APPENDIX

A. Supporting papers

A.1 Aobchey, P., Lhieochaiphant, S., and Phutrakul, S. Characterization of major components in the pigments produced by *Morinda angustifolia* Roxb. var. *scabridula* Craib. root cell culture. *Chiang Mai Journal of Science*. 2004, 31, 303-308.

A.2 Aobchey, P., Sinchaikul, S., Phutrakul, S., and Chen, S. T. Simple purification of indirubin from *Indigofera tinctoria* Linn. and inhibitory effect on MCF-7 human breast cancer cells. *Chiang Mai Journal of Science*. 2007, 34, 1-9.

B. Proceeding of international conferences

B.1 Aobchey, P., Tungphadit, R., and Phutrakul, S. High-performance liquid chromatographic method for the analysis of indigo dye in *Indigofera tinctoria* Linn. (Karm) and *Baphicacanthus cusia Brem*. (Hom). The 15th Annual Meeting of the Thai Society for Biotechnology Sustainable Development of SMEs Through Biotechnology and The JSPS-NRCT Symposium on The Forefront of Bioinformatics Application, February 3-6, 2004, Pang Suan Kaew Hotel, Chiang Mai, Thailand.

B.2 Aobchey, P., Sinchaikul, S., Phutrakul, S., and Chen, S. T. Inhibitory effect of indirubin from *Indigofera tinctoria* Linn. on human breast cancer (MCF-7) cells. BioThailand 2005 "Challenges in the 21st Century", November 2-5,

2005, Queen Sirikit National Convention Center, Bangkok, Thailand.

B.3 Aobchey, P., Kaojaroen, A., and Phutrakul, S. Antibacterial activity of metranol extracts from *Coscinium fenestratum* (Gaertn) Colebr. The 18th Annual meeting of the Thai Society for Biotechnology "Biotechnology : Benefits & Bioethics", November 2-3, 2006, The Montien Riverside Hotel, Bangkok, Thailand.

C. Oral presentation

C.1 Aobchey, P., Sinchaikul, S., Phutrakul, S., and Chen, S. T. Inhibitory effect of indirubin from *Indigofera tinctoria* Linn. on human breast cancer (MCF-7) cells. BioThailand 2005 "Challenges in the 21st Century", November 2-5, 2005, Queen Sirikit National Convention Center, Bangkok, Thailand.

D. Poster presentation

D.1 Aobchey, P., and Phutrakul, S. A HPLC method to quantify indican from *Indigofera tinctoria* Linn. (KHARM). The 17th FAOBMB Symposium/ 2nd IUBMB Special Meeting/ 7th A-IMBN Conference on "Genomics and Health in the 21st Century", November 22-26, 2004, Bangkok, Thailand.

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Contributed Paper

Characterization of Major Components in the Pigments Produced by *Morinda angustifolia* Roxb. var. *scabridula* Craib. Root Cell Culture

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ABSTRACT

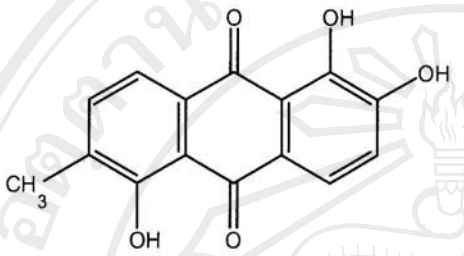
Plants are the origin of most natural dyes. The natural red dyes including the anthraquinones, naphthoquinones and benzoquinones could be found in varieties of plants. *Morinda angustifolia* Roxb. var. *scabridula* Craib. produces pigments in its root. The pigment extracted from the root with methanol and separated by thin layer chromatography (TLC) gave two major components. One component was purified and identified as the anthraquinone pigment morindone. The root cells of this plant cultured on a modified Gamborg's B_5 medium at 25°C for one month produced the pigments which gave two components on TLC using the same solvent system for the separation of the pigment from the plant's root. The red pigment had R_f value the same as morindone and the yellow pigment had the same R_f value as the component from alkali extract of the plant's root. The yellow component was purified and identified as the anthraquinone pigment alizarin 1-methyl ether by spectroscopic spectral comparison with those reported previously.

