An *invitro* study on inhibition of Tyrosinase and Xanthine oxidase by alkaloid rich fraction from *Indigofera aspalathoides*

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**ABSTRACT**

To assay the *in vitro* xanthine oxidase and Tyrosinase inhibitory activity of the alkaloid fractions of the leaves of *Indigofera aspalathoides* and to determine its enzyme inhibition mechanism. Xanthine oxidase and Tyrosinase inhibitory activity was assayed spectrophotometrically under aerobic conditions and the degree of enzyme inhibition was determined by measuring the increase in absorbance at 295nm associated with uric acid and Tyrosine formation respectively. Enzyme kinetics was carried out using Lineweaver-Burk plots using xanthine and Tyrosine as the substrate. The alkaloid fractions exhibited highest potency (82.3±1.5) at 100 µl/ml in Xanthine oxidase followed by Tyrosinase inhibitory diphenolase 50±1.2 and monophenalase 60.05 ± 1.4 fractions. Enzyme inhibition mechanism indicated that the mode of inhibition was of a mixed type. These results suggest that the use of *Indigofera aspalathoides* for the treatment of gout could be attributed to its xanthine oxidase inhibitory activity and Tyrosinase inhibitors have become increasingly important as cosmetic and medicinal products, primarily to control melanin pigmentation.

**Keywords:** *Indigofera aspalathoides*, Xanthine oxidase, Tyrosinase, alkaloid

**INTRODUCTION**

Xanthine oxidase is a vital enzyme catalyzing the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid which is excreted by kidneys. Excessive production and/or inadequate excretion of uric acid results in hyperuricemia (Lespade and Bercion, 2010). Xanthine oxidase is distributed most abundantly in the liver and intestine (Battelli et al.,1972), situated at the end of a catabolic sequence of the purine nucleotide metabolism in humans and few other uricotelic species (Unno et al., 2004). The catalysis of xanthine by xanthine oxidase enzyme can lead to the accumulation of uric acid and ultimately cause gout. Gout has heterogeneous clinical symptoms that which are increasing in levels of serum acid, recurrent attacks of acute inflammation in the joints associated with the accumulation of monosodiumurate (MSU) crystals in synovial fluid. MSU crystal is built up on the tissue or around the joints, kidney disorders and kidney stone formation cause by the buildup of uric acid in kidney tissue (Ernst et al., 2008). It generates superoxide (O2-) during oxidation of substrates (Battelli et al., 1972), subsequently plays an important role in biological source of oxygen derived free radicals that contribute to oxidative damage to living tissues involved in many pathological processes such as inflammation, atherosclerosis, cancer and aging diseases (Havlika et
al., 2010 and Rohman et al., 2010), several types of tissue and vascular injuries (Berry and Hare, 2004), and chronic heart failure hearts have demonstrated that the progressive development of heart failure is associated with increased myocardial xanthine oxidase levels (Ferdinandy et al., 1999, 2000, Pacher et al., 2006). 

Tyrosinase also plays an important role in the formation of melanins, because it facilitates melanization by catalysing reactions from tyrosine to dopa and from dopa to dopaquinone. Tyrosinase inhibitors have become increasingly important as cosmetic and medicinal products, primarily to control melanin pigmentation. Tyrosinase is the key enzyme in the first stage of the melanogenesis pathway, catalysing the conversion of L-tyrosine into L-dopaquinone. Melanin synthesis inhibitors are topically used for treating localized hyperpigmentation in humans such as lentigo, nevus, ephelis, post-inflammatory state and melanoma of pregnancy. Tyrosinase is also one of the key enzymes responsible for controlling the insect molting process and could therefore be used as an insecticide for the control of pests and insects. The development of superoxide scavengers or tyrosinase inhibitors will contribute to the prevention of skin browning (Khanom et al., 2000).

Therefore, this research aims to evaluate xanthine oxidase and Tyrosinase inhibitory activity from Indigofera spalathoides so as to discover a natural substitute of plant origin, which could have a superior effect of inhibiting xanthine oxidase activity and can be used as an alternative to allopurinol for the treatment of gout as well as for the treatment of other inflammatory-related diseases. Optimization helped to increase xanthine oxidase and Tyrosinase inhibitory activity from the selected plant material and potential extraction.

