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ภาคผนวก

1

สัญญาเลขที่ MRG518017

โครงการ: การพัฒนาสาร Neuroprotective ชนิดใหม่กลุ่ม coumarins จากเปลือกรากต้นส่องฟ้า

รายงานสรุปการเงินในรอบ ...24... เดือน

ชื่อหัวหน้าโครงการวิจัยผู้รับทุน : นางสาวเพลินทิพย์ ภูทองกิ่ง

รายงานในช่วงตั้งแต่วันที่ 15 พฤษภาคม พ.ศ. 2551 **ถึงวันที่** 29 มกราคม พ.ศ. 2554

รายจ่าย

หมวด (ตามสัญญา)	รายจ่ายสะสม จากรายงาน ครั้งก่อน	ค่าใช้จ่าย งวดปัจจุบัน	รวมรายจ่าย สะสมจนถึง งวดปัจจุบัน	งบประมาณ รวมทั้งโครงการ	คงเหลือ (หรือเกิน)
ค่าตอบแทน	120,000	60,000	180,000	240,000	60,000
ค่าจ้าง	-	-	-	-	-
ค่าวัสดุ	195,000	15,000	210,000	210,000	-
ค่าใช้สอย	25,000	5,000	30,000	30,000	-
ค่าครุภัณฑ์	-	-	-	-	-
รวม	340,000	80,000	420,000	480,000	60,000

จำนวนเงินที่ได้รับและจำนวนเงินคงเหลือ

จำนวนเงินที่ได้รับ

เงินเดิม (การเปิดบัญชี)	100.00	บาท	เมื่อ 27 มีนาคม 2551
งวดที่ 1	240,000.00	บาท	เมื่อ 20 มิถุนายน 2551
ดอกเบี้ย ครั้งที่ 1	34.71	บาท	เมื่อ 25 มิถุนายน 2551
ดอกเบี้ย ครั้งที่ 2	304.41	บาท	เมื่อ 25 ธันวาคม 2551
ดอกเบี้ย ครั้งที่ 3	40.53	บาท	เมื่อ 25 มิถุนายน 2552
งวดที่ 2 (ครั้งที่ 1)	120,000.00	บาท	เมื่อ 5 สิงหาคม 2552
งวดที่ 2 (ครั้งที่ 2)	59,940.00	บาท	เมื่อ 13 สิงหาคม 2552
ดอกเบี้ย ครั้งที่ 3	93.97	บาท	เมื่อ 25 ธันวาคม 2553
รวม	420,513.62	**บาท**	∂

ค่าใช้จ่าย

งวดที่ 1 เป็นเงิน	240,000.00	บาท
งวดที่ 2 เป็นเงิน	160,000.00	บาท
ฯลฯ		
รวม	420,000.00	**บาท** ❷

จำนวนเงินคงเหลือ ∂ - ❷ 513.62 **บาท**

สำนักงานคณะกรรมการวิจัยแห่งชาติ ห้องสมุดงานวิจัย

นางสาวเพลินทิพย์ ภูทองกิ่ง
ลงนามหัวหน้าโครงการวิจัยผู้รับทุน

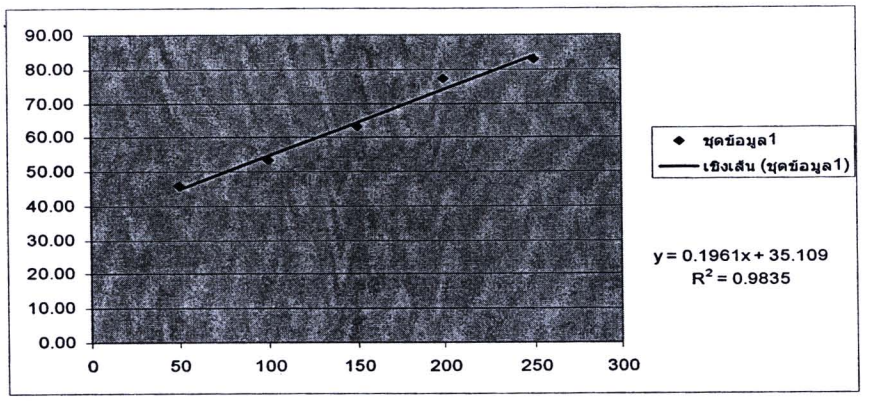
นางพิกุล วิรักษา
ลงนามเจ้าหน้าที่การเงินโครงการ

