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Glandular hair constituents of *Mallotus philippinensis Muell.* fruit act as tyrosinase inhibitors: Insights from enzyme kinetics and simulation study

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ABSTRACT

The glandular hair extracts from the fruit rind of *Mallotus philippinensis Muell.* is employed to treat various skin infections, however the anti-tyrosinase activity remains unknown. Hence the present study inspected on the anti-melanogenic activity of *M. philippinensis* constituents. Lineweaver Burk plot revealed mixed inhibition for Rottlerin; non-competitive type of inhibition for mallotophilippen A and B respectively. Thermodynamic studies resulted in static quenching forming ground state complex with higher binding constant temperature dependently. Fluorescence and circular dichroism study implicated conformational change in secondary and tertiary structure of tyrosinase. Molecular docking suggests rottlerin has high binding affinity to the active site pocket of tyrosinase. Simulation study further proved that the compactness of inhibitor with tyrosinase by hydrogen bonding influenced the stability of the enzyme. Depigmentation efficacy is further proved in *Aspergillus niger* spores. Thus our findings delineate that rottlerin could be utilized as a depigmentation agent in food pharmaceutical and agricultural industries.

1. Introduction

The polymeric pigment 'Melanin' determines the human skin, eye and hair colour [1]. In the biosynthetic pathway of melanin, the first two crucial steps is catalyzed by tyrosinase (EC 1.14.18.1) a copper-containing monooxygenase enzyme in which L-tyrosine is hydroxylated to 3,4- dihydroxyphenylalanine (L-DOPA, monophenolase activity) and the latter is subsequently oxidized to dopaquinone (diphenolase activity) [2,3]. Skin pigmentation is a prerequisite for human health as melanin acts as free radical scavenger and photoprotectant [4]. However excessive accumulation of melanin leads to hyperpigmentation disorders causing consequentiality in aesthetic values, this can be obviously controlled by tyrosinase inhibitors [5]. A large number of moderate to potent tyrosinase inhibitors from natural and synthetic resources has been reported during the last decade [6]. Tyrosinase inhibitor such as arbutin, kojic acid and hydroquinones has been used as whitening or anti hyper pigment agents because of their ability to suppress dermal-melanin production [7]. However, investigations has shown that arbutin and kojic acid hardly showed inhibitory activity against pigmentation in intact melanocytes or in a clinical trial, and hydroquinones are considered to be cytotoxic to melanocytes and potentially mutagenic to mammalian cells [8–10]. Understanding the inhibition mechanism of an inhibitor is essential or else it remains obscure. Therefore, it is necessary to search for natural tyrosinase inhibitors without side effects.

Mallotus philippinensis (Kamala) is a perennial shrub known for its bright orange red dye in the glandular hairs of the fruit [11–15]. The most important constituent of Kamala is a dark brownish red resin wax extracted from the rind of the fruit glands and hairs which is rich in phloroglucinol derivatives like rottlerin, mallotophilippen A and B. Only few reports are available on the biological activity of the glandular hairs of *M. philippinensis.* These phloroglucinol derivatives are reported as the most active anti fertility and anti-allergic agent [15–17]. Rottlerin acts as kinase inhibitor, ameliorates ROS, and inhibits T cell response [18]. Rottlerin mitigates IgE-dependent anaphylaxis and markedly reduces hyperoxaluric outcomes [19].

Traditionally glandular hair extract of *M. philippinensis* is used in oriental medicine to treat skin disorders like parasitic infections [20]. The reported biological activity can be pertained to the presence of phloroglucinol derivatives in the glandular hair of *M. philippinensis*. Though the effect of other phloroglucinol derivatives on tyrosinase activity has been previously described [22,23]. To the

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best of our knowledge no authentic study has been carried out on the tyrosinase interaction with the glandular hair constituents of this plant. Here we report the anti-tyrosinase potential of *M. philippinensis* constituents in order to utilize it as a potential whitening agent to protect against skin disorder.

2. Materials and methods

2.1. Chemicals and materials

All reagents and solvents including ethyl-acetate and hexane were of the highest analytical grade reagents. Tyrosinase was purchased from Sigma (St. Louis, MO, USA), 3,4-dihydroxyphenylalanine (L-DOPA), Silica (60–120 mesh) and dimethyl-sulfoxide (DMSO) were obtained from Himedia, India. De-ionised water was used.

2.2. Plant material and isolation of compounds

The fruit of *M. philippinensis* was collected from Shervaroy Hills, Eastern Ghats, Tamil Nadu, India. The plant material was taxonomically authenticated by Dr. R. Siva [IUCN species survival commission member for Medicinal Plants] and the voucher specimen (VIT-MP-2015) is deposited in VIT herbarium, VIT University, Vellore, India. The red glandular powder from the rind of the fruits was separated from the fruit rind. The crude extract was extracted in ethyl acetate, and then evaporated to dryness at 50 °C under reduced pressure (337 mbar) in a rotavapor R-215 (BUCHI Labortechnik AG, Switzerland). After subjecting the crude extract to silica gel column chromatography, elution was carried out in ethyl acetate: hexane (3:2) collecting approximately 2 mL of the run down solvent in the test tubes. Each of the eluent was further checked for the number of spots it yielded on a TLC sheet. Fractions producing identical spots on the TLC sheet were pooled together and the solvent was evaporated to obtain the isolated compound. The compounds (Rottlerin, mallotophilippen A and mallotophilippen B) thus obtained was weighed, sealed in sample vials and stored for further analysis. The obtained compounds were characterized by UV–vis spectrophotometry, Fourier Transform Infrared (FTIR) and Nuclear Magnetic Resonance (NMR) analysis, and compared with the published data.