Keywords : alizalin 1-methyl ether, anthraquinone, cell culture, *Morinda angustifolia* Roxb. var. *scabridula* Craib., morindone, pigment.

1. INTRODUCTION

Plants produce pigments which have current or potential uses as natural dyes in textile dyeing industries represent potential alternative crops having high value and quality. Textiles are articles of prime necessity with a greater or lesser degree of 'skin proximity' and regulations have been legislated which seek to avoid risks to the textile end-users. On the other hand, textile dyeing is characterised by high environmental pollution and high health risk to personel handling harmful substances. Many colorants from synthetic sources can be harmful and cause allergics in humans [1], therefore interest in natural dyes has increased considerably during the last few years. *Morinda angustifolia* Roxb. var. *scabridula* Craib. produces anthraquinones pigment in its root, one of them has been found to be morindone [2]. Plant cell culture has been considered as an alternative technology to obtain natural dyes [3-5]. We investigated to culture the root cell of this plant species on Gamborg's B_5 medium for one month which could produce the pigments. One major component (the red pigment) was morindone **1** [2]. The present

work deals with the identification of another major component of the pigments produced by the root cell cultures.



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2. Materials and Methods

2.1 General Procedures

^{1}H- and ^{13}C-NMR spectra were recorded on a Jeol-JNM LA 400 spectrometer, with $CDCl_3$ as solvent. Fast Atom Bombardment Mass Spectrometry (FAB-MS) was taken in positive ion mode on a Jeol Automass 20 instrument. The melting point was determined on a Leitz wetzlar apparatus.

2.2 Plant material

The plants were collected from the upper north of Thailand. The seeds of two to three years old plant of *Morinda angustifolia* Roxb. var. *scabridula* Craib. were used to prepare sterilized root cell culture.

2.3 Cell Culture

The *Morinda* root cell cultures were obtained by growing the *Morinda* seed on Murashige and Skoog (MS) medium [6]. The roots were cut into 2.5 mm. pieces and grown in fresh MS medium to get callus. The root cell of this plant was cultured on a modified Gamborg's B_5 medium [7] supplemented with 1 mgl^{-1} 2,4-dichlorophenoxy-acetic acid (2,4-D), 0.2 mgl^{-1} kinetin and 30 gl^{-1} sucrose at 25°C for one month in the solid medium.

2.4 Extraction and Characterization of the Pigments from Root Cell Culture

The root cell culture was grown on B_5 medium at 25°C for one month. The wet cells (59 g) were extracted with 1.4 litres of chloroform. The crude extract was evaporated to dryness under reduced pressure to obtain the concentrated dyestuff. The chloroform extract was monitered by TLC on silica gel 60 developed by chloroform-methanol (9:1). The crude extract was separated by preparative TLC using chloroform-methanol (9:1). The yellow component characterized by comparison of melting point, ^{1}H- NMR, ^{13}C-NMR and mass spectra with the reported values.

2.5 Extraction and Isolation

The dry *Morinda* root (1.5 kg) was extracted with 6 litres of 95% methanol. The methanol extract was chromatographed on silica gel 40 column, eluted with chloroform, chloroform-methanol (9:1) and chloroform-methanol (1:1), respectively. The major component obtained from column chromatography by eluting with chloroform-methanol (9:1) was rechromatographed on silica gel 40 column. Elution with hexane-chloroform (9:1) yielded the pigment which was characterized by the same method mentioned above.

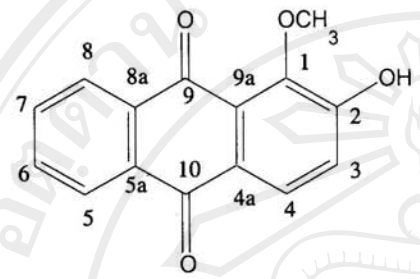
3. Results and Discussion

In previous work, we have extracted the pigments from the root of *Morinda* plant. At least two components have been separated from the crude extract. One component was found to be the anthraquinone pigment morindone. Another component could be extracted but has not yet been identified [2].

