INHIBITOR XANTHINE OXIDASE OF EXTRACT BLUMEA BALSAMIFERA L.(DC) LEAVES (ASTERACEAE)

Le Nguyen Tu Linh¹, Bui Dinh Thach¹, Tran Thi Linh Giang¹, Vũ Quang Dao¹, Trịnh Thi Ben¹, Nguyễn Pham Ai Uyen², Diệp Trung Cang³, Nguyễn Thanh Huy², Ngô Ke Suong¹
(1) Institute of Tropical Biology, Vietnam Academy of Science and Technology; Nong (2) Lam University

ASTRACT

Xanthine oxidase is an enzyme responsible for catalyzing the oxidation of hypoxanthine to xanthine and of xanthine to formation of uric acid. This study aimed to identify the xanthine oxidase inhibitors and decrease of serum uric acid levels from extract methanol Blumea balsamifera L.(DC) leaves in hyperuricaemic. Results were showed methanol extract of B. balsamifera leaves have shown total flavonoid contents was 72.2 mg/g dw, promising inhibited xanthine oxidase activity with IC₅₀ = 27.6 µg/ml, significant decreased in the serum urate level (3.9 and 3.56 mg/dL), and reduced of xanthine oxidase activities in the mouse liver (1.22 and 1.01 nmol acid uric/min. mg protein) at dosage 1.25 and 2.5/kg wb, respectively. These results may explain and support the dietary use of the methanol extracts from B. balsamifera leaves for the prevention and treatment of Gout.

Keywords: Flavonoid, inhibitor, xanthine oxidase, hyperuricaemic, Blumea balsamifera L.(DC).

INTRODUCTION

Xanthine oxidase plays an important role in the purine nucleotide metabolism in humans. Its main function is to catalyze the oxidation of hypoxanthine to xanthine and xanthine to uric acid (Rundes and Wyngaarden, 1969; Owen and Johns, 1999; Ramallo et al., 2006). Gout is a condition caused by the over-production or under-excretion of uric acid, resulting in the deposition of monosodium urate crystals in the joints or soft tissue leads to sudden onset, intense pain, swelling, warmth and erythema (Klippel, 2008). The peak incidence occurs in patients 30 to 50 years old, and the condition is much more common in men than in women (Kramer et al., 2002; Pacher et al., 2006).

The treatment for hyperuricaemic and gout is reducing the uric acid production (Emmerson, 1996). Xanthine oxidase inhibitors are very useful for this (Kong et al., 2001; Unno et al., 2004). Xanthine oxidase inhibitors acts by blocking the biosynthesis of uric acid from purine in the body (Unno et al., 2004). A representative drug widely used to treat hyperuricaemic and gout is allopurinol. Allopurinol, which works by preventing the formation of uric acid through the inhibition of the enzyme xanthine oxidase (Collin et al., 2006). However, allopurinol can cause hazardous side effects such as fever and rash, progressively developing leukocytosis, eosinophilia, vasculitis, aseptic meningitis, nephritis and renal dysfunction, and hepatic dysfunction (Boyer et al., 1977; Duchene et al., 2000).

Thus, the search for novel xanthine oxidase inhibitors with higher therapeutic activity and fewer side effects are desired not only to treat gout but also to combat various other diseases associated with xanthine oxidase activity, including focus the medicinal herbs from plant sources (Kong et al., 2000).
In this present study we report that effect of extract methanol of *Blumea balsamifera* L.(DC) leaves to oxonate –pretreated hyperuricaemic mice in the reduction of serum uric acid levels and decrease of xanthine oxidase activities in the mouse liver.

**MATERIALS AND METHODS**

**Materials and chemicals**

*Blumea balsamifera* L.(DC) leaves was taken from nature. Xanthine, xanthine oxidase, allopurinol from company HIMEDIA, all other chemicals were of the highest analytical grade available.

**Animals for experiment**

Male mice (30-35 g) were purchased from The Pasteur Institute in HCM City. They were allowed one week to adapt to their environment before used for experiments. All animals were maintained on a 12 hr/12 hr light/dark cycle at a constant temperature room.

**Methods**

**Preparation of sample**

Sample was washed cleanly, then dried at 50°C, after that milled into powder for using in later tests.

*B. balsamifera* extract was prepared with ultrasonic method. Powder *B. balsamifera* leaves were extract twice with methanol at 50°C, by ultrasonic. The methanol extract was filtered and concentrated to under vacuum. Extract was store at 4°C for using in later test.

