การประเมินการผลิตสารมัลเบอร์โรไซด์เอและฤทธิ์ทางชีวภาพจากเขียวอะโนโต้ไฟล์และเนื้อเยื่อเพาะเลี้ยงของหม่อน

จักรพันธ์ ไชยทอง 1,2, บุญชู ศรีลาภวัฒน์ 3, ธีรภัทร นาทนารี 3, ธีระชัย กิติรัตนญา 1,2, กรวิทย์ อยู่สกุล 1,2, เจริญพร ทรงสุทธิตก 4, อัมนาน สADB ม่า ไชย 5, วาสนาภรณ์ ภูติลุน 1,2, 5

บทคัดย่อ
การประเมินการผลิตสารมัลเบอร์โรไซด์เอและฤทธิ์ทางชีวภาพจากเขียวอะโนโต้ไฟล์และเนื้อเยื่อเพาะเลี้ยงของหม่อน

จักรพันธ์ ไชยทอง 1,2, บุญชู ศรีลาภวัฒน์ 3, ธีรภัทร นาทนารี 3, ธีระชัย กิติรัตนญา 1,2, กรวิทย์ อยู่สกุล 1,2, เจริญพร ทรงสุทธิตก 4, อัมนาน สADB ม่า ไชย 5, วาสนาภรณ์ ภูติลุน 1,2, 5

สารที่จากหม่อนมีการรายงานฤทธิ์ทางชีวภาพในการยับยั้งเอนไซม์และอาการใกล้เคียง 8.0 ส่วนปริมาณสารที่สำคัญของยาเตรียมทารับที่แขวนตะกอนและการกระจายตัวที่ดีกว่า รวมถึงมีค่าพีเอชที่พัฒนาขึ้นของน้ำกระสายยาและผงยาที่แยกกันอยู่ และทดสอบความคงตัวภายใต้สภาวะเหมือนการใช้งานจริงของน้ำกระสายยา ระยะที่ 1 ถึงระยะที่ 10 กายภาพ เคมี และจุลชีววิทยา

ผล
ยาเตรียมสําหรับผู้ป่วยเฉพาะราย

pheritinum extracts were reported to have some bioactivities such as α-glucosidase and tyrosinase inhibitory activities. Root part of M. alba contains high level of mulberroside A, a glycoside form of oxyresveratrol. This major compound shows many pharmacological activities including anti-tyrosinase activity, anti-viral activity, antioxidant activity, hepatoprotective effect, and neuroprotective effect. In this study, mulberroside A accumulation, α-glucosidase and tyrosinase inhibitory activities of endophytic fungus, in vitro cultures from Morus alba extracts and intact M. alba were determined and compared to evaluate them as alternative sources of secondary metabolites. The results show that mulberroside A concentrations in cell suspension and root cultures of M. alba were higher than the intact fibrous root (10.31±0.76, 19.34±0.53 น ้าหนักแห้ง)

คำสั่งพิเศษ: หม่อน, สารมัลเบอร์โรไซด์เอ, ฤทธิ์ยับยั้งเอนไซม์กลูโคซิเดส, ฤทธิ์ยับยั้งเอนไซม์ไทโรซิเนส, เอนโดไฟต์, เอนโดไฟต์ของน ้าหนักแห้ง น ้าหนักแห้ง}

Abstract
Evaluation of Mulberroside A Production and Bioactivities from Endophytic Fungus and In Vitro Cultures of Morus alba

Jukrapun Komakul 1,*,2, Boonchoo Sritularak 1, Hiroyuki Tanaka 1, Tharita Kittisripunya 1,2, Gorawit Yusakul 1,2, Phetcharawarin Wangsuphadilok 4, Angkana Samaengfaul 1, Waraporn Putalan 1,2, 5

