SUPPLEMENTARY MATERIAL

Title: Tyrosinase inhibition kinetic studies of standardized extract of *Berberis aristata*.

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Abstract

The stem bark and wood of *Berberis aristata* DC (Daruharidra) are one of the principal ingredients of traditional skin lighting and exfoliating a scrub preparation in India. The standardized extract of *B. aristata* was screened to evaluate their *in-vitro* anti-tyrosinase activity and inhibition kinetics. **Phytochemical and pharmacological studies were carried out with different solvent fractions of the methanol extract of *B. aristata* (MEBA). RP-HPLC analysis was used to determine the berberine content in extract and fractions of *B. aristata*. MEBA showed maximum berberine content. Extract and fractions of *B. aristata* contain the maximum amount of alkaloids than other constituents.** In tyrosinase inhibition assay, MEBA was found to possess highest dose-dependent monophenolase and moderate diphenolase activity. The enzyme kinetic study revealed that MEBA possessed mixed type inhibition of monophenolase activity of tyrosinase. These bioactivities indicate that the methanol extract of *B. aristata* has anti-hyperpigmentation potential in human skin.

Keywords: *Berberis aristata*, Berberine, RP-HPLC, Anti-tyrosinase, Mixed-inhibition Kinetics.

1. Experimental

1.1 Materials

Solvents used for chromatography were methanol, acetonitrile and glacial acetic acid (all were HPLC grade) procured from Merck (Darmstadt, Germany). A 0.45 µm pore size membrane filter was used for mobile phase filtration and Whatman NYL 0.45 µm syringe filter was used for the filtration of samples. All other reagents and chemicals used in this study were of analytical grade. Berberin (B3251-5G), Tyrosinase (T3824-25KU), Kojic acid (K3125-5G), L-Tyrosine and L-DOPA were purchased from Sigma-Aldrich, Inc (St. Louis, MO, USA).

1.2 Plant material collection & extraction

The stem bark and wood of *B. aristata* DC (family, Berberidaceae), was purchased from the local market at Kolkata. Plant material was authenticated by Dr. S. Rajan, Field botanist, the medicinal plant collection unit, Ooty, Tamilnadu, Govt. of India. A voucher specimen number (SNPS-JU/2013/1083) of the plant has been preserved in the School of Natural Product Studies, Jadavpur University, Kolkata for further reference.

The collected samples (dried stem bark and wood) were made into small pieces by cutter mill and powdered. 500 g of the powdered sample was extracted with methanol, consecutively for three days at room temperature and the process repeated for three times. The collected solvent extract was filtered and evaporated under vacuum at 45-50°C, to get crude 90 g methanol extracts of *B. aristata* (MEBA). 70 g MEBA was then suspended in water and partitioned successively with hexane, ethyl acetate, and water. The yield of each of the solvent fraction was 11 g of hexane fraction of *B. aristata* (HFBA), 20 g ethyl acetate fraction of *B. aristata* (EFBA), and 39 g aqueous fraction of *B. aristata* (AFBA) respectively. Each sample was stored in borosilicate glass vials at 4°C prior to analysis.

1.3 Estimation of total phenolics, flavonoids, and alkaloid content

The total phenolic content was analysed according to the Folin - Ciocalteu method (FCM) described by Siddhuraju & Becker, (2007). The total flavonoids were calculated by a spectrophotometric assay. Alkaloid was confirmed by Dragendroff's method. Alkaloid content was measured by the method of Sreevidya & Mehrotra, (2003).

1.4 HPLC standardization of *B. aristata* extract

The HPLC system (Waters, Milford, MA, USA) used for the analysis consisted of a 600 controller pump, a dual-wavelength ultraviolet-visible (UV-Vis) detector equipped with an on-line degasser AF 2489 and a rheodyne 7725i injector with a 20 µL loop. Separation was achieved using Waters Spherisorb ODS2 C₁₈, 5 µm, 250 × 4.6 mm (Ireland) columns. A Milli-Q academic water purification system (Bedford, MA, USA) equipped with 0.22 mm Millipak Express filter used. A mobile phase consisting of solvent A (0.1 M acetic acid, pH 3.0, in water) and solvent B (methanol) run through C₁₈ HPLC column with the security guard column system. The elution runs at a flow rate of 1.0 ml/min with gradient flow 80 - 20 % A in B over 30 min and ultraviolet (UV) detection at 290 nm. Peak identification made by comparison of the retention time of standard. Quantitative estimation performed with the Empower2 software program using an external calibration method. This procedure was repeated three times for each sample (Abourashed & Khan 2001).

