15.00
6.0 - 9.5	Ph-H; Ph-CH=O	0.13

Substances that were assessed positively in the AM07Ac crude extract on HPTLC were identified in comparison with ^{1}H NMR spectroscopic data compared to reports in the scientific literature. Correlation between HPTLC analysis and ^{1}H NMR spectra resulted in identifying the chemical markers β-amyrin, kaempferol, and brucine, as shown in Figure 4.

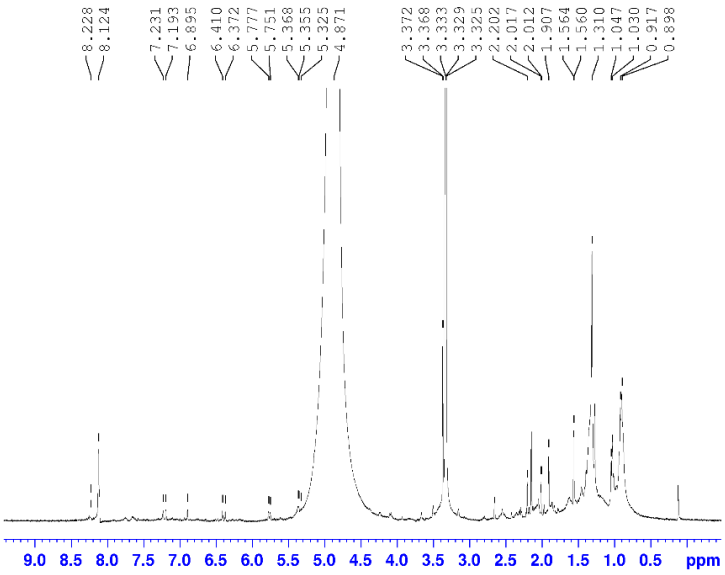


Figure 4. ^{1}H NMR spectrum of AM07Ac extract at 400 MHz.

The analysis indicated a double-doublet in the region of δH 3,325 ppm, characteristic of carbinolic hydrogen in 3β-OH triterpenes, a signal at δH 4,871 ppm typical of olefinic hydrogen, and peaks at δH 2,202 ppm indicating allelic hydrogen and δH 8,228 ppm confirming the presence of hydroxyl on the C-3 carbon of the A ring, allowing us to suggest a triterpene structure with a bear skeleton equivalent to β-amyrin (Figure 5A). [44]

Spectroscopic analysis also indicated a pair of doublets at δH 6,372 and 6,410 ppm of meta-positioned hydrogens, characteristic of the AB system of flavonols, and two pairs of doublets, δH 7,193 and 7,231 ppm, typical of a para-substituted aromatic ring. These chemical shifts allow for inferring the presence of kaempferol (Figure 5B) in the crude extract. [54]

The 1H NMR spectrum also indicates a singlet δH 8,124 ppm characteristic of hydrogen bound to the aromatic ring of indomethanolic alkaloids derived from the strychnine skeleton. Another crucial point is the presence of a singlet at δH 3,372 ppm, resulting in the deshielding of proton 12 by the ether ring of 7 members. Thus, it is possible to infer that it is the brucine alkaloid (Figure 5C). [55]

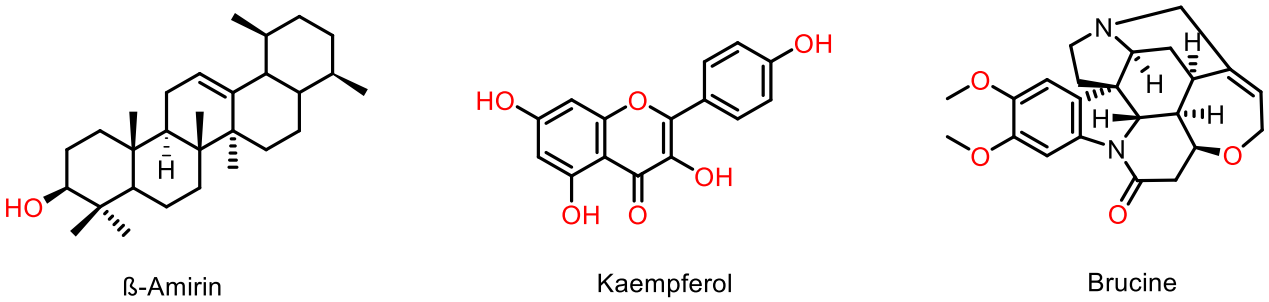


Figure 5. Molecular structures of secondary metabolites identified in the AM07Ac extract of the fungus *Trichoderma asperellum*.