ภาคผนวก

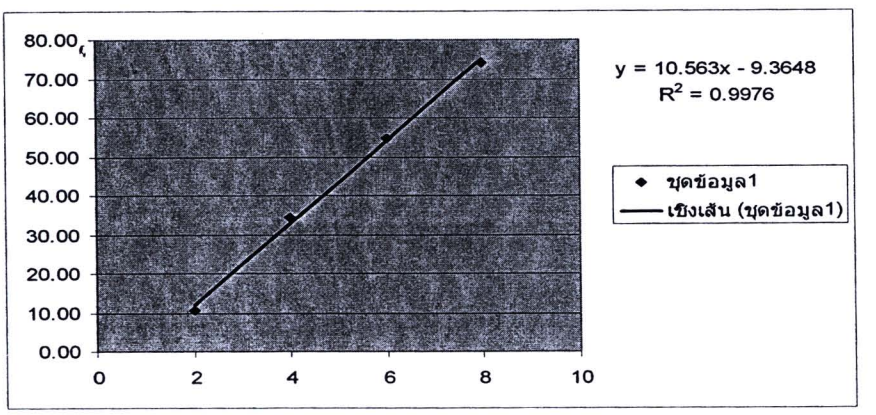
2

TBAR Assay

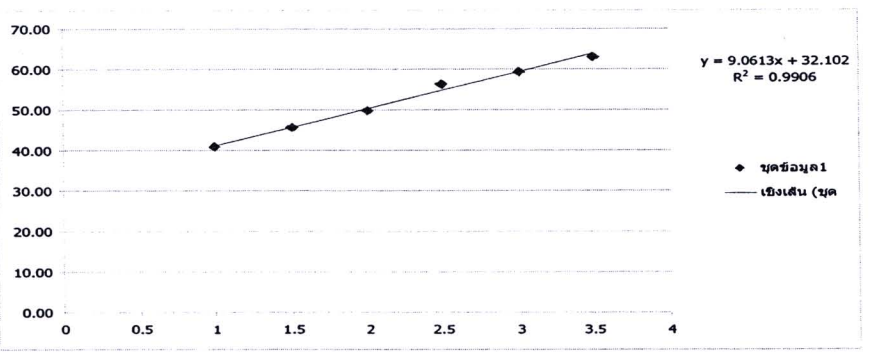
Vit E



Heptaphylline



7-OMe Heptaphyline



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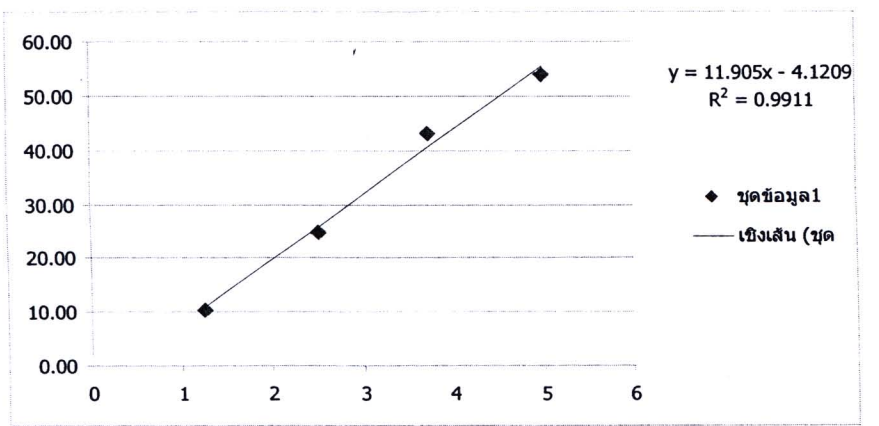

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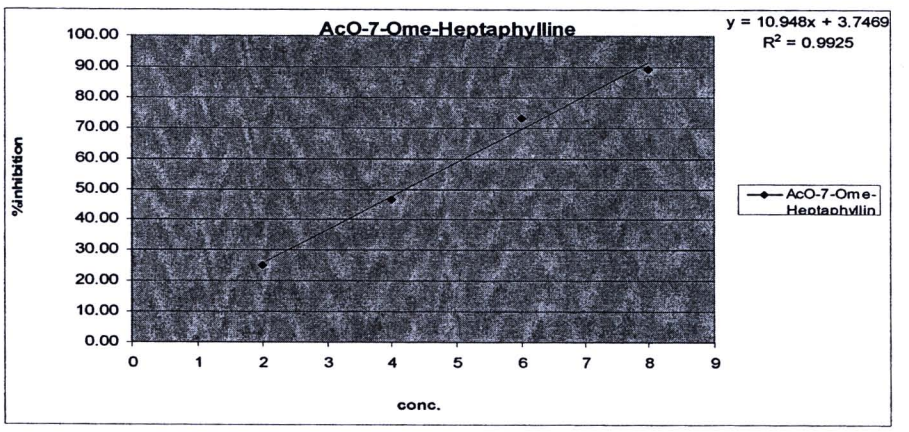

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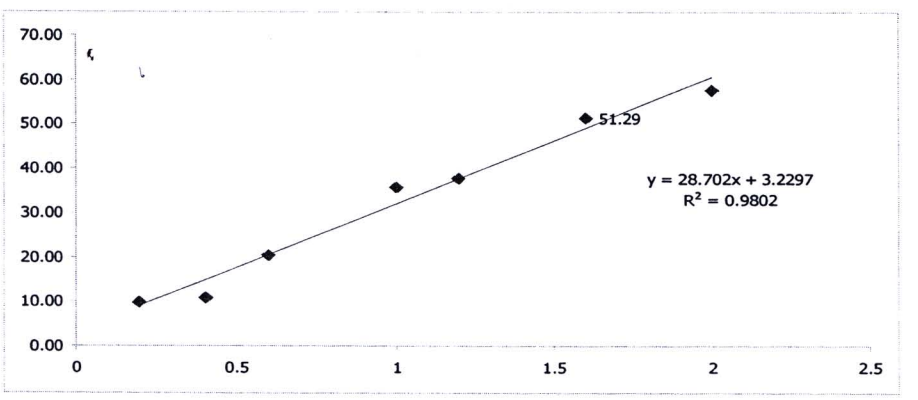

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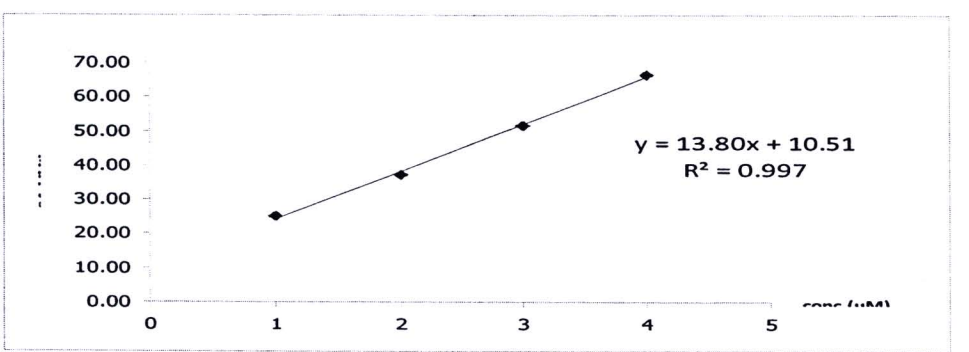