**Determination of Total Flavonoid Content**

0.25 ml of the extract solution or catechin standard solution was mixed with 1.25 ml of distilled water in a test tube followed by addition of 75 μl of a 5% NaNO₂ solution. After 6 min, 0.15 ml of a 10% AlCl₃ solution was added and allowed to stand for another 5 min before 0.5 ml of 1 M NaOH was added. The absorbance was measured immediately against the blank at 510 nm (Dewanto et al., 2002). Results showed as mg/g dw. Standard substance was catechin.

**In vitro xanthine oxidase inhibitory activity**

The assay mixture consisted of 1mL of the test compound (5 – 100 μg/mL), 2.9 mL of phosphate buffer (pH 7.5) and 0.1 mL of xanthine oxidase enzyme solution (0.1 units/mL in phosphate buffer, pH 7.5), which was prepared immediately before use. After preincubation at 25 ºC for 15 min, the reaction was initiated by the addition of different concentration (5 – 100 μg/mL) of the substrate solution. The assay mixture was incubated at 25 ºC for 30 min. The reaction was stopped by adding 1 mL of 1 N HCl and the absorbance was measured at 290 nm using an UV spectrophotometer (Owens and Johns, 1999; Remya et al., 2011). Allopurinol (5 – 100 μg/mL) was used as the standard. The percentage inhibition was calculated by:

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

Where A is the activity of the enzyme without the compound, B is the control of A without the compound and enzyme, C and D are the activities of the compound with or without xanthine oxidase respectively.
The assay was done in triplicate and IC$_{50}$ values were calculated from the percentage inhibition.

**Effects of Xanthine Oxidase Inhibition in Hyperuricaemic**

The experimental animal model of hyperuricaemic induced by the uricase inhibitor potassium oxonate (Stavric et al., 1975; Hall et al., 1990) was adopted. Animal randomly divided into the experimental groups, containing 6 mice per group Briefly, all mice except the control group (group I) were injected intraperitoneally with potassium oxonate (280 mg/kg) 1 hr before the final tested drug administration to increase the serum urate level.

Methanol extract *B. balsamifera* leaves at various concentrations and allopurinol dissolved in CMC 0.5% were given orally to study groups. The volume of the suspension administered was based on body weight measured immediately prior to each dose.

Animals in group I (control group) and group II were orally administered with CMC 0.5% for 7 days. The groups III and groups IV were orally received methanol extract *B. balsamifera* leaves with dose 1.25 or 2.5 g/kg bw, respectively. For groups V received allopurinol at 10 mg/kg.

After 7 days, blood samples were collected from the mice by tail vein bleeding or cardiac puncture. The blood was allowed to clot for approximately 1 hr at room temperature and then centrifuged at 2500 x g for 10 min. to obtain the serum which was stored at −20°C until use.

**Enzyme preparation.** Mouse liver was excised and homogenized in 5 volumes of 80 mM sodium pyrophosphate buffer (pH 7.4) at 4°C. The homogenate was then centrifuged at 3000 x g for 10 min, the lipid layer carefully removed, and the resulting supernatant further centrifuged at 10,000 x g for 60 min at 4°C. The final supernatant was stored at −80°C until enzyme assays.

**Assays of xanthine oxidase activities.**

The activities of xanthine oxidase were assayed by monitoring uric acid formation using a spectrophotometric method described in previous studies (Hall et al., 1990; Kong et al. 2002). The reaction mixture contained 50 mM phosphate buffer (pH 7.5), 100 ml of the mouse liver supernatant prepared in the previous section, and 1 mM potassium allantoxanate to prevent oxidation of uric acid to allantoin, in a final volume of 5.5 ml. After preincubation for 15 min. at 37 °C, the reaction was initiated by the addition of 1.2 ml of 250 mM xanthine. After 10 min, the reaction was stopped by the addition of 0.5 ml of 0.58 M HCl. The solution was then centrifuged at 3000 x g for 5 min, and use commercial kit to determination uric acid after reaction. Xanthine oxidase activities were expressed as nmol uric acid/min per mg protein.

**Uric acid assay.**

The effects of methanolic extract of *B. balsamifera* leaves were measured on blood uric acid level and on liver homogenate xanthine oxidase activity by commercial kits (Abbot, USA).

**Protein determination.**

Protein concentration was determined by the method of Lowry (1951) using bovine serum albumin as the standard.

**Data analysis:** Each treatment was repeated 3 times. Data were analyzed by Microsoft Excel 2013 and SAS 9.1 softwares.
RESULTS AND DISCUSSIONS

Total flavonoid contents
The total flavonoid content was expressed as mg of catechin equivalent per gram of dry weight. The total flavonoid contents in this medicinal plants was 72.2 mg/g dw (Table 1).

