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Short communication

Tyrosinase inhibitory mechanism of betulinic acid from *Dillenia indica*

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ABSTRACT

The fruit of *Dillenia indica* L. is extensively used as a food additive. Betulinic acid (BA) is the most prominent secondary metabolite present in *D. indica*. This study screened the bioassay guided isolation of BA from *D. indica* and explored its tyrosinase inhibitory mechanism. Half maximal inhibitory concentration (IC_{50}) of BA were calculated as 13.93 μM and 25.66 μM for diphenolase and monophenolase. Enzyme kinetic analysis revealed that BA inhibited tyrosinase activity non-competitively. Further, conformational analysis of tyrosinase with BA was measured by fluorescence and circular dichroism spectroscopy. These results implied that diminish rigidity of enzyme might disturb the catalytic conformation of tyrosinase. Moreover, *In-silico* analysis confirmed probable binding polar and non-polar region on the active site of tyrosinase. Based on these findings, we suggest that BA from *D. indica* may be useful in preventing enzymatic browning reactions in food products.

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1. Introduction

Tyrosinase (EC 1.14.18.1) is a multifunctional copper-containing enzyme, which regulates melanogenesis within melanocytes. Oxidation of L-tyrosine and l-DOPA is the first step of melanogenesis catalyzed by tyrosinase. Tyrosinase catalyzed quinone formation, which is used further for the synthesis of melanin. This biosynthetic step is the rate-limiting step in the melanin biosynthesis. Many tyrosinase inhibitors from both natural and synthetic sources have been identified. Tyrosinase inhibitors are examined in the presence of L-tyrosine or L-DOPA as the enzyme substrate, and activity is assessed regarding dopachrome formation. True tyrosinase inhibitors are classified into four types, including competitive inhibitors, uncompetitive inhibitors, mixed type (competitive/uncompetitive) inhibitors, and non-competitive inhibitors (Chang, 2009). For example, Aloesin and 2″-*o*-feruloylaloesin isolated from *Aloe arborescens* Mill (Candelabra aloe) showed non-competitive inhibition to mushroom-tyrosinase (Yagi, Kanbara, & Morinobu, 1987). Thus, overproduction and accumulation of melanin pigments in the skin lead to the development of dermatological 'hyperpigmentation' in clinical conditions like solar lentigo, melasma, post-inflammatory hyperpigmentation (PIH), and linea nigra. Arbutin and kojic acid are known tyrosinase inhibitors. However, Kojic acid causes dermal sensitization at therapeutic concentration whereas arbutin has potential cytotoxicity (Burnett et al., 2010). Utilization of plant extracts and its derived phytoconstituents has a likely future for controlling the hyperpigmentation (Sarkar, Arora, & Garg, 2013). Tyrosinase is also responsible for enzymatic browning reactions in fruits and vegetables. Browning reaction usually damages the color and appearance of fruits, vegetables and plant-derived food products, which may indicate the spoilage of its nutritional quality. This excessive tyrosinase activity in food can be prevented by using tyrosinase inhibitors (Zhu & Gao, 2008).

The fruit of *Dillenia indica* L. (Family: Dilleniaceae), commonly known as elephant apple is native to southeastern Asia, usually grows in tropical forests of India. The fruit taste is sour, and it is extensively utilized as flavoring agents in the preparation of the foodstuff, e.g. jam, jellies, pickle, etc. *D. indica* fruit extracts to possess potent antioxidant activity (Abdille, Singh, Jayaprakasha, & Jena, 2005). It's methanol, and ethyl acetate fraction showed anti-cancer activity in three leukemia cell lines HL-60, U-937, and K-562 (Kumar, Mallick, Vedasiromoni, & Pal, 2010). The fruit has been reported to contain ascorbic acid along with vitamin E, betulinic acid, botulin, cycloartenone, *n*-hentriacontanol and β-sitosterol (Abdille et al., 2005; Das, Sarma, Ahmed, Nirmala, & Choudhury, 2012). Betulinic acid (BA) is one important pharmacologically active molecule obtained from the fruit of *D. indica*. Several methods for isolation and estimation of BA from different plants, including *Nelumbo nucifera* (Mukherjee, Pal, & Saha, 2001), *Carissa spinarum* (Chanda et al., 2014) have been reported from

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our laboratory. BA isolated from *Rhododendron collettianum* and Thai Mulberry plant extracts exhibited significant *in-vitro* tyrosinase inhibitory effect (Nattapong & Omboon, 2008; Ullah et al., 2007). On the contrary, tyrosinase activity has not been inhibited by BA on immobilized tyrosinase reactors (de Oliveira, Mischiatti, Fontana, & de Oliveira, 2014). However, its systematic monophenolase and diphenolase inhibitory kinetic studies have not yet been performed. Recently, it has been evident that BA inhibited melanin production by tyrosinase, Tyrosinase Protein-1 (TRP-1), and Tyrosinase Protein-2 (TRP-2) inhibition in B16F10 cell line (Jin, Oh, Hyun, Kwon, & Kim, 2014). Collectively, it is a thrust area for researchers to search for potent and safe tyrosinase inhibitors that can be used in the food industry.

In the present work, fruit extracts of *D. indica* were evaluated for tyrosinase inhibitory activity guided fractionations. Further, the principal active component responsible for the anti-tyrosinase activity was quantified by using RP-HPLC technique and characterized by mass spectrometry. Along with this, we also explored its tyrosinase inhibitory mechanism through enzyme kinetic analysis, Fluorescence and CD spectroscopy analysis and molecular docking study.