1.5 Tyrosinase inhibition assay

This assay developed by Momtaz and co-worker (2008) was used with some modification. In brief, 80 µl of sample was added with 30 µl of tyrosinase (333 U/ml in phosphate buffer) in triplicate reactions in a 96-well plate. After incubation at 25°C for 5 min, 100 µl of substrate

(4 mM L-tyrosine or 6.0 mM L-DOPA in 80 mM phosphate buffer, pH 6.8) was added to each well and incubated again at the same temperature. The optical density (OD) of the samples in each well was determined at 475 nm using a UV-visible spectroscopy (SpectraMax[®] Plus, United States). Kojic acid was used as a positive control. Tyrosinase activity assayed without BA was calculated as 100%. Relative tyrosinase activity (%) = (slope of reaction kinetics equation obtained by reaction with inhibitor)/ (slope of reaction kinetics equation obtained by reaction without inhibitor) × 100. IC₅₀ values of inhibitors were determined by plotting the percentage of activities against the inhibitor concentration.

1.6 Kinetic analysis of the inhibition of tyrosinase

This study was performed as per previously described monophenolase activity assay method with different substrate (L-tyrosine) and sample concentration (MEBA).

Mixed type inhibition mechanism expressed by the Lineweaver-Burk equation was used as follows:

$$1/V = K_m/V_{\max} \times (1+[I]/K_i) \times 1/[S] + 1/V_{\max} \times (1+[I]/\alpha K_i)$$

The α , K_i , K_m and $V_{\max}$ values were determined by the second plot of the slope ($K_m/V_{\max}$) or Y-intercept ($1/V_{\max}$) versus $[I]$.

1.7 Statistical Analysis

The experimental results, including tyrosinase activity assay and kinetic analysis for mixed-type inhibition, were calculated as triplicate determinations $\pm$ standard deviation (SD). The data was analysed using the Graph pad prism 6.0, USA.

References

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Momtaz S, Mapunya BM, Houghton PJ, Edgerly C, Hussein A, Naidoo S, Lall N. 2008. Tyrosinase inhibition by extracts and constituents of *Sideroxylon inerme* L. stem bark, used in South Africa for skin lightening. J. Ethnopharmacol. 119:507-512.

Figure Legends

Figure S1. RP-HPLC chromatogram of standard berberine

Figure S2. Calibration curve of berberine

Figure S3. RP-HPLC chromatogram of methanolic extract of *B. aristata* (MEBA)

Figure S4. RP-HPLC chromatogram of aqueous fraction of *B. aristata* (AFBA)

Figure S5. RP-HPLC chromatogram of ethyl acetate fraction of *B. aristata* (EFBA)

Figure S6. RP-HPLC chromatogram of hexane fraction of *B. aristata* (HFBA)

Figure S7. Inhibition assay of *B. aristata* extracts on monophenolase activity of tyrosinase. [(■) Without inhibitor; (▼) Kojic acid; (●) MEBA; (▲) AFBA; (♥) EFBA; (♦) HEBA. Data are presented as Mean $\pm$ SEM (n = 3)].

Figure S8. Lineweaver-Burk plots for inhibition of MEBA on monophenolase activity of tyrosinase.

[The MEBA concentrations were 0 (●), 15 (○), 35 (▼) and 45 (▽) $\mu\text{g/ml}$].

Figure S9a. Plot of Slope ($K_m/V_{\max}$) versus MEBA concentration. S9b. Plot of Y-intercept ($1/V_{\max}$) versus MEBA concentration. Secondary replots of data collected from Lineweaver-Burk plots.

[The MEBA concentrations were 0, 15, 35, 45 $\mu\text{g/ml}$.]

Table S1. IC₅₀ value of tyrosinase inhibition assay with L-Tyrosine and L-DOPA.

Plant Extract	IC ₅₀ value of monophenolase μg/ml (L-Tyrosine)	IC ₅₀ value of diphenolase μg/ml (L-DOPA)
AFBA	45.45 ± 1.064	431.11 ± 0.551
MEBA	31.98 ± 2.238	412.01 ± 0.371
EFBA	68.87 ± 1.691	598.50 ± 1.087
HFBA	109.23 ± 1.569	654.01 ± 1.569
Berberine	<1000	NA
Kojic Acid	5.01 ± 0.200	52.08 ± 0.005

[All values are expressed as mean ± S.D (n = 3). AFBA-Aqueous fraction of *B. aristata*; MEBA-Methanolic Extract of *B. aristata*; EFBA-Ethyl Acetate fraction of *B. aristata* ; HFBA-Hexane fraction of *B. aristata*; NA- Not Active]

Figure S1

Figure S2

Figure S3

Figure S4

Figure S5

Figure S6

Figure S7

Figure S8

Figure S9a.

Figure S9b.