MATERIALS AND METHODS

Preparation of extracts

Organic solvents (methanol) extract of the Indigofera aspalathoides leaf were prepared according to the method described by Boaky- Yiadon (1979) with little modifications. Twenty grams of Indigofera aspalathoides leaf extracts were air-dried, crushed and blended into powder using an electric blender for each solvent. The blended material was transferred to a beaker and soaked separately in 100 ml of the organic solvent at room temperature. The mixture was extracted by agitation on a rotary shaker. The extract obtained was vacuum-dried and used for further test.

Extraction of Alkaloid

Ground leaf material was extracted with cold distilled methanol (CH$_3$OH) with occasional swirling. After filtration, the solvent was removed under reduced pressure at 40°C, to minimise any thermal degradation of the alkaloids. The crude alkaloid mixture was then separated from neutral and acidic materials, and from water solubles, by initial extraction with aqueous acetic acid (CH$_3$CO$_2$H) followed by dichloromethane. Then basification was done on the aqueous solution and further the organic layer of dichloromethane contained crude alkaloid extract (Surya Hadi and John, 2001).

Assay of xanthine oxidase activity

The xanthine oxidase activity with xanthine as the substrate was assayed spectrophotometrically (EC 1.1.3.22) (Owen and Johns, 1999). Mixture consisted of 1 mL plant extract solution (100 µg/mL), 2.9 mL 50 mM potassium phosphate buffer (pH 7.5 at 25°C) that were initiated by adding to 2 mL of the substrate solution (xanthine 0.15 mM). Xanthine 0.15 mM was prepared by dissolving it in 100 µL NaOH and the pH was adjusted to 7.5. The mixture was incubated at 25°C for 15 min. After pre incubation, the reaction was initiated by the addition of 0.1 mL (0.1 units/mL in phosphate buffer, pH 7.5 at 25°C) xanthine oxidase enzyme (from bovine milk, Sigma X1875). Xanthine oxidase was prepared in cold potassium phosphate buffer immediately before used. The mixture was incubated at 25°C for 30 min, for stopping reaction 1 mL HCl 1 N was added. The absorbance was recorded at 290 nm using an UV spectrophotometer. Allopurinol (100 µg/mL) was used as positive control (Nguyen et al., 2004; Umamaheswari et al., 2007).

One unit will convert 1.0 µmol of xanthine to uric acid per minute. Xanthine oxidase activity was expressed as the percentage inhibition of XO, which was calculated as,

\[
\%\text{Inhibition} = \frac{(A - B) - (C - D)}{A - B} \times 100
\]

where \(A\) is the activity of the enzyme without test extract, \(B\) the control of \(A\) without test extract and enzyme, \(C\) and \(D\) are the activities of the test extract with and without xanthine oxidase. Plant extract (10, 25, 50 and 100 µg/mL), a known inhibitor of xanthine oxidase, was used as a positive control. IC$_{50}$ values were calculated from the mean values of data.

Assay Procedure

The assay was performed as previously described (Masamoto et al., 1980) with slight modifications. The reaction media (3 ml) for x-
diphenolase activity contained 0.5 mM L-DOPA in 50 mM sodium phosphate buffer (pH 6.8), the indicated concentration of inhibitor, and 3.3% DMSO. The final concentration of mushroom tyrosinase was 6.67 µg/ml.

In this method, 0.1 ml of different concentrations of effectors dissolved in DMSO solution was added into the test tube. Then, 2.8 ml substrate solution in sodium phosphate buffer preincubated at 30°C was mixed and 0.1 ml of the aqueous solution of mushroom tyrosinase (20 µg) added. This solution was immediately monitored for 1 min (after a lag period of 5 sec) for the formation of dopachrome by measuring the linear increase in absorbance at 475 nm. The reaction was carried out at constant temperature of 30°C. Absorption was recorded using a Beckmann UV 600 spectrophotometer (Germany). The kinetic and inhibition constants were obtained by the method previously described (Chen et al., 2000).