3.3. Melanogenesis Studies in Zebrafish Embryos

3.3.1. Effect of treatment with fungal extract (AM07Ac) on melanin synthesis in Zebrafish

Our ZF phenotype-based screening model demonstrated that developing melanophores inhibit melanin synthesis after treatment for 24 h (between 24 and 96 hpf). All extract concentrations significantly affected pigmentation in developing melanophores in ZF embryos (Figure 6) without developmental disturbances compared to control groups. The anti-melanogenic effect was compared using known melanogenic inhibitors, kojic acid and PTU, [56-57] as positive controls. Treatment with AM07Ac extract in ZF embryos resulted in anti-melanogenic effects in a dose-dependent manner (Figure 6E-G).

Treatment with the 30 mg/L concentration (Figure 6G) produced a remarkable inhibition of pigmentation in ZF embryos, similar to that of the PTU-treated group (Figure 6A) at 24 hpf.

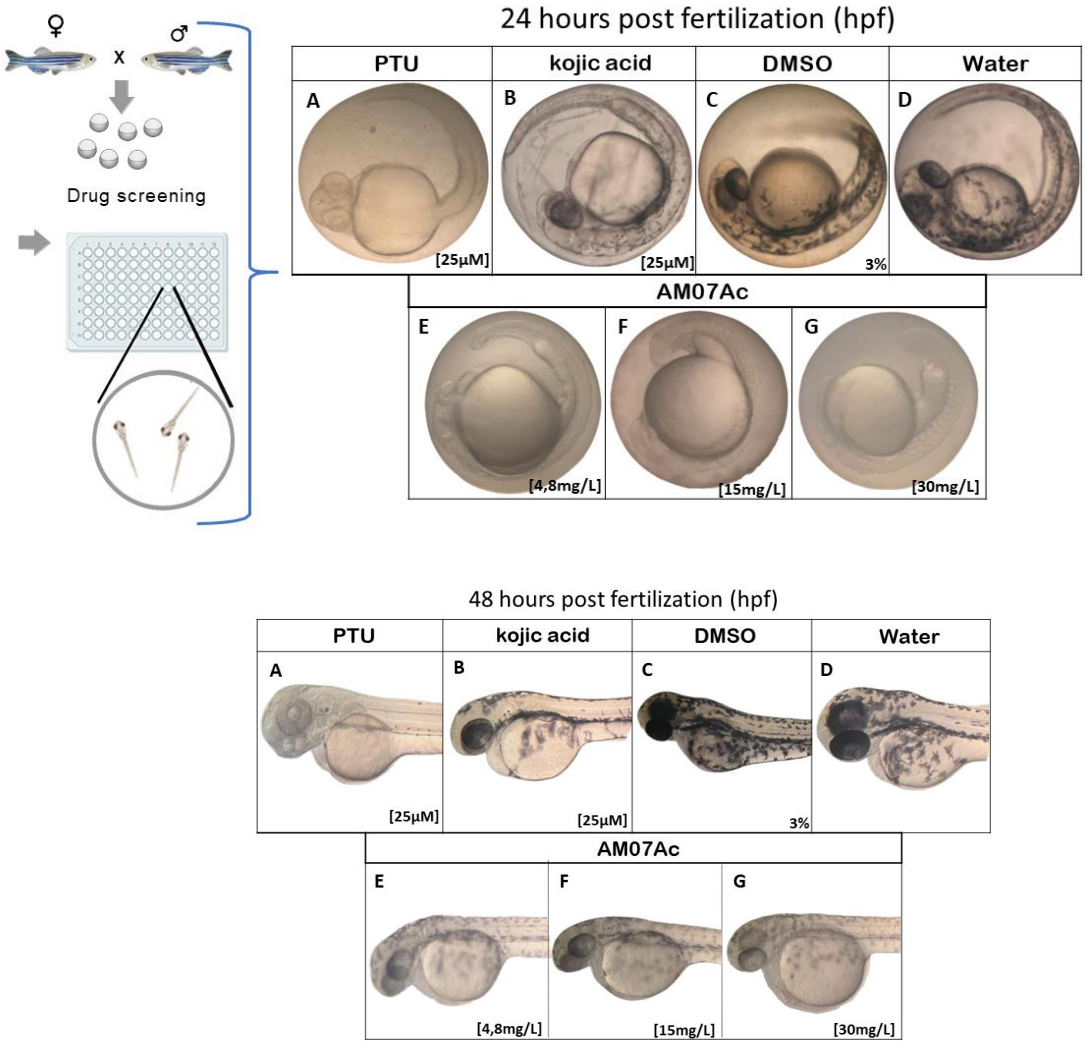


Figure 6. Effect of treatment with extract AM07Ac (E–G) on ZF embryos between 24 and 48 hpf. Embryos A-D were treated with positive and negative controls: (A) 0.25 µM PTU; (B) 0.25 µM kojic acid; (C) 3% DMSO; and (D) water.