2. Material and method

2.1. Chemicals and reagents

Solvents used for chromatography were methanol and glacial acetic acid (all are HPLC grade) procured from Merck (Darmstadt, Germany). All other chemicals used in this study were of analytical grade. Mushroom tyrosinase from *Agaricus bisporus*, kojic acid, L-tyrosine and L-DOPA (3,4-dihydroxyphenylalanine), 1-Anilino-8-Naphthalene Sulfonate (ANS) and betulinic acid (purity ≥98%) were purchased from Sigma-Aldrich, Inc (St. Louis, MO, USA).

2.2. Plant material collection and extraction

The fruits of *D. indica* were procured from a local market of Jadavpur, Kolkata, India in August 2012. Plant material was authenticated and identified by a field botanist, and a voucher specimen number (SNPS-JU/2012/2102). One kg of the dried fruits was extracted with methanol, three times at room temperature. The collected solvent extracts were filtered and concentrated under the reduced pressure through a rotary evaporator. The yield of the crude methanol extracts of *D. indica* (MEDI) was 392.4 g (39.24% w/w). Afterward, a suspension of MEDI in water was partitioned successively with chloroform and ethyl acetate. Each fraction was evaporated under vacuum in order to obtain (i) 106 g of chloroform fraction (CFDI, 27.09% w/w), (ii) 153 g of ethyl acetate fraction (EAFDI, 21.53% w/w) and (iii) 35 g of aqueous fraction (AFDI, 38.99% w/w).

2.3. Bio- assay guided isolation and characterization of betulinic acid

The tyrosinase inhibitory activity guided assay was performed with every fraction of the extract as reported earlier (Biswas, Mukherjee, Dalai, Mandal, & Nag, 2015). The isolation of bioactive compound was carried out with the most active EAFDI using a published method (Kumar et al., 2010) with modifications. Briefly, from fractionation of EAF (40 g), BA was isolated using silica gel column eluted with the mixtures of petroleum ether (PE): chloroform gradient to yield six fractions (F1-6). Further, the bioactive fractions F1, F2 and F3 were combined and subjected to flash chromatography (Biotage-Isolera One, Sweden) using a silica gel cartridge (Biotage® SNAP cartridge KP-Sil 30 g) with a step gradient of the toluene-methanol solvent system. The yielded sub-fractions F′1 (0.75 g), F′2 (0.9 g) F′3 (0.72 g) and F′4 (0.61 g) showed tyrosinase inhibition. Potent bioactive sub-fractions 1–4 were combined and subjected to repeat flash chromatography (Silica gel Cartridge KP-Sil 10 g). A white color BA crystal was obtained after recrystallization with methanol. Scheme-I (Supplementary document) represented the bioassay-guided isolation of BA from *D. indica*.

Waters (Milford, MA, USA) HPLC system with spherisorb column (C18; 250 × 4.6 mm, 5 μm particle size) was used to identify and quantify the percentage of purity of BA (isolated). Peak integration was calculated with Empower 2 software program. The mobile phase was optimized by acetonitrile and Milli-Q water (85:15) with phosphoric acid at pH 3. A calibration curve was prepared by diluting standard BA to different concentration (1–500 μg/ml) range. The sample was also prepared in the same way. The entire standard and the sample solution were sonicated and filtered before injection into the HPLC column. Detection was done at 210 nm (Kumar et al., 2010). Additionally, BA was identified with the help of molecular ion peak by a mass spectrometry. ESI-MS (positive ion mode) spectrum was recorded with a High-Resolution Mass spectrometry (QTOF Micro YA263, Waters, Milford, MA, USA).

2.4. Mushroom tyrosinase inhibition kinetic analysis

The tyrosinase inhibition assay method developed by Biswas et al. (2015), was used with modification. One mg/ml stock solution of BA was prepared with 2% dimethyl sulphoxide (DMSO) in 50 mM potassium phosphate buffer (pH 6.5). 200 μl of reaction mixture was prepared by addition of 13.12 μg/ml (0.11 μM) tyrosinase in a 96-well plate. After incubation at 25 °C for 10 min, 2.5 mM L-tyrosine and 1 mM L-DOPA (in phosphate buffer) were added to a 96-well plate. Subsequent absorbance was recorded by using UV–visible spectroscopy (SpectraMax® M2e, Molecular Devices LLC, Sunnyvale, CA, USA) at 475 nm ($\varepsilon = 3600\ M^{-1}\ cm^{-1}$). The reaction was stopped by addition of 0.1N HCl in the reaction mixture, after 15 min of addition of substrate. IC_{50} values and relative tyrosinase activity (%) were calculated (Biswas, Mukherjee, & Chaudhary, 2016).

A non-competitive type inhibition mechanism was expressed by the Lineweaver-Burk equation as follows: $1/V = K_m/V_{max} \times (1 + [I]/K_i) \times 1/[S] + 1/V_{max} \times (1 + [I]/\alpha K_i)$. Noncompetitive inhibition is the special case of mixed inhibition where $\alpha K_i = K_i$. The Ki, K_m and V_{max} values were determined by software SigmaPlot version 12.

2.5. ANS-binding extrinsic fluorescence measurements

ANS-binding extrinsic fluorescence measurements were carried out as per method described by Biswas et al. (2015). ANS-binding fluorescence assay methodology has been discussing thoroughly in the Supplementary document.

2.6. Circular dichroism spectroscopy

Effect of BA on the secondary structure of tyrosinase was evaluated by using a Circular dichroism (CD) spectroscopy (J-815-1508, JASCO, Tokyo, Japan). The deconvolution software CDNN was used to analyze the results (Böhm, Muhr, & Jaenicke, 1992). CD spectroscopy methodology has been discussing thoroughly in the Supplementary document.

2.7. Molecular docking study

The programs Autodock 4.2 (Morris et al., 2009) was used for the docking simulations between tyrosinase and BA. The 3D struc-

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