Table 1. Total flavonoid contents.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total flavonoid contents (mg/g dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. balsamifera</td>
<td>72.2 ± 0.84</td>
</tr>
<tr>
<td>leaves</td>
<td></td>
</tr>
</tbody>
</table>

Extract from B. balsamifera effected high total flavonoid contents, flavonoids were found widely in plant, has been shown effect inhibitory xanthine oxidase (Chrysoula et al., 2012).

Inhibitor xanthine oxidase in vitro
Xanthine oxidase is an enzyme responsible for catalyzing the oxidation of hypoxanthine to xanthine and of xanthine to formation of uric acid (Rundes and Wyngaarden, 1969; Owen and Johns, 1999; Ramallo et al., 2006). Inhibitor xanthine oxidase was resulted decrease acid uric concentration, so useful in treatment for Gout.

Effect inhibitor xanthine oxidase based on levels acid uric were created in the same reaction time. The absorbance changes can be quantified at wavelength 290 nm.

Xanthine oxidase inhibitory activity for both allopurinol and optimized extract of B.balsamifera leaves were also expressed in term of IC$_{50}$, the concentration of positive control and optimized sample needed to achieve 50% inhibition of xanthine oxidase under experimental conditions. IC$_{50}$ values of standard substance and extract were presented in table 2.

Table 2. Inhibitor xanthine oxidase in vitro.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage inhibition (%)</th>
<th>IC$_{50}$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration µg/mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>B. balsamifera</td>
<td>47.93 ± 0.49</td>
<td>68.29 ± 0.92</td>
</tr>
<tr>
<td>leaves</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allopurinol</td>
<td>3.56</td>
<td></td>
</tr>
</tbody>
</table>

To have a basic for the comparison of the activities of tested samples, we used allopurinol as positive control substance due to its strong inhibitor xanthine oxidase activity. Results showed effect xanthine oxidase inhibitory increase base on concentration sample. The IC$_{50}$ values of B. balsamifera leaves extract and allopurinol were 27.6 and 3.56 µg/mL, respectively. In concentration 100 µg/mL, B. balsamifera leaves extract had the most potent xanthine oxidase inhibitory (93.8%).

Many studies had analyzed the chemical composition of B. balsamifera leaves contain tannin compounds, phenols, glycosides, saponins, steroids, terpenoids and antharaquinon (Siddesha et al., 2011). Phenol compounds, especially flavonoids had a strong inhibitor xanthine oxidase activity and against antioxidant potential. Many studies prove that xanthine oxidase activity causes the creation of more free radicals (Cotelle, 2001). Research has demonstrated...
that the inhibitory xanthine oxidase enzyme activity can reduce levels of uric acid in the blood, but also reduce the production of free radicals (Kong et al., 2002). So, the addition of inhibitors xanthine oxidase enzyme not only effect inhibitory uric acid to prevent gout, has also effect against the oxidative stress that causes damage to cells and tissues in the body.

**Effects of Xanthine Oxidase Inhibition in Hyperuricaemic**

The hypouricaemic effects of the orally administered methanol extract of *B. balsamifera* leaves on serum uric acid levels in oxonate-pretreated mice are also shown in Table 3.

**Table 3.** Hypouricaemic effects of methanol extract *B. balsamifera* leaves and allopurinol on serum urate levels in oxonate-pretreated mice.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Dosage (g/kg bw)</th>
<th>Concentration of acid uric in serum (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mice dosed with vehicle</td>
<td>-</td>
<td>3.14 ± 0.06</td>
</tr>
<tr>
<td>Hyperuricaemic mice dosed with vehicle</td>
<td>-</td>
<td>5.16 ± 0.13</td>
</tr>
<tr>
<td>Hyperuricaemic mice dosed with methanol extract <em>B. balsamifera</em> leaves</td>
<td>1.25</td>
<td>3.90 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>3.56 ± 0.04</td>
</tr>
<tr>
<td>Hyperuricaemic mice dosed with Allopurinol</td>
<td>0.01</td>
<td>1.25 ± 0.05</td>
</tr>
</tbody>
</table>

Each value in the table was the average of 3 repetitions ± SE (Mean ± SE). The different letters (a, b, c...) showed significant difference at p = 0.05 according to Duncan’s method.