Extraction, Purification and Identification of the Pigments

Extraction of pigments from the root cell culture of *Morinda angustifolia* Roxb. var. *scabridula* Craib. with chloroform gave two components monitored by TLC. The red pigment had R_f value the same as morindone **1** (0.80 in chloroform-methanol 9:1) and the yellow pigment had R_f value (0.33) the same as the component from alkali extract of the plant's root. The crude extract was purified by preparative TLC. The yellow band was

collected, dissolved in methanol and recrystalized in cold methanol to yield alizarin 1-methyl ether **2**.



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The crystal of compound **2** was obtained as needles shape , m.p.178 -179°C. The FAB-MS (positive ion mode) spectrum exhibited a psuedomolecular ion peak at m/z 255 corresponded to a molecular formula of $C_{15}H_{10}O_4$ (Figure 1). The ^{1}H- and ^{13}C-NMR data (Figure 2, Table 1 and Figure 3, Table 2, respectively) were consistent with those reported for alizarin 1-methyl ether **2** (1-methoxy-2-hydroxyanthraquinone)[8]. This compound has given ^{1}H-NMR (400MHz, $CDCl_3$) data at δ 4.02 (s, 3H), 6.65 (br d s, 1H), 7.34 (d,1H), 7.72-7.78 (m, 2H), 8.13 (d, 1H), 8.22-8.28 (m, 2H). The yellow compound isolated from the root of *Morinda angustifolia* Roxb. var. *scabridula* Craib. was thus concluded to be alizarin 1-methyl ether **2**. Callus of *Galium verum* established under a variety of culture conditions readily produced anthraquinones. One of them was fully identified as compound **2** [8]. From *Cinchona ledgeriana* callus cultures, 3 anthra-quinones (alizarin, alizarin 1-methyl ether and 1,8-dihydroxyanthraquinone) have been reported [9].

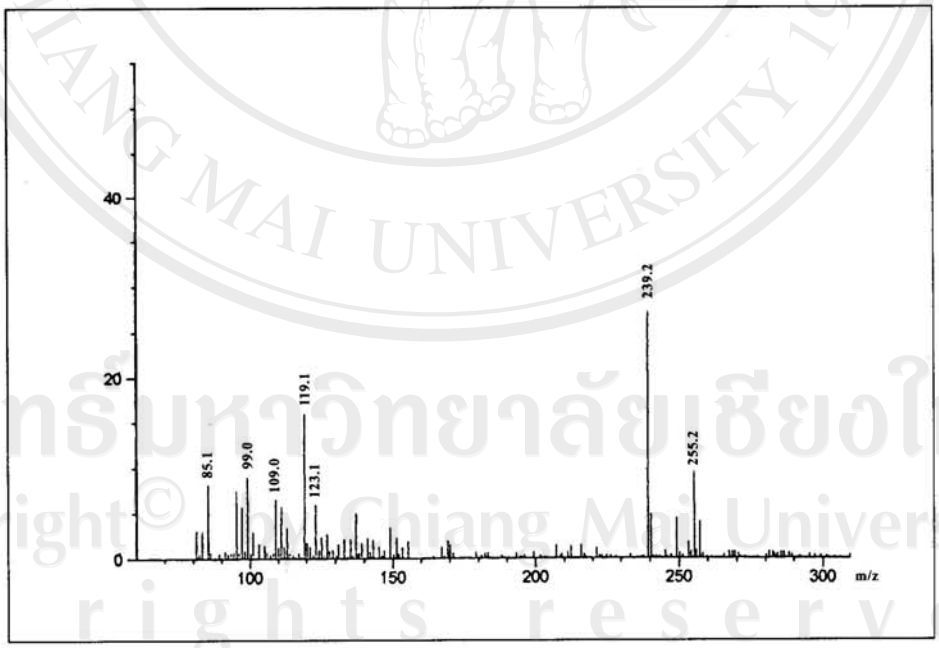

Figure 1. FAB-Mass spectrum of the yellow pigment **2**.