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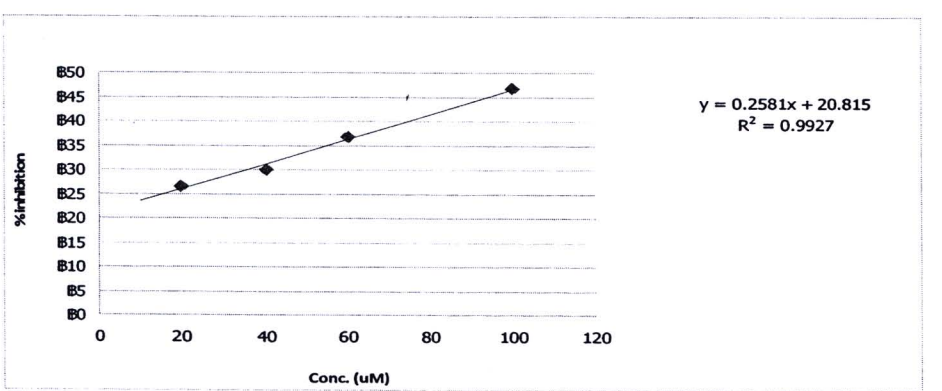

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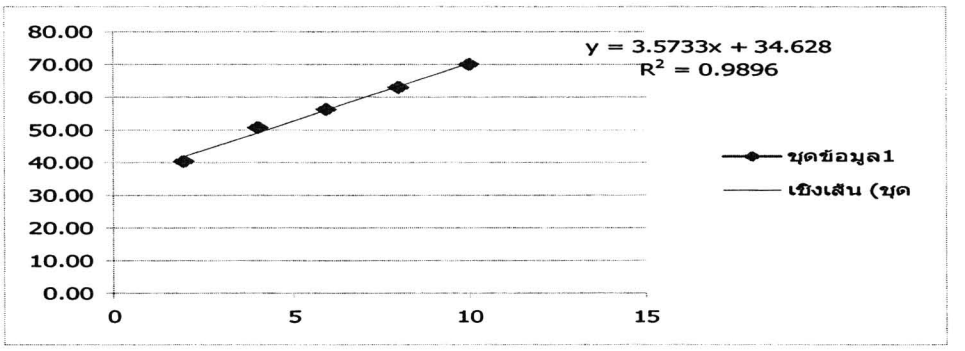

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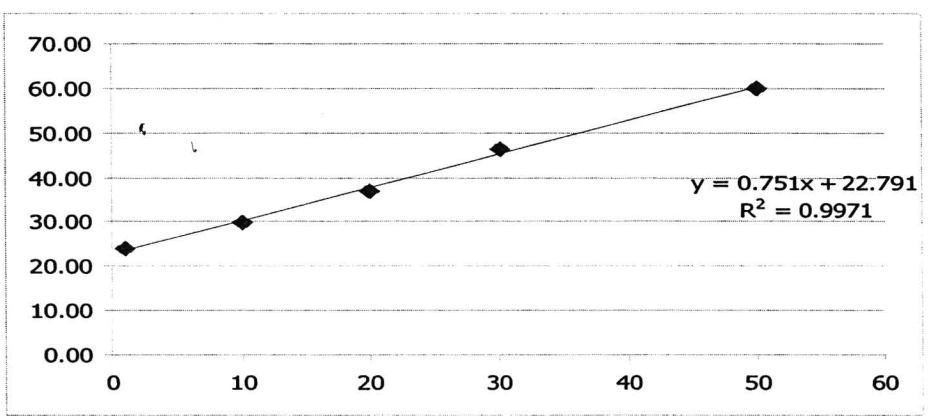

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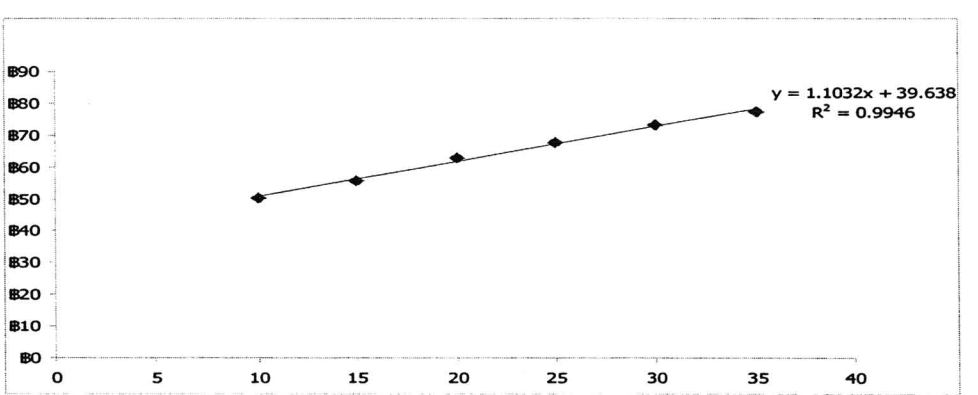