Initial mean serum uric acid levels in the normal mice was 3.14 mg/dL. Treatment with oxonate resulted in a marked elevation of serum uric acid level. After induction of hyperuricaemic with potassium oxonate, the serum urate levels of the control mice was 5.16 mg/dL (Table 3). After 7 days treatment, compared with group II, the serum uric acid levels reduced 3.9 and 3.56 mg/dL with dosage 1.25 and 2.5 g/kg bw, respectively. Parallel studies involving oral dosing with allopurinol at 0.01 g/kg bw also elicited significant reductions in serum urate levels in the hyperuricaemic mice (1.25 mg/dL). Allopurinol effected reducing of the serum urate levels stronger than methanol extracts of *B. balsamifera* leaves. However, allopurinol can cause hazardous side effects such as fever and rash, progressively developing leukocytosis, eosinophilia, vasculitis, aseptic meningitis, nephritis and renal dysfunction, and hepatic dysfunction (Boyer et al., 1977; Duchene et al., 2000). Elevated levels of uric acid in the circulation could give rise to gout and possibly other pathological conditions (Liese et al., 1999; Fang and Alderman, 2000; Tomita et al., 2000; Puddu et al., 2001). But on the other hand, the antioxidant action of uric acid is also well documented, particularly its ability to inhibit DNA damage (Burkhardt et al., 2001; Singh et al., 1998). Thus, excessive lowering of the uric acid level in the circulation beyond that of the normal range might even be counter productive.
Table 4. Inhibition of hepatic xanthine oxidase activities in oxonate-pretreated hyperuricaemic mice by administration of methanol extracts of B. balsamifera leaves and allopurinol.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Dosage (g/kg)</th>
<th>Xanthine oxidase activity (nmol acid uric/min. mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperuricaemic mice dosed with vehicle</td>
<td>-</td>
<td>1.38 ± 0.06</td>
</tr>
<tr>
<td>Hyperuricaemic mice dosed with methanol extract B. balsamifera leaves</td>
<td>1.25</td>
<td>1.22 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1.01 ± 0.06</td>
</tr>
<tr>
<td>Hyperuricaemic mice dosed with Allopurinol</td>
<td>0.01</td>
<td>0.37 ± 0.03</td>
</tr>
</tbody>
</table>

Each value in the table was the average of 3 repetitions ± SE (Mean ± SE). The different letters (a, b, c...) showed significant difference at p = 0.05 according to Duncan's method. It can be seen that both methanol extracts of B. balsamifera leaves at the two dosage levels used and allopurinol were able to produce significant decreases xanthine oxidase activities in the mouse liver. Compare with control group, the reduction in enzyme activities were 1.22 and 1.01 nmol acid uric/phút. mg protein with dosage 1.25 and 2.5 g/kg bw, respectively by methanol extracts of B. balsamifera leaves and 0.37 nmol acid uric/phút. mg protein for allopurinol. Therefore, as far as in vivo inhibition of enzyme activities is concerned, allopurinol appears to be much more potent than that of methanol extracts of B. balsamifera leaves. Although in vitro inhibition of xanthine oxidase by methanol extracts of B. balsamifera leaves have been reported previously (Nguyen et al., 2012, Dung et al., 2012), the present study is the demonstrating clearly the in vivo hypouricaemic action of methanol extracts of B. balsamifera leaves as well as their ability to decrease tissue levels of xanthine oxidase in animals orally administered.

Xanthine oxidase enzyme is the key in purines' hepatic metabolic pathways. The reaction is the conversion of hypoxanthine to xanthine and uric acid. Controlling uric acid levels is the most important factor in prevention and treatment (Umamaheswari et al., 2007, Kong et al., 2001; Unno et al., 2004). Allopurinol uric acid lowering was the first drug for the past 4 decades in the treatment of hyperuricaemic utilized and gout (Wallach, 1998). Unfortunately allopurinol causes some deleterious side effects. Thus, the search for novel xanthine oxidase inhibitors with higher therapeutic activity and fewer side effects are desired not only to treat gout but also to combat various other diseases associated with xanthine oxidase activity, including focus the medicinal herbs from plant sources, including the B. balsamifera, especially the leaves shown effect inhibitory xanthine oxidase and reduce uric acid, this result can support the treatment of gout.

CONCLUSIONS

Total flavonoid content of Methanol extracts of B. balsamifera leaves was 72.2 mg/g dw and effect inhibitor xanthine oxidase IC$_{50}$ = 27.6 μg/mL.
Methanol extract of *B. balsamifera* leaves effected inhibitory xanthine oxidase activity and reduced uric acid in serum in hypouricaemic oxonate-pretreated mice with dosage 1.25 and 2.5 g/kg bw.

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**REFERENCES**