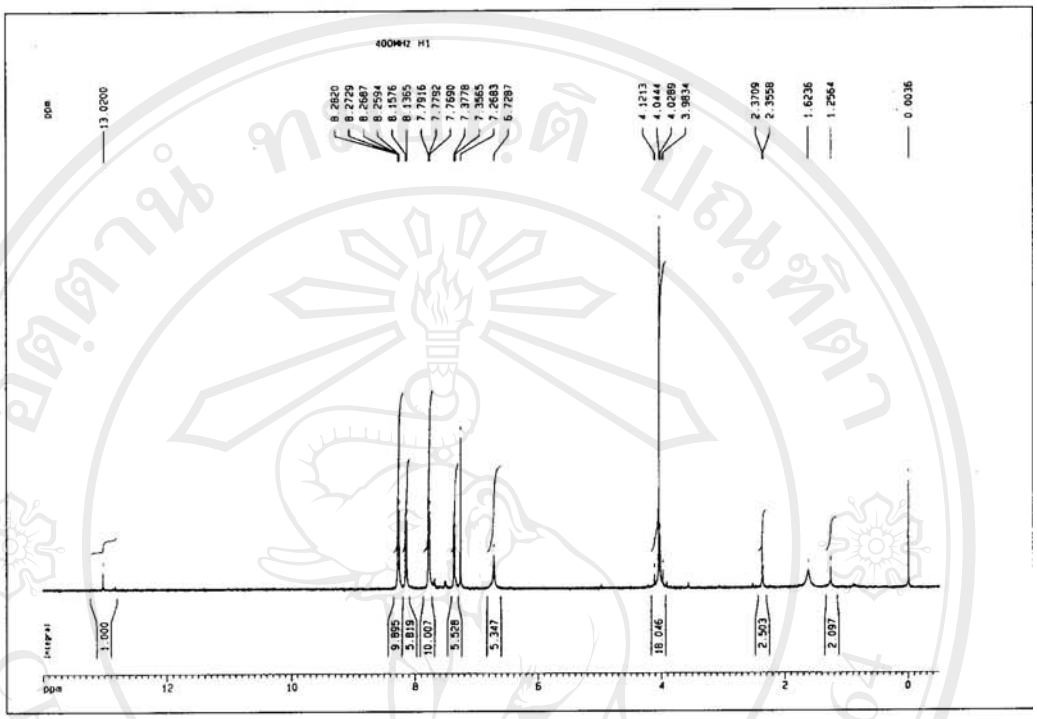

Figure 2. ^{1}H-NMR spectrum of the yellow pigment **2**.

Table 1. ^{1}H-NMR data of the yellow pigment **2** in $CDCl_3$.

^{1}H	Chemical shift (ppm)	H-Coupling
$-OCH_3$	4.03	s, 3H, - OCH_3
-OH	6.73	s, 1H, -OH
H-4	7.36	d, $1H_a$, CH
H-6, H-7	7.80	d, $2H_{d,f}$, CH
H-3	8.14	d, $1H_b$, CH
H-5, H-6	8.29	d, $2H_{c,e}$, CH

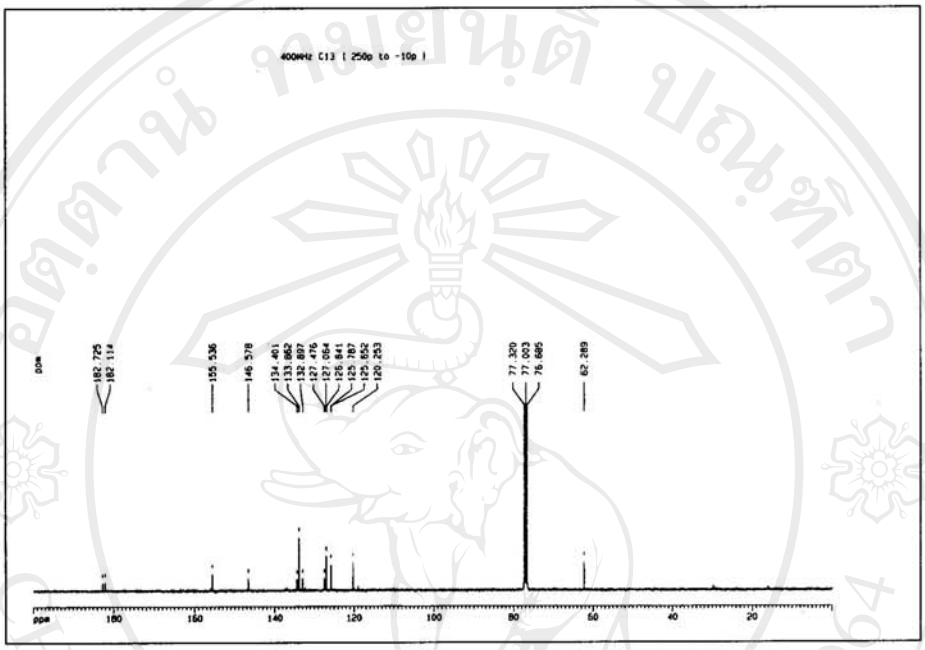

Figure 3. ^{13}C-NMR spectrum of the yellow pigment **2**.