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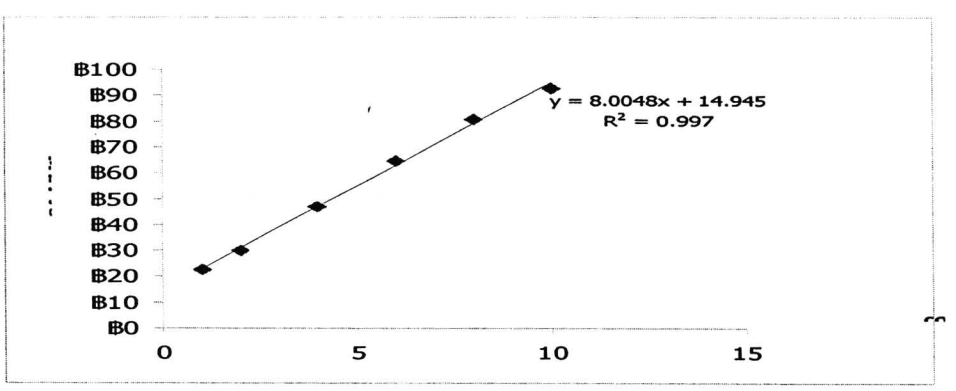

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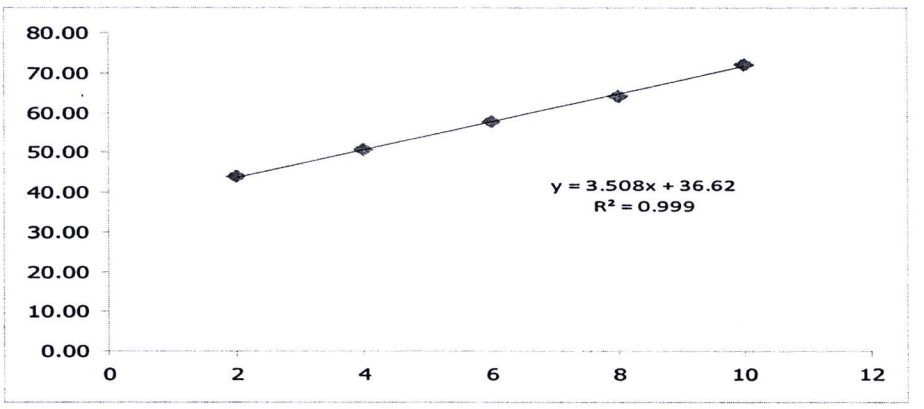

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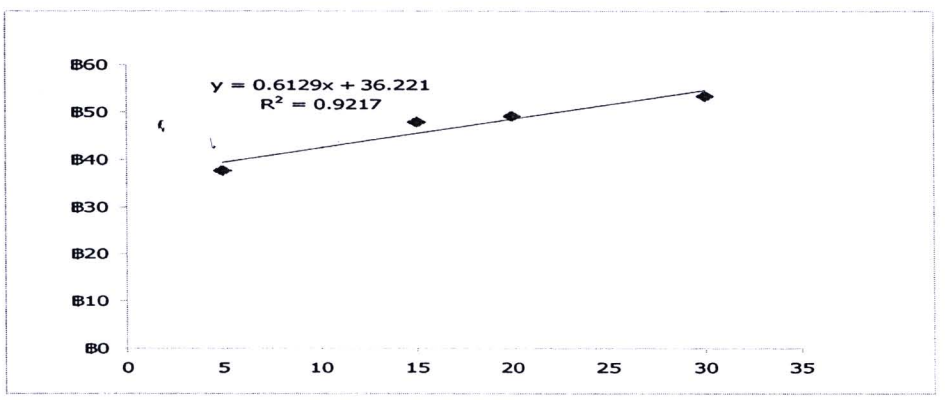

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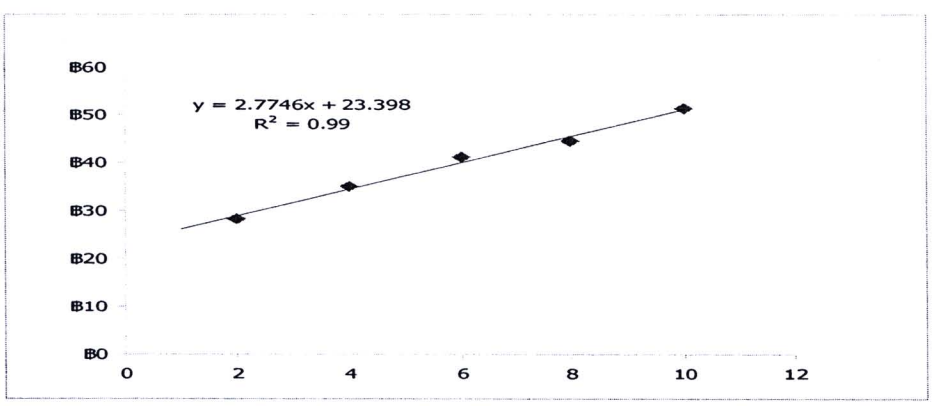

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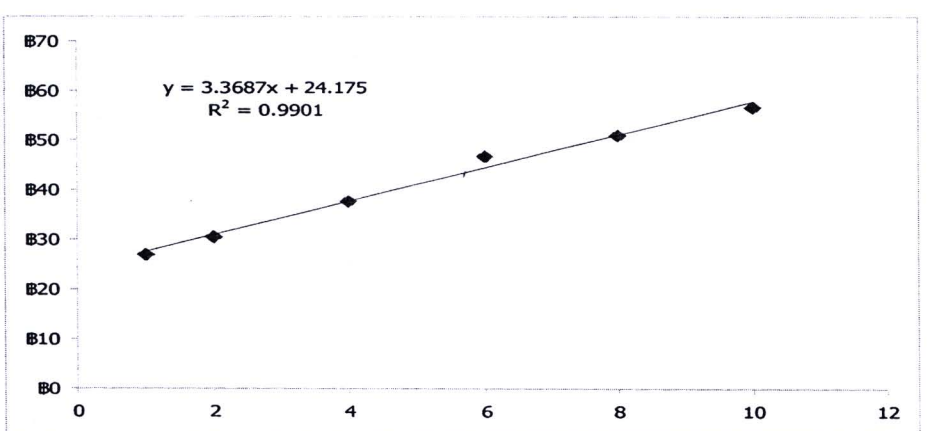

