RESEARCH ARTICLE

Neuroprotective Effect of *Bacopa Monnieri* Extract on Oxidized Low Density Lipoprotein-Induced Neurotoxicity in SH-SY5Y Neuroblastoma Cells

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Abstract

Oxidized low density lipoprotein (LDL)-mediates neurotoxicity and possibly plays some roles in the pathological process of Alzheimer’s disease (AD). Neuroprotection of *Bacopa monnieri* (L.) Wettst (BM) has been studied in various cells and animal models but not in oxidized LDL-induced toxicity. This study aimed to investigate the neuroprotective effects of BM extract and four components including three bacoside A; bacopaside I (BA-I), II (BA-II), and bacoside A3 (BA-A3), and one flavonoid; luteolin (Lut) in SH-SY5Y neuroblastoma cells. The results demonstrated that BM extract diminished the neurotoxicity of oxidized LDL in a dose dependent manner, potentially by suppression of cellular oxidative stress. Among four tested compounds, Lut exhibited the most effect on preventing the cell injury mediated by oxidized LDL. These compounds also suppressed the increment of cellular acetylcholinesterase (AChE) activity mediated by oxidized LDL, possibly as the consequence of oxidation suppression. These data support the beneficial effect of BM as a neuroprotective agent and this effect is possibly a combined result of its constituents.

Keywords: *Bacopa monnieri*, Alzheimer’s disease, oxidized low density lipoprotein, Bacoside A, Luteolin
ฤทธิ์ปกป้องประสาทของสารสกัดสมุนไพรพบมิจากพิษที่ถูกเหนี่ยวเนื้อตัวของออกซิไดซ์ไลโปโปรตีนชนิดความหนาแน่นต่ำในเซลล์ประสาทเลี้ยงชนิด SH-SY5Y blastoma

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บทคัดย่อ

ออกซิไดซ์ไลโปโปรตีนชนิดความหนาแน่นต่ำส่งผลให้เกิดพิษต่อเซลล์ประสาทและอาจมีบทบาทในการกระบวนการทางพยาธิวิทยาของการเกิดโรคอัลไซม์เมอร์ ที่ผ่านมามีการศึกษาถึงฤทธิ์ปกป้องประสาทของสมุนไพรพบมิ (Bacapa monerri (L.) Wettst) ด้วยวิธีการต่าง ๆ ทั้งในเซลล์ประสาทและในสัตว์ทดลอง แต่ยังไม่มีการศึกษาถึงตัว параพิษจากออกซิโดซ์ไลโปโปรตีนชนิดความหนาแน่นต่ำในศึกษาเจ้าจึงมุ่งเน้นทดสอบลูกย์ปริปักษ์ของสารสกัดหยาบจากสมุนไพรพบมิ และสารประกอบที่พบมากในสมุนไพรนี้คือ 4 ชนิด โดยเป็นสารกลุ่มบาโคไซด์เอ (bacoside A) 3 ชนิด ได้แก่ bacopasid I (BA-I), II (Ba-II) และ bacoside A3 (Ba-A3) และสารกลุ่มฟลาโวนอยด์ ได้แก่ ลูทิโอลีน (luteolin) โดยทำการทดลองในเซลล์ประสาทเลี้ยงชนิด SH-SY5Y ผลการทดลองพบว่าสารสกัดหยาบพบมิแสดงฤทธิ์ยับยั้งพิษที่เกิดจากออกซิโดซ์ไลโปโปรตีนชนิดความหนาแน่นต่ำได้ โดยลูทิโอลีนเป็นสารที่มีประสิทธิภาพสูงที่สุดในการป้องกันเซลล์จากพิษของออกซิโดซ์ไลโปโปรตีนชนิดความหนาแน่นต่ำ นอกจากนี้ยังพบว่าสารที่ทำการทดสอบทุกชนิดสามารถยับยั้งการเพิ่มขึ้นของการทำงานของเอนไซม์อะเซทิลโคลีนที่ถูกเหนี่ยวเนื้อแบบที่นำเสนอได้ด้วยออกซิโดซ์ไลโปโปรตีนชนิดความหนาแน่นต่ำ ซึ่งคาดว่าเป็นผลที่เกิดจากสารประกอบของสารประกอบที่พบมากในสมุนไพรพบมิเป็นสารปกป้องประสาทโดยถูกเหนี่ยวเนื้อต่ำกว่าผลรวมของการออกฤทธิ์ของสารประกอบชนิดต่าง ๆ ในการสกัด