Table 2. ^{13}C-NMR data of the yellow pigment **2** in $CDCl_3$.

^{13}C	Chemical shift (ppm)
C-1	155.54
C-2	146.58
C-3	120.25
C-4	125.79
C-4a	132.90
C-5	127.06
C-5a	134.40
C-6	127.47
C-7	127.47
C-8	126.84
C-8a	133.86
C-9	182.11
C-9a	125.78
C-10	182.73
CH_3	62.29

4. CONCLUSION

Morinda angustifolia Roxb. var. *scabridula* Craib. produced the same pigments in both its root and root cell culture. Two major components have been isolated and identified as the anthraquinone pigment morindone **1** and alizarin 1-methyl ether **2**. In further study, increase production of both pigments from root cell culture will be investigated and the content of both pigments will be analyzed by high-performance liquid chromatographic method.

ACKNOWLEDGEMENTS

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Contributed Paper

Simple Purification of Indirubin from *Indigofera tinctoria* Linn. and Inhibitory Effect on MCF-7 Human Breast Cancer Cells

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ABSTRACT

Indirubin is a naturally occurring pigment and widely used as natural dye for local handicraft and industrial application. Although there are many extraction and purification methods of indirubin, the complex and several methods were used and the purified indirubin still contained impurity. In this study, we purposed to improve the extraction and purification methods of indirubin and study its anticancer property. A methanol extract of *Indigofera tinctoria* Linn. (Thai name - 'kram') powder was purified by two steps of silica gel 60 column chromatography and reverse phase high performance liquid chromatography (RP-HPLC) using C18 column. The purified indirubin has the high purity with exactly molecular mass of 263 Da. In addition, the inhibitory effect of indirubin on MCF-7 human breast cancer cells showed that 30 mM indirubin strongly inhibited the cell growth of MCF-7 cells about 42% within 24 h. A longer time of treatment and a higher concentration of indirubin could reduce the cell growth inhibition of MCF-7 cells, in which it may be caused by degradation and crystallization of indirubin. Thus, we suggest that the incubation time of treatment and indirubin concentration are the essential factors of the inhibition of indirubin on MCF-7 cell growth.

Key words: *Indigofera tinctoria* Linn., kram, indirubin, purification, MCF-7.

1. INTRODUCTION

The blue dye indigo is one of the oldest natural dyestuffs and has been obtained from a variety of plant sources, such as *Indigofera tinctoria* (Africa, Asia, East India, South America), *Polygonum tinctorium* (China, Korea) and *Isatis tinctoria* (Europe), since ancient times [1-3]. Indigo is formed in damaged leaves by oxidation of products from the hydrolysis of indigo precursor; indican (indoxyl-β-D-glucoside) or isatan B (indoxy-α-ketogluconate) to indoxyl. The by-products of biosynthetic indigo formation which is the

reaction between indoxyl and isatin C (obtained in a side-reaction) are indigotin, indirubin, isoindigotin, isoindirubin, isoindigo and indigo yellow [4].