In this study, melanogenesis in ZF embryos (Figure 7) was shown to be effectively inhibited. Treatment with AM07Ac extract produced a concentration-response effect with statistically significant differences when compared to control groups ($p < 0.05$). Effects of melanogenic inhibitors on tyrosinase activity and melanin synthesis in ZF embryos were observed, and both tyrosinase activity and total amount of inhibited melanin content after treatment with the fungal extract were evaluated to estimate inhibitory activities.

Results indicated a significant reduction in tyrosinase activity and total melanin content after treatment with 30 $mg.L^{-1}$ of extract, demonstrating an inhibition potential similar to that of the melanogenic inhibitors PTU and kojic acid at 96 hpf. Kojic acid reduced the pigmentation of ZF embryos up to 48 hpf, while concentrations of 4.8 and 15 mg/L of the AM07Ac extract showed a lesser effect at 96 hpf. In particular, yolk sac pigmentation was inhibited after treatment with the extract at all concentrations.

3.3.2. Toxicity of melanogenic inhibitors

No significant changes in mortality were observed in groups of ZF embryos treated with PTU 25 μM, kojic acid 25 μM, DMSO 3%, or AM07Ac at 4.8, 15, or 30 mg.L^{-1}; however, the mortality rate at 30 mg.L^{-1} was higher compared to the other groups. Treatments did not show morphological malformations, except for the AM07Ac extract at 30 mg.L^{-1}, which showed abnormality in the size of the yolk sac. In the heart rate test, kojic acid produced a slight disturbance.