คำสำคัญ: สมุนไพรพบมิ, โรคอัลไซม์เมอร์, ออกซิโดซ์ไลโปโปรตีนชนิดความหนาแน่นต่ำ, สารประกอบฟลาโวนอยด์, ลูทิโอลีน
Introduction

Alzheimer’s disease (AD) is the most common cause of dementia and pathologically characterized by accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles containing hyperphosphorylated tau. AD neurodegeneration carries multiple risks including hypercholesterolemia. The role of cholesterol is controversial but a key link to AD is oxidized cholesterol (oxysterols) leading to microvascular dysregulation and blood brain barrier (BBB) leakage. Thus, cerebrospinal fluid (CSF) of AD patients contain increased oxidized lipoprotein and has antibodies against oxidized low density lipoprotein (LDL), suggesting links between oxidative stress, oxidized cholesterol/lipoprotein, and AD pathogenesis.

In cultured neurons, oxidized LDL induced cell death by generating reactive oxygen species (ROS), caspase-3 activation, and glutathione depletion. Oxidized LDL also activated BACE-1, thereby promoting Aβ accumulation while killing cells via toll-like receptor-4 (TLR4). The increased ROS via oxidized LDL promoted another AD pathology by enhancing acetylcholinesterase (AChE) activity in culture neurons. Thus, oxidized LDL-mediated neurotoxicity provides an accessible model on which interventions leading to reduced AD pathogenesis can be tested.

Bacopa monerri (L.) Wettst (BM) is used as a nootropic agent in Ayurvedic medicine, an idea supported its antiepileptic, anxiolytic, antidepressant, and sedative actions. Furthermore, BM reduced β-amyloid levels and protected neurons against Aβ-induced toxicity. Limited clinical trials suggest some potential to improve cognition possibly through reducing AChE activity and β-amyloid, increasing antioxidant function, improving cerebral blood flow, and neurotransmitter modulation.

BM extract contains tetracyclic triterpenoid saponins called bacoside A, which is a mixture of saponins including bacoside A3 (Ba-A3), bacopaside I (Ba-I), bacopaside II (Ba-II), bacopasaponin C and jujubogenin isomer of bacosaponin C (bacopaside X) and two common flavonoids, luteolin (Lut) and apigenin. These compounds are considered to provide the neuroprotective and precognitive functions of BM by testing in various in vitro and in vivo models, while Lut also prevented Aβ-induced neuronal death. BM enjoys global commercialisation as a dietary supplement by promoting its perceived pro-cognitive effects, but there lacks a clear understanding about its cellular mechanism of action.

In this study, we aimed to show that BM extract and four components (Ba-I, Ba-II, Ba-A3 and Lut) can improve the viability of neurons (SH-SY5Y neuroblastoma cells), by reducing intracellular ROS, lipid peroxidation, and downregulating AChE provoked by oxidized LDL.

Materials and Methods

Materials

Dulbecco’s modified eagle’s medium (DMEM)/F12, retinoic acid, 5,5'-dithio-bis (2-nitrobenzonic acid) (DTNB), acetylthiochloine iodide (ATCI), 2',7'-dichlorofluorescein diacetate (DCFH-DA), neostigmine, and luteolin were purchased
from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), trypsin/EDTA and penicillin-streptomycin were purchased from Gibco (Grand Island, NY). Amicon Ultra-4 centrifuge filters were purchased from Merck Millipore (Darmstadt, Germany). The BCA protein assay kit was purchased from Thermoscience (Rockford, IL). Ba-I, Ba-II, and Ba-A3 were purchased from Natural Remedies Private Limited (Bangalore, India).