ภาคผนวก

3

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ

1. **Puthongking P.**, Jantakoon P., Bootchan T., Mari T., Yokoya M., Saito N. and Patarapanich C. "Antilipid peroxidation of bioactive compounds and derivatives from Thai medicianal plant, *Clausena Harmandiana*" The 9st NRCT-JSPS Joint seminar. December 7-9, 2010, Bangkok, Thailand.
2. Mari T., **Puthongking P.**, Yokoya M., and Saito N. "Preparation and biological activites of nordentatin derivatives" The 9st NRCT-JSPS Joint seminar. December 7-9, 2010, Bangkok, Thailand.
3. **Puthongking P.**and Noytong S. "Antilipid peroxidation activity of carbazole alkaloids and its derivatives from Thai medicianal plant, *Clausena Harmandiana*" International conference on Flok and Herbal medicine. November 25-28, 2010, Udaipur(Rajathan), India.
4. **Puthongking P** and Yusakul G. "Coumarins and carbazole with antioxidant activity from *Clausena Harmandiana*" The 22nd Federation of Asia Pharmaceutical Association congress Translation Research. November 7-10, 2008, Singapore.

ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

ขณะนี้กำลังเตรียม manuscript สำหรับตีพิมพ์ในระดับนานาชาติโดยจะรวมเนื้อหาทั้งวิธีการสังเคราะห์สาร nordentatine ด้วยวิธีทางเคมี และการแยกสารสำคัญจากธรรมชาติ รวมถึงผลการต้านไลปิดเปอร์ออกซิเดชั่น และฤทธิ์ต้านมะเร็งซึ่งรอผลการทดสอบจากประเทศญี่ปุ่น (ส่งทดสอบฤทธิ์ต้านมะเร็งที่ประเทศญี่ปุ่น)

การนำผลงานวิจัยไปใช้ประโยชน์

- การฝึกอบรมเรื่อง การพัฒนาสาร Neuroprotective ชนิดใหม่กลุ่ม coumarins จากเปลือกรากต้นส่องฟ้า ณ ประเทศญี่ปุ่น ด้วย ทุน Thai visiting scholarship 2552 (แหล่งทุน มหาวิทยาลัยขอนแก่น)
- ได้มีการนำงานวิจัยดังกล่าวมาพัฒนาการเรียนการสอนโดยให้เป็นหัวข้อวิทยานิพนธ์ แก่นักศึกษาระดับปริญญาโทจำนวน 2 เรื่อง เพื่อให้นักศึกษาได้เรียนรู้งานวิจัย และเป็นหัวข้อโครงการปัญหาพิเศษในระดับปริญญาตรี จำนวน 2 เรื่องดังนี้

- ได้เป็นหัวข้อวิทยานิพนธ์ระดับปริญญาโท 2 เรื่อง คือ

1) นายพงศกร จันทะคุณ ซึ่งจะทำการสังเคราะห์อนุพันธ์เพิ่มเติมและศึกษาฤทธิ์การเป็น neuroprotective agent โดยใช้เซลล์ P19 (กำลังดำเนินการ โดยเริ่มดำเนินการเมื่อ มิถุนายน 2553)

2) Miss Mari Tatsukawa จาก Meiji Pharmaceutical University, Tokyo, Japan ซึ่งจะศึกษาวิธีการสังเคราะห์ nordentatin (กำลังดำเนินการ โดยเริ่มดำเนินการเมื่อ เมษายน 2553)

- ได้เป็นหัวข้อโครงการพิเศษระดับปริญญาตรี 2 เรื่อง คือ

1) การสังเคราะห์อนุพันธ์ของสารกลุ่ม coumarin จากเปลือรากต้นส่องฟ้า โดยนายพงศกร จันทะคุณ และนาย ธีรภัทร บุตรจันทร์ แล้วเสร็จเมื่อ เดือน ตุลาคม พ.ศ. 2552

2) การสังเคราะห์อนุพันธ์ของสารกลุ่ม carbazoleต่อฤทธิ์จ้านอนุมูลอิสระ โดยนายทรงกลด น้อยตั้ง แล้วเสร็จเมื่อ เดือน ตุลาคม พ.ศ. 2553

ANTILIPID PEROXIDATION OF BIOACTIVE COMOUNDS AND DERIVATIVES FROM THAI MEDICINAL PLANT *CLAUSENA HARMANDIANA*

Ploenthip Puthongking[1)], Pongsakorn Jantakoon[1)], Teerapat Bootchan[1)], Tatsukawa Mari[2)], Masashi Yokoya[2)], Naoki Saito[2)], and Chamnan Patarapanich[3)]

1) Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand 2) Meiji Pharmaceutical University, 2-522-1, Nishio, Kiyose, Tokyo 204-8588, Japan

3) Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

KEYWORDS: Anti-lipid peroxidation, carbazole alkaloid, *Clausena harmandiana*, coumarin, neuroprotective agent

