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การประเมินการผลิตสารมัลเบอร์โรไซด์เอและฤทธิ์ทางชีวภาพจากเชื้อราเอนโดไฟต์และเนื้อเยื่อเพาะเลี้ยงของหม่อน

จักรพันธ์ โคมัยกุล[*,1,2], บุญชู ศรีตุลารักษ์[3], ฮิโรยูกิ ทานากะ[4], ธฤตา กิติศรีปัญญา[1,2], กรวิทย์ อยู่สกุล[1,2], เพชรวรินทร์ หวังศุภดิลก[6], อังคณา สำแดงไฟ[6], วราภรณ์ ภูตะลุน[,2,5]**

บทคัดย่อ

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สารสกัดจากหม่อนมีการรายงานฤทธิ์ทางชีวภาพในการยับยั้งเอนไซม์แอลฟากลูโคซิเดสและเอนไซม์ไทโรซิเนส โดยสารที่ออกฤทธิ์ทางชีวภาพหลักที่พบในรากหม่อนได้แก่ มัลเบอร์โรไซด์เอ สารชนิดนี้เป็นโครงสร้างไกลโคไซด์ของออกซีเรสเวอร์ราทรอลซึ่งมีฤทธิ์ทางเภสัชวิทยาได้แก่ ฤทธิ์ต้านเอนไซม์ไทโรซิเนส ฤทธิ์ต้านไวรัส ฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ปกป้องตับ และ ฤทธิ์ปกป้องประสาท การศึกษานี้จึงทดลองหาปริมาณสารมัลเบอร์โรไซด์เอ ฤทธิ์ยับยั้งเอนไซม์กลูโคซิเดส และเอนไซม์ไทโรซิเนส จากสารสกัดเอนโดไฟต์และเนื้อเยื่อเพาะเลี้ยงต้นหม่อน เปรียบเทียบกับหม่อนในธรรมชาติเพื่อประเมินคุณค่าของเอนโดไฟต์และเนื้อเยื่อเพาะเลี้ยงต้นหม่อนสำหรับการเป็นแหล่งผลิตสารทุติยภูมิ จากการทดลองพบว่า ความเข้มข้นของมัลเบอร์โรไซด์เอในรากและในเซลล์เพาะเลี้ยงหม่อนสูงกว่าในรากฝอยหม่อนจากธรรมชาติอย่างมีนัยสำคัญทางสถิติ (10.31±0.76, 19.34±0.53 และ 5.32±0.37 มก./ก. น้ำหนักแห้ง ตามลำดับ) รวมถึงมีฤทธิ์ยับยั้งเอนไซม์กลูโคซิเดส (IC_{50} = 0.11±0.01 มก. น้ำหนักแห้ง/มล., 86±0.9 นก. น้ำหนักแห้ง/มล. และ 0.34±0.02 น้ำหนักแห้ง/มล., ตามลำดับ) และ เอนไซม์ไทโรซิเนสสูงกว่ารากหม่อนจากธรรมชาติ (IC_{50} = 0.70±0.05, 0.76±0.06 and 3.20±0.19 มก./ก. น้ำหนักแห้ง/มล. ตามลำดับ) จากผลการทดลองสรุปได้ว่า เนื้อเยื่อเพาะเลี้ยงหม่อนเป็นแหล่งทางเลือกในการผลิตสารออกฤทธิ์ทางชีวภาพที่น่าสนใจ นอกจากนี้ยังพบว่า สารสกัดจากอาหารเลี้ยงเชื้อราไตรโคเดอร์มา ซึ่งเป็นเอนโดไฟต์จากต้นหม่อนให้ผลบวกเมื่อทดสอบหามัลเบอร์โรไซด์เอ (0.40±0.03 มก./ก. ของน้ำหนักสารสกัด) รวมถึงมีฤทธิ์ยับยั้งเอนไซม์แอลฟากลูโคซิเดส (IC_{50} = 0.81±0.12 มก. ของน้ำหนักสารสกัด/มล.) และ เอนไซม์ไทโรซิเนส (IC_{50} = 3.72±0.58 มก. ของน้ำหนักสารสกัด/มล.)

คำสำคัญ: หม่อน, สารมัลเบอร์โรไซด์เอ, ฤทธิ์ยับยั้งเอนไซม์กลูโคซิเดส, ฤทธิ์ยับยั้งเอนไซม์ไทโรซิเนส, เอนโดไฟต์

Abstract

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

Jukrapun Komaikul *[,1,2], Boonchoo Sritularak[3], Hiroyuki Tanaka[4], Tharita Kitisripunya [1,2], Gorawit Yusakul [1,2], Phetcharawarin Wangsuphadilok[6], Angkana Samdaengfai[6], Waraporn Putalun[**,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

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

1) Student, Master of Pharmacy in Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Khon Kaen University Thailand
2) Research Group for Pharmaceutical Activities of Natural Products using Pharmaceutical Biotechnology (PANPB), National Research University-Khon Kaen University, Thailand
3) Assoc.Prof.Dr., Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand
4) Assoc.Prof.Dr., Graduate School of Pharmaceutical Sciences, Kyushu University, Japan
5) Assoc.Prof.Dr., Department of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand
6) Student, Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand
Corresponding author: (*, **) Faculty of Pharmaceutical Sciences, 123 Khon Kaen University, Naimaung, Muang, Khon Kaen, Thailand, 40002. Tel: (6643) 202378. Fax: (6643) 202379. *E-mail address: jk_juk@hotmail.com ** E-mail address: waraporn@kku.ac.th

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 el 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 el 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. Oxyresvertrol aglycone form of MuA has been reported to possess many pharmacological effects including anti-tyrosinase activity (Tengamnuay *et al.*, 2006), anti-viral activity (Galindo *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 endodophytes 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 thiadiazuron (TDZ) and 1 mg/L naphthaline 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 re-dissolved 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 1 Mulberroside A accumulation in samples. (n=3)

Sample	Mulberroside A concentration (mg/g dry weight)
Intact fibrous root	5.32±0.37
Intact root	22.39±1.06
Intact root bark	26.86±2.69
Cell culture	10.31±0.76
Root culture	19.34±0.53
Trichoderma sp. medium	0.40±0.03 (mg/g crude extract)

Table 2 shows that the cell suspensions and root cultures also exhibited a higher level of α-glucosidase inhibition (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 tyrosinase inhibition compared with intact root (IC_{50} = 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)

Sample	IC_{50} of anti-α-glucosidase activity (mg dry weight/mL)	IC_{50} of anti-tyrosinase activity (mg dry weight/mL)
Intact fibrous root	0.09±0.01	0.32±0.01
Intact root	0.34±0.02	3.20±0.19
Intact root bark	0.07±0.01	0.90±0.09
Intact twig	0.76±0.07	-
Intact leaf	2.61±0.11	-
Cell culture	0.11±0.01	0.70±0.05
Root culture	$86x10^{-6}$±$0.9x10^{-6}$	0.76±0.06
Trichoderma sp. medium	0.81 (mg crude extract/mL)	3.72 (mg crude extract/mL)
1-Deoxynojirimycin	$5x10^{-3}$±$0.43x10^{-3}$	-
Oxyresveratrol	-	$2.68x10^{-3}$±$0.35x10^{-3}$

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 (IC_{50} = 0.81±0.12 mg crude extract/mL), tyrosinase enzymes (IC_{50} = 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.

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