**Preparation and standardization of BM extract**

BM was collected from Phitsanulok province, Thailand. It was identified by Associate Professor Wongsatit Chuakul, Faculty of Pharmacy, Mahidol University, Thailand. The voucher specimen, Phrompittayarat001, was kept at the Pharmaceutical Botany Mahidol Herbarium, Mahidol University, Thailand. BM was cut into small pieces and dried in hot-air oven at 50ºC for 12 h. The dried plant was coarsely powdered and percolated twice with circulating 95% ethanol for 8 h. The extract was then filtrated and dried under reduced pressure. Constituent analysis of BM extract was analyzed by HPLC with some modification from previously described method. Briefly, the separation was performed using a Shimadzu LC-20AT liquid chromatography equipped with a SPD-20A UV/VIS detector. The mobile phase consisted of 0.2% orthophosphoric acid and acetonitrile (65:35 V/V) adjusted to pH 3.0 with 5M NaOH, and chromatography was run at 1.0 mL/min flow rate. All peaks were integrated at detector wavelength 205 nm and were assigned by retention times compared with standards. Calibration curves of seven standards (Ba-A3, Ba-I, Ba-II, bacopasaponin C isomer, bacosaponin C, Lut, and apeginin) were prepared based on peak areas of seven concentrations (7.8-500 µg/mL).

**Isolation and oxidation of human LDL**

LDL isolation and oxidation were performed as previously described. The protocol was approved by Naresuan University Institutional Review Board (IRB No. 148/56). Briefly, LDL was isolated from plasma provided by Blood Bank, Naresuan University Hospital by density gradient ultracentrifugation. LDL was collected and dialyzed in 1xPBS containing 10 µM EDTA for 24 h at 4ºC. LDL (1 mg/mL) was oxidized by 10 µM CuSO4 at 37ºC for 2 h and the reaction was terminated by adding EDTA (0.3 mM final concentration). The EDTA and CuSO4 were removed by passing LDL sample through a 30 kDa cut-off Amicon filter. The degree of LDL oxidation was measured as lipid peroxidation product using thiobarbituric acid reactive substance (TBARs) assay.

**Cell culture preparation**

The human neuroblastoma cell line (SH-SY5Y) (American Type Culture Collection, Rockville, MD) was cultured in DMEM/F12 containing 10% FBS and 0.01% penicillin-streptomycin at 37ºC in 95 % air and 5% CO2 incubator. Cells were seeded into 96-well plates (2x10^4 cells/well), allowed to attach for 24 h, and differentiated in low serum culture medium (2% FBS) containing 10 µM retinoic acid for 6 days. Before treatment, cells were incubated in serum-free medium for 24 h.
**Determination of cell viability by MTT assay**

Differentiated SH-SY5Y cells were treated with BM extract or tested compounds for 2 h. Then oxidized LDL was added (200 µg/mL final concentration) and incubated for 24 h. MTT (5 mg/mL in PBS) was added to each well 2 h prior to the end of treatment. The medium was then removed and 200 µL of DMSO:ethanol (1:1) was added to dissolve formazan crystals. The absorbance was measured at 595 nm by a microplate reader.

**Determination of intracellular reactive oxygen species (ROS)**

Cells were incubated with 10 µM DCHF-DA in serum- and phenol red-free DMEM/F-12 for 30 min and culture medium was then removed. Cells were then pretreated with BM extract or tested compounds for 2 h before adding 200 µg/mL oxidized LDL and then incubated for 24 h. The fluorescence intensity was measured by a microplate reader with excitation wavelength at 485 nm and emission wavelength at 535 nm. Data from each experiment were normalized by number of viable cells.

**Determination of lipid peroxidation by TBARs assay**

At the end of treatment, 100 µL of TBARs reagent containing 0.4% TBA, 1.4% TCA, and 8% HCl (1:2:1) was directly added to each well. The mixtures were incubated at 90°C for 1 h and cooled to room temperature. The fluorescence intensity was measured by a microplate reader with excitation wavelength at 535 nm and emission wavelength at 595 nm.