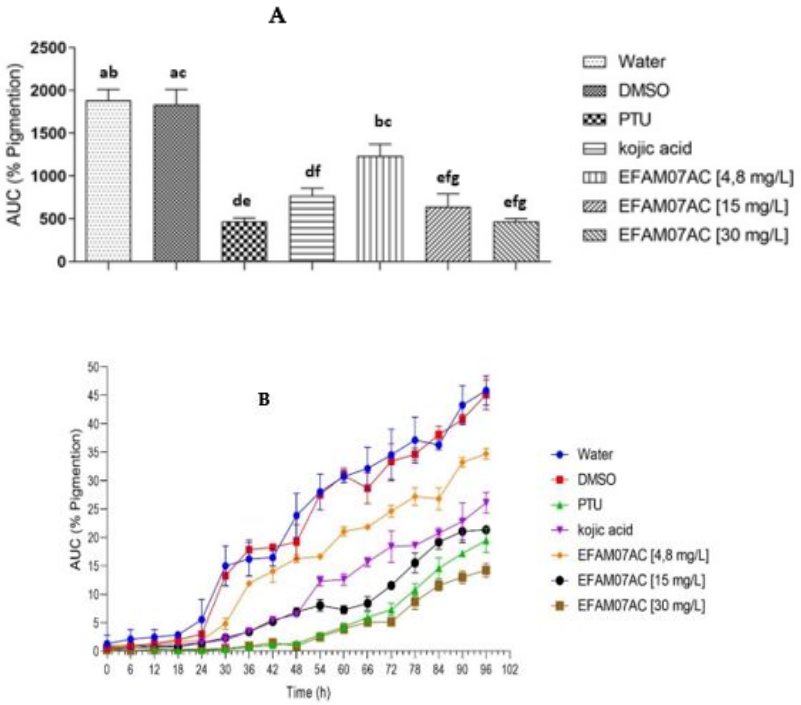


Figure 7. Effect of treatment with AM07Ac extract (4.8 mg.L^{-1}, 15 mg.L^{-1}, and 30 mg.L^{-1}) on melanin synthesis and tyrosinase activity in Zebrafish embryos. (A) Percentage of melanogenesis inhibition as a function of time in different fungal extract concentrations compared with positive and negative controls. (B) expresses the Area Under the Curve (%AUC) obtained by pigments in the embryos as a function of time. Results are presented as mean ± SD (n = 50 embryos/group). Differences between groups were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test ($p < 0.05$).

3.4. Inhibition potential of secondary metabolites of AM07Ac extract on the enzyme tyrosinase

Molecular docking was used to evaluate tyrosinase inhibition through the secondary metabolites β-amyrin, kaempferol, and brucine, as identified in the AM07Ac extract. Protocol validation was conducted by superimposing the crystallographic structure of MMS (positive control) on the biological target in order to reproduce in the *in silico* model bioactive conformation similar to that of tyrosinase co-crystallized with MMS (PDB ID: 5M8N) (Figure 8).

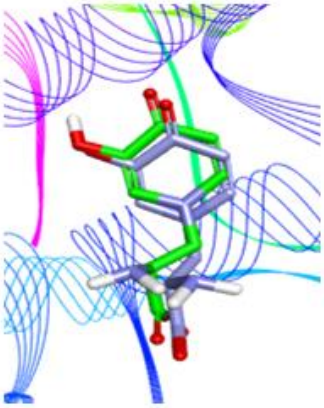


Figure 8. Superimposition of the docked MMS positive control (green) with the corresponding pose of the co-crystallized ligand in the enzymatic site of human tyrosinase (Lilac).

Validation of the *in silico* model, as proposed in this study, was considered satisfactory owing to similarity between the crystallographic ligand pose and the molecular docking poses through the root-mean-square deviation (RMSD) value of 0.96 Å. According to the literature, the docking protocol is similar to the experimental model in RMSD values ≤ 2 Å. [58-61]

The interactions observed at the MMS binding site with tyrosinase (Figure 9A) are around the α-helix (between amino acid residues His381 and Ser394) and in the β-sheet (among amino acid residues Tyr362, Arg374, and Thr391). In MMS, hydrogen bonds with residues Tyr362, Thr391, and Ser394 can be observed.

Among the secondary metabolites evaluated, kaempferol (Figure 9D) showed the best binding affinity value (-6.8 Kcal/mol), followed by brucine (-5.7 Kcal/mol) (Figure 9E). These secondary metabolites have enough potential binding affinity energy to act as tyrosinase enzyme inhibitors compared to positive controls (Figure 9A-C).

Figure 9- Hydrogen bonding or lipophilic interactions of positive controls MMS (A), kojic acid, (B) and PTU (C) and the secondary metabolites kaempferol (D) and brucine (E) identified in the extract (AM07AC) with the catalytic site of human tyrosinase.

Kaempferol (C) and brucine (D) exhibited interactions similar to those observed in the positive controls MMS, kojic acid and PTU for the amino acid residues Arg374, His381, and Leu382. These interactions reproduce those described in the literature for anti-melanogenic activity in an *in silico* model, [46,49,62] as shown in Table 2.

Table 2- Results of binding affinities and their main interactions at the human tyrosinase receptor.