Tyrosinase inhibition was calculated as follows:

Tyrosinase inhibition (%) = \((A - B)/A \times 100\)

Where \(A\) represents the difference in the absorbance of the solution in the absence of the test sample and the presence of the enzyme; and \(B\) represents the difference in the absorbance of the solution in the presence of the test sample and the enzyme. Each experiment was carried out in triplicate.

RESULT

The Partial characterization of methanol petal extract of Indigofera aspalathoides by TLC

The methanol extract of Indigofera aspalathoides loaded on Pre-coated TLC plates (60 F254 Merck) and developed with a solvent system of hexane, chloroform and methanol in the ratio of 1:0.5:0.1 was efficient to extract the antibacterial compound it is used for further studies. The developed plate was viewed under UV 240nm and 360nm (Table-1).

Table-1 Partial characterization of methanol leaf extract of Indigofera aspalathoides by TLC

<table>
<thead>
<tr>
<th>Component No.</th>
<th>UV Light 360nm RF value</th>
<th>UV light 240 nm RF value</th>
<th>Normal Light RF value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.80</td>
<td>0.80</td>
<td>0.80</td>
</tr>
<tr>
<td>2</td>
<td>0.76</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>0.66</td>
<td>0.66</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.58</td>
<td>-</td>
<td>0.58</td>
</tr>
<tr>
<td>5</td>
<td>0.56</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>0.45</td>
<td>0.45</td>
<td>-</td>
</tr>
</tbody>
</table>

In vitro xanthine oxidase inhibitory activity of alkaloid extract of Indigofera aspalathoides

The four different concentrations of Indigofera aspalathoides alkaloid extract were tested for the inhibition activity of xanthine oxidase. Indigofera aspalathoides extracts in different concentrations inhibited the xanthine oxidase activity. The maximum inhibition was found at 100 µg/ml concentration (Table-2).

Table. 2 In vitro xanthine oxidase inhibitory activity of Indigofera aspalathoides

<table>
<thead>
<tr>
<th>Different concentrations of Indigofera aspalathoides alkaloid extract</th>
<th>Inhibition percentage of Xanthine oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µg/ml</td>
<td>22.21±1.8</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>38.1±0.8</td>
</tr>
<tr>
<td>75 µg/ml</td>
<td>68.21±1.2</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>82.3±1.5</td>
</tr>
</tbody>
</table>

Values are mean±S.E.M. of three parallel measurements.

Concentration Effects of Indigofera aspalathoides alkaloid extract on the Diphenolase Activity of mushroom Tyrosinase

The alkaloid extract of Indigofera aspalathoides were tested for the effects on the oxidation of DOPA by mushroom tyrosinase. The inhibitory course is shown in Fig-8. With increasing the concentrations of the alkaloid extract of Indigofera aspalathoides, the diphenolase activity of mushroom tyrosinase markedly decreased concentration-dependently. The values of IC50, the inhibitor concentration leading to 50% activity lost, of alkaloid extract of Indigofera aspalathoides were estimated to be 25, 50, 75 and 100 µg/ml, respectively (Table-3).

Table-3. The inhibitions of alkaloid extract of Indigofera aspalathoides on the diphenolase activity of mushroom tyrosinase for the catalysis of DOPA at 30°C

<table>
<thead>
<tr>
<th>Different concentrations of Indigofera aspalathoides alkaloid extract</th>
<th>Inhibition of Diphenolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µg/ml</td>
<td>14.75±2.6</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>18.85±0.1</td>
</tr>
<tr>
<td>75 µg/ml</td>
<td>38.52±1.2</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>50.00±2.1</td>
</tr>
</tbody>
</table>

Results are expressed as percentage inhibited diphenolase formation with respect to control. Each value represents the mean+SD of five experiments.
Inhibitory Effects of *Indigofera aspalathoides* alkaloid extract extract on the Monophenolase Activity of Mushroom Tyrosinase

Tyrosinase can catalyze the hydroxylation of monophenols (monophenolase activity) and the oxidation of o-diphenols to o-quinones (diphenolase activity), both depending on molecular oxygen. On the other hand, the steady-state rate decreased with increasing the concentration of *Indigofera aspalathoides* alkaloid extract as shown in Table-4. When the concentration of this inhibitor reached 100 µg/ml, the remaining enzyme activity was determined to be 60%. The inhibitor concentration leading to 50% activity lost (IC$_{50}$) was estimated to be 25 µg/ml. The results obtained are summarized in Table 4. The results indicated that 4-halobenzoic acids inhibited the monophenolase activity of mushroom tyrosinase.