Morus alba (mulberry) extracts were reported to have some bioactivities such as α-glucosidase and tyrosinase inhibitory activities. Root part of M. alba contains high level of mulberroside A, a glycoside form of oxyresveratrol. This major compound shows many pharmacological activities including anti-tyrosinase activity, anti-viral activity, antioxidant activity, hepatoprotective effect, and neuroprotective effect. In this study, mulberroside A accumulation, α-glucosidase and tyrosinase inhibitory activities of endophytic fungus, in vitro cultures from Morus alba extracts and intact M. alba were determined and compared to evaluate them as alternative sources of secondary metabolites. The results show that mulberroside A concentrations in cell suspension and root cultures of M. alba were higher than the intact fibrous root (10.31±0.76, 19.34±0.53 น ้าหนักแห้ง)
and 5.32±0.37 mg/g dry weight, respectively). Cell suspension and root cultures also showed higher anti α-glucosidase activity (IC_{50} = 0.11±0.01 mg DW/mL, 86±0.9 ng DW/mL and 0.34±0.02 mg DW/mL, respectively) and anti-tyrosinase activity than that found in intact root (IC_{50} = 0.70±0.05, 0.76±0.06 and 3.20±0.19 mg dry weight/mL, respectively). It is concluded that in vitro cultures of M. alba are potential sources of bioactive secondary metabolites. Additionally medium extract of endophytic fungus isolated from M. alba (identified as Trichoderma sp.) show positive mulberroside A accumulation (0.40±0.03 mg/g crude extract), an anti α-glucosidase activity (IC_{50} = 0.81±0.12 mg crude extract/mL) and anti-tyrosinase activity (IC_{50} = 3.72±0.58 mg crude extract/mL)

Keywords: Morus alba, mulberroside A, anti α-glucosidase activity, anti-tyrosinase activity, endophytes

Introduction

Morus alba or mulberry has been used as herbal medicine for various therapeutic purposes, including anti-inflammation, anti-asthmatic, function tonic and anti-diabetic. Moreover, its root extract, fruits and leaves are also used in cosmetic industry as antioxidant and whitening agent. (Kim et al., 2002; Kumar and Chauhan, 2008; Piao et al., 2010; Hunyadi et al., 2012).

Iminosugars or piperidine alkaloids and polysaccharides isolated from mulberry were reported to have α-glucosidase inhibition, which is one of anti-diabetic mechanism (Hunyadi et al., 2012). The secondary metabolites extracted from mulberry species, including anthocyanins, stilbenes and flavonoids have been reported to act as anti-tyrosinase agents (Ryu et al., 2008; Aramwit, 2010; Piao et al., 2010). Mulberroside A (MuA) is a major stilbene glycoside from root bark of mulberry. Oxyresveratrol aglycone form of MuA has been reported to possess many pharmacological effects including anti-tyrosinase activity (Tengamnuay et al., 2006), anti-viral activity (Gалиндо et al., 2011; Lipipun et al., 2011), antioxidant activity (Aftab et al., 2010), hepatoprotective effect (Shi et al., 2008) and neuroprotective effect (Horn et al., 2004).

Plant tissue cultures can be growth with short growth cycles in the laboratory and they can also accumulate high amount of the chemicals found in the parent plant (Rao and Ravishankar, 2002). Some endophytes can also produce some secondary metabolites similar as their host (Strobel, 1996; Yin, 2011). Therefore, this study was aim to investigate MuA production, α-glucosidase and tyrosinase inhibitory activities from root culture, cell culture and endophytes of M. alba compared with intact plant.

Materials and Methods

Plant materials, sample preparation and determination of MuA by indirect competitive ELISA

M. alba samples were collected from faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand in April 2011. Cell suspension cultured was obtained from leaf-derived callus of M. alba cultured on Murashige and Skoog (MS) liquid medium supplemented with 0.1 mg/L thidiazuron (TDZ) and 1 mg/L naphthalene acetic acid (NAA). Root culture was obtained from root of M. alba
cultured on half strength MS liquid medium supplemented with 1 mg/L NAA.