INTRODUCTION

Various pathological conditions including cancer, arthritis, arteriosclerosis, heart disease, inflammation, brain dysfunction and acceleration of the ageing process appear to have etiological relation to the active oxygen-induced and free radical-mediated oxidation of biomolecules. Antioxidant agents are substances that can prevent or delay oxidative damage of lipids, proteins and nucleic acids by reactive oxygen species, which hydroxyl, peroxyl, hypochlorous, etc. The most abundant antioxidants in plants are polyphenol compounds. These polyphenols, most of which are flavonoids, are present mainly in ester and glycoside form. Thus interest in natural antioxidant has increased considerably. And various plants are proposed to be antioxidant activity according to isolated bioactive compounds. *Clausena harmandiana* (Rutaceae) is distributed mainly in the northeast of Thailand, whose roots and root bark of this plant are reputedly used in folk medicine for stomach ache, and fever, revealed the presence of coumarin and carbazole alkaloids.[1,2] Although coumarins and carbazoles isolated form *C. harmandiana* and other species of the genus *Clausene* have been reported to exhibit diverse biological activity, such as antiplasmodial activity,[3] antimicrobial and antifungal activities.[4] The screening data for antioxidant capacity of some coumarins and carbazole alkaloids from *C. harmandiana* were done by Dr. Chantana boonyarat group (data unpublished). The primary data revealed that one of coumarin compound exhibit significant antioxidant activity by using DPPH assay. And there are a few previous phytochemistry report on any part of *C. Harmandiana*[5-7]. Furthermore, in recently report revealed that pyranocoumarin derivative showed anti-HBV and cytotoxic activity[8]. These evidence together with our previously research attempt us aimed to isolated bioactive compounds from the root bark of *C. harmandiana* and screen for their anti-lipid peroxidation.

MATERIALS AND METHODS

Plant material : The roots bark of *C. harmandiana* (Rutaceae) were collected from Yangtalad district, Kalasin province, Thailand, in May of 2008.

Extraction and Isolation : The root bark of *Clausena harmandiana* was macerated with methanol and further partitioned with hexane and methylene chloride sequentially. The biological assay revealed that the methylene chloride soluble fraction possessed high antioxidant activity in DPPH assay (data is not shown). Methylene chloride soluble fraction (20 g) have led to the identification of 4 known cabazole alkaloids and 3 coumarin compounds.

Chemistry : All reaction were prepared in milligram scale and conducted under nitrogen atmosphere. The derivatives were prepared via the reaction between acetic anhydride and starting material from naturals. The reaction was reacted under room temperature and used 4dimethylaminopyridine (DMAP) or pyridin as catalyst substance to corresponding ester derivatives. ^{1}H and ^{13}C-NMR spectra were recorded in CDCl3 and DMSO at 400 MHz and 125.65 MHz, respectively, on a JEOL-JNM-LA 500 FT NMR spectrometer and at 270 spectrometer (ppm, *J* in Hz with TMS as internal standard). Dry solvents and reagents were obtained using standard procedures. Analytical thin-layer chromatography was performed on silica gel 60 F254 plates. Silica gel chromatography was performed with the indicated solvent on silica gel 60 (70-230 mesh). All organic solvent extracts were dries over anhydrous sodium sulfate.

Bioactivities testing : All these compounds were studied for the ability to scavenge the 1,1diphenyl-2-picrylhydrazyl (DPPH) radical and thiobarbituric acid reactive substances (TBARs) by comparison with a well known synthetic antioxidant, -tocopherol (Vitamin E). The IC50 value was calculated from the concentration at which 50 % inhibition.

RESULTS

The root bark, leave and stem of *C. harmandiana* were extracted by refluxing with hexane, methylene chloride and methanol for 24 h respectively. The nine resulting fractions were tested in DPPH assay. The biological assay revealed that the methylene chloride soluble fraction of root bark and methanol soluble fraction of leaves possessed high antioxidant activity with IC50 = 88.3 and 53.8 mg/mL, respectively (data not shown). Then the root bark underwent activity-guided fractionation in this work, First we attempt to use methylene chloride as a solvent. Our work, the root bark of *C. harmandiana* was macerated with methanol and further partitioned with methylene chloride to give crude extract about 50 g. The methylen chloride soluble fraction has led to the isolation of 4 known carbazole alkaloids and 3 coumarins, heptaphylline [**1**], 7-methoxyheptaphylline [**2**], 2-hydroxy-3-formyl-7-methoxycarbazole [**3**], mukonal [**4**], xanthoxyletin [**5**], dentatin [**6**], and nordentatin [**7**], respectively. All known compounds were identified structure by comparison of their spectroscopic and physical data with those reported in the literature. The primary data revealed that one of coumarins, nordentatin, and two carbazole alkaloids, heptaphyline and 7-methoxyheptaphyline exhibit significant antioxidant activity by using DPPH ans TBAR assays. Then ester derivatives were synthesized via acetylation reaction to be acetate-heptaphylline [**8**], acetate-7-methoxyheptaphylline [**9**] and acetate-nordentatin [**10**], respectively. Our result had shown that, in the DPPH assay, compounds **1**, **3**, **4**, **5** and **6** showed less activity with IC50 > 1000 μM, while compounds **2**, **7**, **8** and **9** showed antioxidant property with IC_{50} = 810.7 μM, 84.3 μM, 264.8 μM and 915.0 μM, respectively (Vit E; IC_{50} = 17.90 μM). In the TBAR assay, all compounds excepted compounds **3**, **4**, **5** and **6** showed anti-lipid peroxidation activity better than standard vitamin E (IC_{50} = 75.9 μM) which compound **1** showed IC_{50} = 5.6 μM, **2** showed IC_{50} = 1.9 μM, **7** showed IC_{50} = 1.7 μM, **8** showed IC_{50} = 4.5 μM, **9** showed IC_{50} = 4.2 μM and **10** showed IC_{50} = 2.8 μM, respectively.