Table 4: The inhibitions of alkaloid extract of *Indigofera aspalathoides* on the monophenolase activity of mushroom tyrosinase.

<table>
<thead>
<tr>
<th>Different concentrations of <em>Indigofera aspalathoides</em> leaf alkaloid extract</th>
<th>Inhibition of Monophenolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µg/ml</td>
<td>16.39±2.6</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>24.59±0.2</td>
</tr>
<tr>
<td>75 µg/ml</td>
<td>49.18±1.5</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>60.05±1.4</td>
</tr>
</tbody>
</table>

Results are expressed as percentage inhibited diphenolase formation with respect to control. Each value represents the mean±SD of five experiments.

**DISCUSSION**

Recent findings show that the occurrence of gout is increasing worldwide, possibly due to the changes in dietary habits like intake of food rich in nucleic acids, such as meat and seafoods. Hypouricemic agents are commonly employed for the treatment of chronic gouty arthritis, which includes xanthine oxidase inhibitors and uricosuric agents (Pacher et al., 2006). The inhibition of XO has been recognized as one of the promising targets for the treatment of hyperuricemia since allopurinol, a potent XO inhibitor with a purine backbone, has been used clinically for many years (Yasuda et al., 2008). In general, allopurinol is the drug of choice; however it has serious side effects. Thus, new alternatives with increased therapeutic activity and lesser side effects are desired. Hydroxychavicol was a more potent xanthine oxidase inhibitor than allopurinol, which is clinically used for the treatment of hyperuricemia (Kazuya et al., 2009). In the present study delivered for xanthine oxidase inhibitors of phytochemical origin from the various fractions of the hydromethanolic extract of the leaves of xanthine oxidase.

*Indigofera aspalathoides* alkaloid extract fractions inhibited xanthine oxidase in a concentration-dependent manner. The *in vitro* inhibition of xanthine oxidase by the chloroform fraction is moderate when compared to allopurinol. However, at higher doses of the fraction, xanthine oxidase would be significantly inhibited. Alkaloid extract are a group of nitrogenous and heterocyclic compounds, which have been reported to possess xanthine oxidase inhibitory activity (Costantino et al., 1992). Hence, the presence of *Indigofera aspalathoides* alkaloid extract would have contributed towards xanthine oxidase inhibition.

Skin whitening agents bring the attention of researchers in recent days in order to find the solution for hyperpigmentation disorders. Many tyrosinase inhibitors and antioxidant agents have been tested as a way of preventing overproduction of melanin in epidermal layers (Cabanes et al., 1994). The current study employs the antityrosinase efficacy of *Indigofera aspalathoides* alkaloid extract, DOPA as substrate for the diphenolase activity and Tyrosinase for the monophenolase activity of the enzyme. The effects of *Indigofera aspalathoides* alkaloid extract on the diphenolase activity and the monophenolase activity of tyrosinase were studied. The results showed that *Indigofera aspalathoides* alkaloid extract could inhibit the diphenolase activity and the monophenolase activity of mushroom tyrosinase. For the diphenolase activity, the inhibition displayed as reversible, and the inhibition types were determined to be noncompetitive.

**CONCLUSION**

The alkaloid extracts of *Indigofera aspalathoides* possess strong xanthine oxidase and tyrosinase inhibitory activity; therefore it could be applied as a natural source of bioactive compounds applicable in food, pharmaceutical and cosmetic industries. However, the bioactive compounds responsible for the observed biological activities should be identified and activity of the isolated compounds should also be compared with that of the crude extract to reveal possible synergistic interaction. Hence, the further experiment on isolation, structure elucidation and identification of bioactive compounds are in progress.
REFERENCE