Dried powdered of plant samples (10 mg) were weighted, extracted with 500 μL methanol and then sonicated for 15 min. The extracts were centrifuged at 3,000 rpm for 3 minutes to collect the supernatant. This extraction procedure was repeated four times. The combined extract were evaporated at 50°C and redissolved in 1 mL methanol. Consequently, sample solutions were diluted into appropriated concentrations. The MuA concentrations were determined by indirect competitive ELISA using polyclonal antibody against MuA.

**Endophytes isolation and extraction**

*M. alba* explants were rinsed with tap water, dipped in 2% sodium hypochlorite (10 min), 70% ethanol for 30 seconds and washed with sterilized distilled water several times. Surfaces of explants were removed by cutting under sterile conditions. Small pieces of surface sterilized explants were plated on potato dextrose (PDB) and Lysogeny broth (LB). Difference characteristic microorganism were selected and subcultured at least 3 times to obtained a single strain. Isolated microorganisms were transferred onto liquid potato dextrose (PDB) or Lysogeny broth (LB). Endophytes medium were harvested, and then separated with ethyl acetate. Ethyl acetate parts were kept and evaporated. Dried crude extracts were re-dissolved in methanol, then diluted into appropriated concentrations.

**Anti-Alpha-glucosidase activity**

α -glucosidase enzyme (100 μL, 0.7 units/mL) was mixed with 10 μL methanol or samples and incubated at 37°C for 10 minutes in 96-well plate. After that 20 mM p-nitrophenyl-α-D-glucopyranoside in phosphate buffer saline (PBS) were added, mixed and incubated at 37°C for 15 minutes, and then 100 μL sodium carbonate were added to stop the reaction. Produced p-nitrophenyl was measured by microplate reader at the absorbance of 405 nm. Acarbose (1 mg/mL) was used as the positive control.

**Anti-tyrosinase activity**

Mushroom tyrosinase enzyme (20 μL, 31 unit/mL in 0.1 M PBS) or PBS were mixed with 50% methanol or samples in 50% methanol (20 μL) and 160 μL PBS in 96-well plate. The mixtures were incubated for 10 minutes, and then 20 μL L-Dopa (2 mg/mL) were added. Amount of dopachrome was measured by using spectrophotometer at the absorbance of 490 nm.

**Statiscal analysis**

One-way analysis of variance (ANOVA) was performed to check different accumulations of MuA in samples and compared with Duncan at 0.05 level of significant.

**Results and Discussion**

The results showed that the MuA concentrations (table 1) in cell suspensions and root cultures of *M. alba* were significantly higher than those found in the intact fibrous root (10.31±0.76, 19.34±0.53 and 5.32±0.37 mg/g dry weight, respectively) but these level were lower than intact root and the root bark (22.39±1.06 and 26.86±2.69 mg/g dry weight, respectively). The concentrations range of MuA in mulberry root bark of *M. alba* were reported from lower than 0.09 to 53.97 mg/g dry weight, approximately (Piao et al., 2010).

<table>
<thead>
<tr>
<th><strong>Sample</strong></th>
<th><strong>MuIbroside A concentration (mg/g dry weight)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact fibrous root</td>
<td>5.32±0.37</td>
</tr>
<tr>
<td>Intact root</td>
<td>22.39±1.06</td>
</tr>
<tr>
<td>Intact root bark</td>
<td>26.86±2.69</td>
</tr>
<tr>
<td>Cell culture</td>
<td>10.31±0.76</td>
</tr>
<tr>
<td>Root culture</td>
<td>19.34±0.53</td>
</tr>
<tr>
<td><em>Trichoderma</em> sp. medium</td>
<td>0.40±0.03 (mg/g crude extract)</td>
</tr>
</tbody>
</table>
endophytic fungus may produce compounds with the
Trichoderma microorganisms can produce secondary metabolites
indicated that the false positive from the ELISA method can be occurred
while others microbial medium showed the inhibition (IC50 = 0.11±0.01 mg DW/mL, 86±0.9 ng DW/mL and 0.34±0.02 mg DW/mL, respectively) and tyrosinase inhibition compared with intact root (IC50 = 0.70±0.05, 0.76±0.06 and 3.20±0.19 mg dry weight/mL, respectively).