DISCUSSION AND CONCLUSION

The data showed that the neuroprotective components from natural are heptaphylline, 7methoxyheptaphylline and nordentatin which displayed potent in the primary screening with DPPH and TBAR assays, in addition, nordentatin was the most potent anti-lipid peroxidation in both assays. The most interesting result attempts our group decided to synthesize additional derivatives of the isolated nordentatin, heptaphylline and 7-methoxyheptaphylline to comparison with the parent compounds. Therefore, the starting compound were prepared to ester derivatives under the condition of acid chlorides in pyridine to give the yield of products in the range of 85-95 %. In our results all acetate ester derivatives which tested by TBAR assay were show the best of antioxidant activity and better than vitamin E, all above suggest we can be developed the carbazoles core structure as heptaphylline and 7-methoxyheptaphylline and coumarin core structure as nordentatin to have strong activity than the substrate which isolated and extracted from natural products cause develop to effectiveness drugs in the future.

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Natural Medicine Research for the Next Decade: New Challenges and Future Collaboration

PP 4

PREPARATION AND BIOLOGICAL ACTIVITIES OF NORDENTATIN DERIVATIVES

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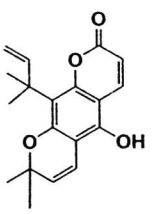
KEY WORKS: preparation, nordentatin, biological evaluation, *Clausena harmandiana*

INTRODUCTION

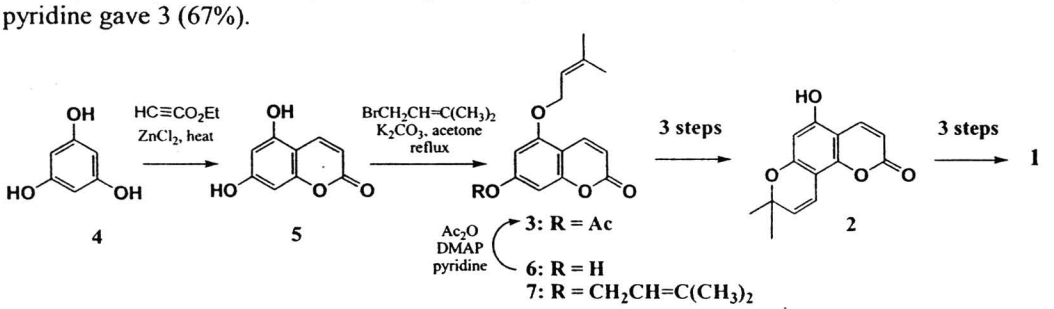
Clausena harmandiana (Rutaceae) is districted mainly in the northeast of Thailand, whose roots and root bark reputedly used in folk medicine for stomach ache and fever, revealed the presence of coumarines and carbazole alkaloids. Although coumarines and carbazoles from *Clausena harmandiana* and other plants have been reported to exhibit diverse biological activities, such as antiplasmodial activity, antimicrobial and antifungal activity, antioxidant activity have not been studied, yet.[1] Recently, we found nordentatin **1** possessed *in vitro* highly antioxidant activity, which was comparable to vitamin E. Thus, we might prepare a large amount of **1** by chemical synthesis, followed by transformation it to several kinds of nordentatin analogue to evaluate structure-activity relationship studies. In this paper, we report practical synthesis of **1**.



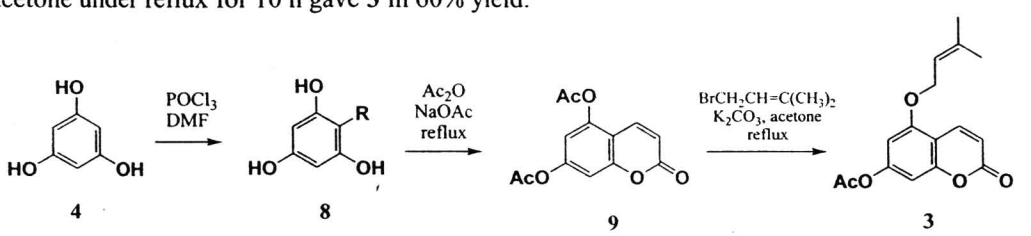
nordentatin 1

RESULT and DISCUSSION

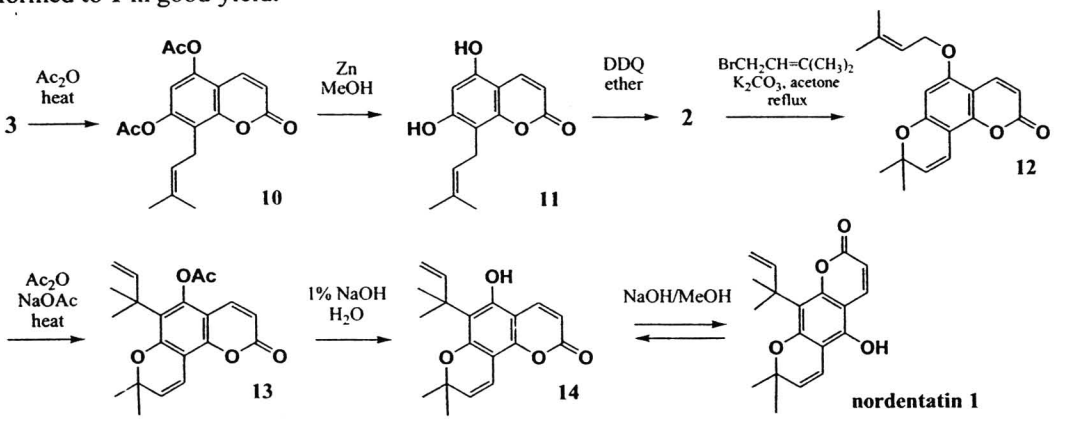
Synthesis of **1** from 5-hydroxyseselin **2**, which was prepared by the Claisen rearrangement of the acetate **3**, have already been reported by Murray in 1983.[2] Before starting to synthesis of **1**, we need access to **3** in quantities. It is well know that three step synthesis of **3** from the commercially available phloglucinol **4**, thus, $ZnCl_2$ catalyzed transformation of **4** with ethyl propiolate afforded **5** including transesterification and cyclization sequence.[3] Reaction of **5** with 1-bromo-3-methylbut-2-ene and K_2CO_3 in acetone gave the mono-alkyl compound **6** (25%) along with the bis-alkyl compound **7** (26%) according to the procedure of Murray.[2b] Acetylation of **6** with Ac_2O and DMAP in pyridine gave 3 (67%).