**Cellular acetylcholinesterase (AChE) activity assay**

Cells were lysed by lysis buffer containing 15 mM tris (pH 7.4), 150 mM NaCl and 1% triton X-100. AChE activity was determined by Ellman’s colorimetric method with minor modification. The yellow reaction is formed by the reaction of DTNB (1.5 mM) and thiocholine released from ATCI by enzymatic hydrolysis, and measured by a microplate reader at 405 nm. Neostigmine, an AChE inhibitor, was used as a positive control. Data were normalized by numbers of viable cells.

**Ferric iron reducing antioxidant power (FRAP) assay**

Reducing activities were assessed by their ability to reduce Fe$^{3+}$ to Fe$^{2+}$ using the FRAP assay. FRAP reagent (180 µL) containing 3 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, 20 mM FeCl$_3$ (10:1:1) was mixed with 20 µL of BM extract (250 µg/mL) or tested compounds (250 µM). The absorbance was measured at 595 nm.

**DPPH radical scavenging capacity assay**

DPPH solution (180 µL of 0.2 mM in methanol) was mixed with 20 µL of BM extract (250 µg/mL) or tested compounds (250 µM). The absorbance was measured by a microplate reader at 510 nm. Data were expressed as percent of radical scavenging activity.
**Statistical analysis**

Data from all experiments were presented as mean ± standard errors of mean (SEM). The data were analyzed by analysis of variance (ANOVA) with a LSD test between groups. Comparisons between two groups used student’s *t*-test and differences were considered to be significant when *p* ≤0.05.

**Results**

**Chemical constituents of BM extract and their cytotoxicity**

BM ethanolic extract used in this study contained at least five saponin glycosides and two flavonoids (Table 1). Four standard compounds including Ba-I, Ba-II, Ba-A3 and Lut were selected to study their neuroprotective actions and compare with BM extract. At concentrations ranging from 0.1 to 25 µM, most compounds showed no toxicity to differentiated SH-SY5Y cells (Table 2), except Ba-II which induced cell death at 25 µM. BM extract at 25 µg/mL (containing approximately 1 µM bacoside A mixture) and lower were not toxic to cells. So the extract at 25 µg/mL and standard compounds at 10 µM were used for further studies.

**Table 1.** Content of certain saponin glycosides and flavonoids in BM extract.

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Mean ± SD (% w/w)</th>
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</thead>
<tbody>
<tr>
<td>Glycosides</td>
<td></td>
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<tr>
<td>Ba-A3</td>
<td>0.74 ± 0.06</td>
</tr>
<tr>
<td>Ba-II</td>
<td>0.98 ± 0.06</td>
</tr>
<tr>
<td>Bacopaside X</td>
<td>0.53 ± 0.04</td>
</tr>
<tr>
<td>Bacopasaponin C</td>
<td>1.00 ± 0.06</td>
</tr>
<tr>
<td>Ba-I</td>
<td>0.93 ± 0.06</td>
</tr>
<tr>
<td>Flavonoids</td>
<td></td>
</tr>
<tr>
<td>Lut</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Apigenin</td>
<td>0.07 ± 0.00</td>
</tr>
</tbody>
</table>

**Table 2.** Effect of BM and constituent bacosides on SH-SY5Y cell viability.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>0.1 µM</th>
<th>1 µM</th>
<th>10 µM</th>
<th>25 µM</th>
<th>50 µg/mL</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Cell viability (% of control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ba-I</td>
<td>100.9 ± 2.0</td>
<td>100.4 ± 3.5</td>
<td>95.8 ± 3.7</td>
<td>95.6 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>Ba-II</td>
<td>103.4 ± 3.1</td>
<td>106.7 ± 5.0</td>
<td>91.9 ± 5.3</td>
<td>26.2 ± 8.9*</td>
<td></td>
</tr>
<tr>
<td>Ba-A3</td>
<td>104.4 ± 10.9</td>
<td>96.9 ± 7.7</td>
<td>101.2 ± 5.5</td>
<td>89.6 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>Lut</td>
<td>99.6 ± 4.2</td>
<td>102.4 ± 4.5</td>
<td>131.0 ± 2.3*</td>
<td>102.0 ± 6.6</td>
<td></td>
</tr>
<tr>
<td>BM extract</td>
<td>102.1 ± 4.1</td>
<td>107.3 ± 4.2</td>
<td>111.0 ± 3.7</td>
<td>72.4 ± 6.5*</td>
<td></td>
</tr>
</tbody>
</table>