21-day-old cell and root cultures of M. alba contained lower level of MuA than that found in intact root of M. alba. However, higher MuA yield may be obtained by elicitation and condition optimization, including inoculums density, nutrient content and light condition. In vitro cultures of M. alba especially root culture dry powders exerted a strong anti α-glucosidase activity compared with 1-deoxynojirimycin, one of the most effective iminosugars isolated from M. alba (Hunyadi et al, 2012). Moreover, both in vitro cultures showed strong tyrosinase inhibition. These results conclude that in vitro cultures of M. alba are potential sources of bioactive secondary metabolites.

Table 2 Inhibitory effects on α-glucosidase activity and tyrosinase activity of the samples. (n=3)

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC50 of anti-α-glucosidase activity (mg dry weight/mL)</th>
<th>IC50 of anti-tyrosinase activity (mg dry weight/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact fibrous root</td>
<td>0.09±0.01</td>
<td>0.32±0.01</td>
</tr>
<tr>
<td>Intact root</td>
<td>0.34±0.02</td>
<td>3.20±0.19</td>
</tr>
<tr>
<td>Intact root bark</td>
<td>0.07±0.01</td>
<td>0.90±0.09</td>
</tr>
<tr>
<td>Intact twig</td>
<td>0.76±0.07</td>
<td>-</td>
</tr>
<tr>
<td>Intact leaf</td>
<td>2.61±0.11</td>
<td>-</td>
</tr>
<tr>
<td>Cell culture</td>
<td>0.11±0.01</td>
<td>0.70±0.05</td>
</tr>
<tr>
<td>Root culture</td>
<td>86×10^2±9×10^-6</td>
<td>0.76±0.06</td>
</tr>
<tr>
<td>Trichoderma sp. medium</td>
<td>0.81 (mg crude extract/mL)</td>
<td>3.72 (mg crude extract/mL)</td>
</tr>
<tr>
<td>1-Deoxynojirimycin</td>
<td>5×10^-3±0.43×10^-3</td>
<td>-</td>
</tr>
<tr>
<td>Oxyresveratrol</td>
<td>-</td>
<td>2.68×10^-3±0.35×10^-3</td>
</tr>
</tbody>
</table>

After 10 different of endophytic microbial were isolated from M. alba explant. Medium extract of endophytic fungus isolated from M. alba (identified as Trichoderma sp.) can inhibit α-glucosidase (IC50 = 0.81±0.12 mg crude extract/mL), tyrosinase enzymes (IC50 = 3.72±0.58 mg crude extract/mL). It also showed a positive mulberroside A accumulation (0.40 mg/g crude extract). While others microbial medium showed the negative results. This study can’t be concluded that Trichoderma sp. can produce mulberroside A, because of the false positive from the ELISA method can be occurred by the cross reactivity of polyclonal antibody. The endophytic fungus may produce compounds with the similar structures as MuA. However, These results still indicated that Trichoderma sp. is the interesting source of bioactive secondary metabolites because some microorganisms can produce secondary metabolites related to plants and level of the secondary metabolites can be improved by optimization of culture conditions. For an example Bacillus sp. can produce a 1-deoxynojirimycin and yield of the compound was increased by sorbitol supplementation (Onose et al., 2012).

Conclusion

In vitro cultures of M. alba and Trichoderma sp. isolated from M. alba are potential sources of the secondary metabolites. In future studies, improvement of MuA accumulation in in vitro cultures by elicitation and optimization culture condition as well as isolation of bioactive secondary metabolites from Trichoderma sp. will be investigated.

Acknowledgements

This work was supported in part by Kanae Foundation for the Promotion of Medical Science, Tokyo, Japan, Graduate School, Khon Kaen University, Thailand and Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand.
References