The yield of the above three steps from **4** into **3** led to surprising low, and this problem was solved using modified Roux procedure.[4] Formylation of **4** with $POCl_3$ in DMF afforded **8** in 87% yield. Heating **8** with Ac_2O and NaOAc at 185°C for 21.5 h gave the diacetate **9** in 88% yield. Treatment of **9** with 1-bromo-3-methylbut-2-ene and K_2CO_3 in acetone under reflux for 10 h gave **3** in 60% yield.[2b, 5]



Having established synthetic process for the precursor of Claisen rearrangement **3**, we then attempted to convert **3** into the final goal **1**. Heating **3** with Ac_2O to give the diacetate **10** was followed by deacetylation with zinc dust in MeOH afforded **11** in excellent yield.[2a] Oxidative cyclization of **11** with DDQ in ether gave the chromene **2**, which was transformed to **1** in good yield.[2c]



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ABSTRACTS

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Anti-lipid peroxidation activity of carbazole alkaloids and its derivatives from thai medicinal plant, *Clausena Harmandiana*

PYS-4

Noytung S. and Puthongking P.

Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand.

Clausena harmandiana is distributed mainly in the northeast of Thailand, whose roots and root bark are reputedly used in folk medicine for stomach ache and fever, revealed the presence of coumarins and carbazole alkaloids. The carbazole molecule and with various hydroxyl and other substituentes were found to inhibit lipid peroxidation and to scavenge hydroxyl radical and superoxide anion. Thus, we found it interesting to screening new antioxidant agents from the isolated bioactive compounds from the root bark of *C. harmandiana*. Therefore, this present investigation aimed to screen for their antioxidant capacities. The activity-guided fractionation of methylene chloride soluble fraction (15 g) of the root bark of *C. harmandiana* have led to the identification of the four known carbazole alkaloids as heptaphylline (**1**), 7-methoxyheptaphylline (**2**), 2-hydroxy-3-formyl-7-methoxycarbazole (**3**) and mukonal (**4**). Their structures were elucidated by comparison with the authentic sample, which were identical in all respects. All isolated compounds and its ester derivatives of heptaphylline (**5**) and 7-methoxyheptaphylline (**6**) were evaluated for their anti-lipid peroxidation with 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and thiobarbituric (TBA) assay. The result reveals that compounds, **1**, **2**, **5** and **6** are promising activity on TBAR assay than that standard antioxidant agent as Vit E (IC_{50} = 75.9 μM) with IC_{50} as 5.6, 1.9, 4.5 and 4.2 μM, respectively. Compound **2** processing strong anti-lipid peroxidation, and ester analogs show slightly reduce and increase activities of **5** and **6**, respectively. Therefore, another substitution on hydroxyl group of carbazole core structure are interesting to study, which might be useful for the next structure modification the new candidate neuroprotective agent from carbazole alkaloids system.

ABSTRACTS

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SCP050

COUMARINS AND CARBAZOLES WITH ANTIOXIDANT ACTIVITY FROM *CLAUSENA HARMANDIANA*

P PUTHONGKING, G YUSAKUL

Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002,Thailand.

Objective: This work aim to isolate bioactive compounds from the root bark of *Clausena harmandiana* and screen for their antioxidant capacities.

Method: The root bark of *Clausena harmandiana* was macerated with methanol and partitioned with methylene chloride respectively. The crude methylene chloride was isolated with colum chromatography to give 2 cabazoles and 2 coumarins. And 3 bioactive compounds were further prepared to acetate derivative and measured antioxidant activity by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay.

Result and Conclusion: Our research, the root bark was macerated with methanol and partitioned with hexane and methylene chloride sequentially. The data revealed that the methylene chloride soluble fraction displayed high antioxidant activity in DPPH assay (IC_{50} = 49 μg/mL). The activity-guided fractionation of methylene chloride soluble fraction (15 g) have led to the identification of 2 cabazoles and 2 coumarins as heptaphylline[**1**] (0.454 g), 7-methoxy heptaphylline[**2**] (0.501 g), dentatine[3] (1.35 g), and nordentatin[**4**] (0.266 g), respectively. In addition, compound **1**, **2** and **4** were prepared to acetate-derivatives under the condition of acetic anhydride in 50-60 % yield. All compounds were evaluated antioxidant activity in DPPH assay. It reveals that **4** shows the highest antioxidant activity in DPPH assay. Moreover, 7 compounds were also evaluated with thiobarbituric acid (TBA) assay to identify

the antioxidant activity and consequently might be useful for the next structural modification the new candidate antioxidant agents from the root bark of *Clausena harmandiana*.

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