*Note: Values are means ± SEM, *p* ≤0.05 compared to control (untreated cells)
**BM extract and its active components protected cells from oxidized LDL-induced oxidative stress**

Oxidized LDL (200 µg/mL) mediated approximately 60% death of SH-SY5Y cells and BM extract, particularly at 10 and 25 µg/mL, could effectively prevent cell death (Figure 1A). Oxidized LDL generated tremendous oxidative stress in cultured neurons expressing as many folds increased intracellular ROS and lipid peroxidation (Figure 1B, C). BM extract effectively suppressed both intracellular ROS and lipid peroxidation in dose-dependent patterns (Figure 1B, C). Vitamin E analog, trolox (25 µM), could also prevent those effects induced by oxidized LDL.

**Figure 1.** Protection against oxidative actions of oxidized LDL by BM extract. Cells were treated BM extract 2 h prior to incubating with oxidized LDL (ox-LDL) for 24 h. Cell viability by MTT assay (A), intracellular ROS (B), and lipid peroxidation (C) were determined. Data are presented as means±SEM from at least four different batches of cells. * p ≤ 0.05, compared to cells treated with ox-LDL.
The effects of four BM active compounds on oxidized-LDL induced cell damage were showed in Figure 2. Compared to trolox (25 µM) and BM extract (25 µg/mL), all three saponins (10 µM); Ba-I, Ba-II, and Ba-A3, moderately prevented cellular oxidative stress mediated by oxidized-LDL. A flavonoid, Lut exhibited better neuroprotection in comparison with saponins at the same concentration (Figure 2).

**Figure 2.** Neuroprotection by Ba-I, Ba-II, Ba-A3 and Lut on oxidized LDL-induced SH-SY5Y cells damage. Cells were treated with tested compounds for 2 h prior to ox-LDL and incubated for 24 h. Cell viability by MTT assay (A), intracellular ROS (B), and lipid peroxidation (C) were determined. Data are presented as means±SEM from at least four different batches of cells. * p ≤ 0.05, compared to ox-LDL-treated cells.
**BM extract and its active components reduced cellular AChE activity**

SH-SY5Y cells treated with oxidized LDL exhibited approximately two folds AChE activity higher than that of control cells (Figure 3). BM extract, Ba-I, Ba-II, Ba-A3, and Lut, significantly diminished such effect of oxidized LDL. Lut showed slightly higher inhibitory activity than the three saponins. We also determined AChE inhibitory activity in the *in vitro* enzymatic assay of BM extract and four active compounds. The results showed that all tested compounds did not directly inhibit the activity of AChE (data not shown). Taken all data together, the effect of BM and its components on cellular AChE activity might possibly be the consequence of their ability to suppress cellular oxidative stress.

**Figure 3.** Effect of BM extract and four constituents on cellular AChE activity. Cells were treated with BM and tested compounds for 2 h prior to ox-LDL and incubated for 24 h. Cells were then lysed and measured for AChE activity. Data are presented as means±SEM from at least four different batches of cells. Neo = neostigmine (100 µM), * p ≤ 0.05, compared to ox-LDL-treated cells

**Free radical scavenging and reducing activities of BM extract and its active components**

From the *in vitro* antioxidant assays of BM extract and its active components, Lut at 25 µM exhibited the highest DPPH free radical scavenging capacity when compared to either trolox at the same concentration or BM extract at 25 µg/mL (Figure 4A). On the other hand, ferric reducing activity of trolox (25 µM) was higher than other compounds (Figure 4B). It should be noted that these three saponins demonstrated very low radical scavenging and ferric reducing activities whereas BM extract generously established those antioxidant activities.
Antioxidant activities of BM extract and its constituents. Metal ion reducing activity by FRAP (A) and free radical scavenging by DPPH assay (B) were measured. BM extract at 25 µg/mL and saponins and Lut at 25 µM were tested. Data are presented as mean±SEM from three experiments. * p ≤ 0.05, versus 1%DMSO.