2.3. Anti tyrosinase assay

Diphenolase assay was performed as previously reported [24]. In this reaction L-DOPA was used as the substrate for diphenolase activity. The reaction media (3 mL) for enzyme activity assay contained 0.5 mM L-DOPA in 50 mM sodium phosphate buffer (pH 6.8) and 100 mL of different concentrations of inhibitor (5, 10 and 15 μM). The final concentration of mushroom tyrosinase was 3.33 μg/mL for diphenolase activity. Enzyme assay was performed by following the increase of optical density at 475 nm accompanying the oxidation of substrates. The reaction was carried out at 30 °C. A Systronics AU-2701 UV–vis double beam spectrophotometer was used. The inhibitor was dissolved in 0.1% DMSO and diluted to appropriate concentration. The control without inhibitor but containing 0.1% DMSO in the reaction media was routinely carried out. The inhibitory effects of inhibitors on the enzyme were expressed as the concentrations that inhibited 50% of the enzyme activity (IC_{50}). The inhibition types were obtained by the Line-weaver Burk plot, and the inhibition constants were determined by the secondary plots of the apparent *Km/Vm* or *1/Vm* versus the concentrations of the inhibitors.

2.4. Intrinsic fluorescence binding

The intrinsic fluorescence quenching of tyrosinase by Rottlerin, mallotophilippen A and mallotophilippen B was recorded using Hitachi F-7000 spectrofluorophotometer at three different temperatures (298, 310 and 313 K). The fluorescence intensities of constant concentration of tyrosinase (0.77 μM) in 0.1 M phosphate buffer (pH 6.8), was recorded initially at excitation wavelength of 290 nm. Increasing concentrations of rottlerin, mallotophilippen A and mallotophilippen B (2–10 μM) was titrated and the change in fluorescence intensity was measured.

2.5. Circular dichroism spectroscopy

Circular Dichroism spectra were recorded using a Jasco J-715 spectropolarimeter, in a 3-cm rectangular cuvette associated with an optical path length of 1 cm. Temperature was synchronized by a Peltier type temperature control system. All spectra were collected three times with a bandwidth of 1.0 nm and a resolution of 0.5 nm at a scan speed of 100 nm/min. All observed Circular Dichroism spectra was recorded in the wavelength range of 200–400 nm at 37 °C under constant nitrogen flush. The Circular Dichroism measurement was recorded for tyrosinase (10 μg/mL) initially to which increasing concentrations of Rottlerin, mallotophilippen A and mallotophilippen B was added and the spectral changes were monitored. The secondary structural content was analyzed from Jasco Secondary Structure estimation software version 1 [9].

2.6. Molecular interaction study

3D coordinates of *Agaricus bisporus* tyrosinase was obtained from Protein Data Bank (PDB ID-2Y9W). The structures of ligands were plotted using ChemDraw12. The SMILES was further generated and they were converted into the 3D structure using OpenBabel [25]. The molecular interaction between protein and ligands were studied using AutoDock. Necessary Hydrogen and charges was added to the protein and torsions were fixed for the ligand. The cubical grid box of size $60 \times 60 \times 60$ was fixed around the active site, and autogrid was performed. Further, dockings was performed using Lamarckian Genetic Algorithm to obtain ten binding poses. The docking was performed twice, and average binding energy was calculated. The pose with best binding affinity was visualized using Maestro [26]. To understand the molecular aspects of protein-ligand complexes, molecular dynamics study was initiated.

2.7. Molecular dynamics study

Molecular dynamics study was carried out using Gromacs v 4.5.6 [27]. GROMOS96 53a6 force field was used to generate protein topology [28]. The topologies for ligands were generated using ATB Server [29]. The complex was solvated in a cubical box containing TIP3P water molecules. Counter ions was added to neutralize the systems. Energy minimization criterion was fixed to 1000 Kcal/mol with the steepest decent algorithm. Further, NVT and NPT were employed to equilibrate the systems at 300 K. Finally, molecular dynamics simulations were performed for 10 ns. Root Mean Square deviation (RMSD), Radius of gyration (Rg) and Intramolecular hydrogens bonds were analyzed using g_rms, g_rmsf and g_gyrate respectively.

2.8. Aspergillus niger *spores: phenotypic evaluation and melanin content*

Aspergillus niger (MTCC 277) was cultured in PDA plates until the mycelium sporulated. The fungal sporulated plate was washed with Tween- 80 in order to collect the spore suspension. The spore