Protease	Binder	Binding Affinity (Kcal/mol)	H-Bond	Lipophilic interactions
Tyrosinase	MMS	-5.9	Arg374, Thr391, Ser394	His192, His215, Leu382, His377, Tyr362, Asn378, His381, Gly388, Thr391, Asn378, Phe400,
	PTU	-5.7	Gly388, Gly 389, Thr391	His381, Leu382

	Kojic acid	-5.7	His215	His381, Thr391
	Kaempferol	-6.8	Tyr362, Arg374, Ser394	His381
	Brucine	-5.7	Tyr362, Arg374	Leu382
	β-Amyrin	-4.5	-	Gln390

5. Discussion

Terpenes represent chemosystematic markers of the genus Trichoderma with chemical diversity of structures ranging from sesquiterpenes to diterpenes and triterpenes, such as the β-amyrin identified in the species *T. asperellum*. [63]

The aglycone flavonoid kaempferol is a metal ion chelator with a complete conjugate linkage system, a tightly coordinated oxygen atom, and an acceptable steric configuration. [64] Kaempferol contains two sites that interact with metal ions: the 3-hydroxyl or 5-hydroxyl of the C ring and the 4-carbonyl of the C ring. [65] The compound has several pharmacological activities, including anticancer, antioxidant, and anti-inflammatory activities. [66]

Alkaloid is another class of metabolite found in *T. asperellum* extract with the potential to influence the activity of the enzyme tyrosinase. Brucine has demonstrated anti-inflammatory and analgesic activity, as well as antitumor potential and antagonism toward arrhythmia with a negative inotropic effect owing to oxygen consumption in the cardiovascular system. [67]

Tyrosinase is the rate-limiting enzyme in melanin biosynthesis. The process of melanin production starts with the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and then the oxidation of DOPA to dopaquinone. Therefore, tyrosinase has been considered a critical target for the development of melanogenic inhibitors. [49,68]

The inhibitory activity of secondary metabolites from species of the genus Trichoderma has been investigated, and the extracts of Trichoderma atroviride, Trichoderma gamsii, Trichoderma guizhouense, and Trichoderma songyi were all demonstrated as potential inhibitors of the enzyme tyrosinase. [69] The present study describes, for the first time, the *in vivo* anti-melanogenic activity of the crude ethyl acetate extract of *T. asperellum* (AM07Ac).

No significant difference was noted between PTU and kojic acid ($p > 0.05$). However, the results show a significant difference between the crude extract of *T. asperellum* (30 mg/L) and the positive controls PTU and kojic acid ($p < 0.05$). The results obtained in the melanogenesis assay in ZF embryos and molecular docking suggest that the inhibitory activity may be related to the chemical markers of the extract, kaempferol and brucine, which showed better binding affinity with the enzymatic site of tyrosinase.

Binding affinity results show selectivity for flavonols and indomethanolic alkaloids derived from the strychnine backbone. Reported studies show the anti-aging potential of a limited group of polyoxygenated xanthones, some of them with anti-tyrosinase activity. The inhibitory activity of enzymes related to skin photoaging (tyrosinase, collagenase, elastase, and hyaluronidase) was investigated for the first time for three simple hydroxylated xanthones considered tyrosinase inhibitors with IC_{50s} on the same order of magnitude, but lower than the IC_{50} obtained for the control kojic acid. [68]

According to Lai et al., [49] L-tyrosine and candidate inhibitors of the tyrosinase enzyme do not interact directly with zinc ions; instead, their aromatic hydroxy and keto groups are linked by hydrogen bonding to the water molecule, which then binds to zinc ions. Additional interactions include tight aromatic stacking bonds with His381 and hydrogen bonds of its carboxylate group with Arg374 and Ser394. Potential inhibitors with better-defined electron density than L-tyrosine have better binding affinities with enzymatic sites, highlighting the importance of electron pair donor groups, such as hydroxyls and carbonyls of kaempferol.

In addition to its antioxidant activity and cytotoxicity, kaempferol's tyrosinase inhibitory activity has been described against B16 melanoma cells. [70] Kaempferol inhibition acts through competitive binding with the enzymatic site of tyrosinase and the metabolite, preventing the catalytic oxidation of L-DOPA to L-DOPAquinone through catecholase activity. The free hydroxyl C-3 of the C ring of kaempferol has been indicated to have an important function in the molecular mechanism of tyrosinase inhibition, as evidenced through the molecular docking assay which showed a hydrogen bond with the residual amino acid Tyr362 of tyrosinase (Figure 2-D). [71-72]

6. Conclusion

In the present study, the chemical markers kaempferol, brucine, and β-amyrin present in the fungal extract of *Trichoderma asperellum* were identified. By using *in vivo* assays in Zebrafish, it was possible to demonstrate the action of the extract on the melanogenic process, which is related to the major markers identified that act by inhibiting tyrosinase activity, as demonstrated in the *in silico* study. The results of the melanogenesis inhibition test in zebrafish embryos treated with the AM07Ac extract produced a concentration-response effect, demonstrating a specific relationship with the compounds identified in this extract, and the results imply the importance of investigating microorganisms and their pharmacological activities, demonstrating the potency of Amazonian diversity in terms of organisms that generate active metabolites, in addition to their preservation.