Discussion

Of the BM extract and four tested constituents, luteolin (Lut) was the most effective compound to prevent a spectrum of oxidative effects of oxidized LDL and 10 µM of Lut had almost the same effect as 25 µM Trolox. Since BM extract contained only low content of Lut, other untested antioxidant compounds might play some roles in this pathway of neuroprotection. While the saponins appeared to be redox inactive, yet they still prevented some of the adverse oxidized LDL action. This suggested that their protection was not directly redox dependent but might promote endogenous anti-oxidant enzyme expression. Previous studies have demonstrated the indirect protective effect of BM extract on certain cell lines through upregulating antioxidant enzymes and glutathione.22, 27 This may occur in vivo where catalase, glutathione peroxidase, and superoxide dismutase are increased.21, 27-29

Our study provided the first evidence describing protective effect of BM extract against oxidized LDL-mediated oxidative pathologies. Similar effects of BM and its saponins have been extensively studied both in vitro and in vivo models.
including preserving mitochondrial function after challenging with \( \text{H}_2\text{O}_2 \) in neuroblastoma cells\(^{10}\), rotenone-induced oxidative stress a dopaminergic cell-line\(^{27}\), and lung epithelial cells (L123) challenged by reactive nitrogen species\(^{22}\). Oxidized LDL-induced neurotoxicity is mediated through elevated cell ROS and \( \text{Ca}^{2+} \), depletion of cellular glutathione, caspase-apoptosis\(^{3, 8-10}\) and lipid peroxidation as shown here.

The protective effect of BM constituents is less clear but a mixture of saponin/bacoside A was considered responsible\(^{18, 20, 21, 23}\) although the effect of each purified bacoside A saponin has not been tested. The bacosides tested in this study (Ba-I, Ba-II, and Ba-A3) were effective at 10 \( \mu \text{M} \) but the BM extract contained only 1 \( \mu \text{M} \) bacoside which unable to reduce DPPH or \( \text{Fe}^{3+} \) at this concentration. All three standard bacoside A compounds demonstrated very low antioxidant activities, suggesting other mechanisms involved in neuroprotection.

The neuroprotective effects of Lut were investigated in various neuronal damage models in primary and neuronal cell lines\(^{31-34}\) as well as animal models.\(^{35-37}\) From our study, among four standard compounds, Lut was the strongest antioxidant and exhibited the highest activity to suppress oxidative stress in cultured neurons. However, the antioxidative activity of Lut at 10 \( \mu \text{M} \) was comparable to that of BM extract at 25 \( \mu \text{g/mL} \) which contained only low amount of Lut (approximately 0.1 \( \mu \text{M} \)). This suggested that other untested BM components in addition to Lut might provide the redox-mediated neuroprotection. Taken all data together, neuroprotective activity of BM could possibly be the result of synergistic effect of its major and minor active constituents via several pathways.

Oxidized LDL also upregulated AChE activity and all the intervention suppressed this augmentation. Although BM was reported to directly inhibit AChE activity tested by \textit{in vitro} enzymatic assay\(^{28}\), BM extract and all tested compounds did not change basal cellular AChE activity. Thus their ability to reduce AChE activity was possibly associated with suppression of cellular oxidative stress.

**Conclusion**

BM ameliorated neurotoxicity induced by oxidized LDL, partly by bacoside saponins and luteolin. The cellular oxidative stress and AChE were attenuated by BM extract and all four standard components (Ba-I, Ba-II, Ba-A3, and Lut). By using oxidized LDL-mediated neuron damage in SH-SY5Y cells, a cellular model never been tested with BM, our results still support the neuroprotection of BM. This plant displays the potential to prevent the adverse effects induced by various types of toxic agents and possibly can be used in various neurological disorders.

**Acknowledgements**

The authors thank Dr. Norman Scholfield for help in manuscript preparation. This work was supported by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0018/2555), the Center of Excellence for Innovation in Chemistry and Naresuan University (Grant No. R2558B027).
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