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CONSIDERAÇÕES FINAIS

Na presente tese foi possível demonstrar evidências da importância que os fungos endofíticos possuem para à descoberta de novos metabólitos bioativos. Muitos destes metabólitos possuem um grande potencial a ser utilizado na medicina, agricultura e indústria. Corriqueiramente, novos antibióticos, imunossupressores e compostos anticâncer são esperados como metabólitos secundários produzidos por endofíticos. Os fungos endofíticos constituem um importante e ainda pouco explorada fonte de novas substâncias químicas com potencial biológico, portanto, devem ser considerados em programas de bioprospecção. Cabe ressaltar que uma das maiores preocupações atualmente envolvem os processos de distúrbios melanogênicos que estão relacionados a geração de câncer de pele, sendo um dos mais perigosos o melanoma, com grande capacidade metastática. Portanto, este estudo abrange vertentes de alta relevância no campo dos produtos naturais e biotecnológico, uma que se os estudos evidenciaram que os metabolitos secundários de fungos endofíticos de uma espécie vegetal de grande importância internacional apresentou atividade anti-melanogenica satisfatória, outra, relacionada a aplicação desses metabolitos em vias fisiopatológicas de expressão na terapêutica farmacológica carente de novos fármacos.

Dessa maneira, no capítulo um da presente tese, que foi publicado na revista Molecules com o título de Anti-Melanogenic Potential of Natural and Synthetic Substances: Application in Zebrafish Model demonstramos o papel que a tirosinase desempenha em distúrbios relacionados a alterações de despigmentação em humanos. Assim, os inibidores de TYR podem ser a melhor opção de tratamento. Muitas pesquisas vêm avançando na descoberta de novos inibidores. Uma variedade de plantas e fungos são importantes produtores de metabólitos bioativos que inibem a tirosinase. *Trichoderma* é o gênero mais estudado quanto à inibição da tirosinase, pois os metabólitos de sua espécie são derivados de isoflavonas e pironas que juntamente com terpenos, esteróides e alcaloides, que podem inativar reversível ou irreversivelmente a enzima. Nos últimos anos, pesquisas nortearam importantes avanços no desenvolvimento de tecnologias e na triagem de compostos bioativos.

Além disso, testes in vivo intensificaram o uso do modelo experimental do peixe-zebra baseado em fenótipos nos quais pigmentos de melanina podem ser observados na superfície do organismo, permitindo a simples observação do processo de pigmentação sem procedimentos experimentais mais detalhados. Por esta razão, o zebrafish está ganhando cada vez mais viabilidade como modelo in vivo para avaliar a atividade despigmentantes de compostos reguladores melanogênicos.

Enquanto no capítulo dois, publicado na revista Microorganisms e intitulado *Trichoderma asperellum* extract isolated from Brazil nuts (*Bertholletia excelsa* BONPL): *in vivo* and *in silico* studies on melanogenesis in zebrafish, detectou se os marcadores químicos kaempferol, brucina e β-amirina presentes no extrato fúngico de *Trichoderma asperellum.* Assim como, por meio de ensaios in vivo em Zebrafish, foi demostrado a ação do extrato no processo melanogênico, que está relacionado aos principais marcadores identificados que atuam inibindo a atividade da tirosinase, conforme demonstrado no estudo *in silico*. Os resultados do teste de inibição da melanogênese em embriões do zebrafish tratados com o extrato AM07Ac produziram um efeito concentração-resposta, demonstrando uma relação específica com os compostos identificados neste extrato, e os resultados indicam a importância de investigar microrganismos e suas atividades farmacológicas, demonstrando o potencial da biodiversidade amazônica em termos de organismos geradores de metabólitos ativos, além de sua preservação.

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