Indirubin is a pink colored pigment and synthesized as a by-product of indigo. Few attempts in extraction and purification of indirubin in plants by TLC and RP-HPLC analyses have been studied [5-6], but all of the methods have used many processes and gave the complex mixture of indirubin that still contained the high impurity. Thus, a simple purification method of indirubin is necessary for reducing the process cost and enhancing the efficiency of purification method, leading to get the high purity of indirubin. On the other hand, the biological activity of indirubin has been widely studied; for example, it is well known that indirubin is an active component in Chinese traditional medicine, namely Danggui Longgui Wan, and used to cure chronic myelocytic leukemia (CML). In clinical trials with patients suffering from chronic myelocytic and chronic granulocytic leukaemia, indirubin could induce complete remission in 26% and partial remission in 33% of the 314 cases, showing only low toxicity and limited side effects [7]. In addition, indirubin is a potent inhibitor of cyclin-dependent kinases (CDKs) and glycogen synthase kinase-3β (GSK-3β), which may play an important role in the development of Alzheimer's disease and leukemia [8-9] and inhibit inflammation in delayed-type hypersensitivity (DTH) reactions [10]. Moreover, indirubin has the immunomodulatory activity on the expression of RANTES, which reduced both the expression and production of regulated on activation, normal T cell expressed and secreted (RANTES) in influenza A/NWS/33-infected H292 cells [11]. Although the biological activities of indirubin have been elucidated, some properties of indirubin and its mechanism are not clear.

In this study, we established a simple purification method of indirubin, which were extracted from *Indigofera tinctoria* Linn. ('Kram' in Thai name). Two steps of silica open column and HPLC were used for purification of indirubin with the high purity. In addition, we studied the inhibitory effect of pure indirubin on MCF-7 human breast cancer cell line that may be useful for further therapeutic application.

2. MATERIALS AND METHODS

2.1 Chemical Materials

Indirubin or indirubin-3′-monoxime was obtained from ALEXIS Biochemicals (Carlsbad, CA, USA). Human breast cancer epithelial cell line (MCF-7, ATCC#CRL1721) was purchased from ATCC (Rockville, MD, USA). DMEM and MEM sodium pyruvate were purchased from Atlanta Biologies (Nocross, GA, USA). Antibiotic-antimycocytic, MTT (methylthiazoletetrazolium) and sodium bicarbonate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA, USA).

2.2 Plant Material

Indigofera tinctoria plants were grown in a field of Lampang province (Thailand) from April to September. The plant material was harvested in the end of September 2004. The aerial parts of *Indigofera tinctoria* Linn. were collected locally and its morphological characteristics were kindly identified by Mr. James F. Maxwell at Department of Biology, Faculty of Science, Chiang Mai University, Thailand.

2.3 Preparation of Kram Powder

Fresh plant materials of *Indigofera tinctoria* Linn. were cut to small pieces, put into cotton

bag and soaked in water for 24 h. The bag was transferred into a new chamber and soaked in water for another 24 h. The soaked solutions from twice sample soaking were combined and twice volumes of $Ca(OH)_2$ solution (pH ~11) were added. After 30 min under air blowing, the extracted indigo was allowed to precipitate overnight. Then, the upper solution was discarded and the precipitated pigment or kram paste was passed through drum dryer to get kram powder.

2.4 Extraction of Indirubin from Kram Powder

Kram powder (100 g) was suspended in methanol (10 l). The red dye methanol solutions were pooled, filtered and evaporated under reduced pressure to dryness. The dried kram powders were re-extracted with methanol for six times. The residue of dry powder was dissolved in small volume of methanol and analyzed by TLC.

2.5 TLC Analysis

Crude indirubin were dissolved in methanol (50 mg/ml) and spotted on silica gel 60F plates (Merck). Elution was carried out with the mixture of dichloromethane: hexane:methanol (7:4:0.3, v/v/v). The commercial indirubin standard was used to compare the purity of indirubin.

2.6 Purification of Indirubin by Silica Gel 60 Column Chromatography

A glass column (2.5´54 cm) containing 120 g of silica gel 60 was used to separate the crude extract from kram powders (60 mg/ml) and eluted with dichlorome-thane: hexane:methanol (7:4:0.3, v/v/v) (240 ml). The eluates were fractionated and the purity of indirubin was determined by TLC and HPLC.

2.7 Purification of Indirubin by RP-HPLC

Purification of indirubin were performed using a reverse-phase HPLC column (9.4´250 mm, ZORBAX ODS) attached to a L-4250 UV-VIS detector and an L-6200A pump (Hitachi, Tokyo, Japan). Two mobile phases, A (5% v/v acetonitrile, 0.1% v/v trifluoroacetic acid) and B (95% v/v acetonitrile, 0.1% v/v trifluoroacetic acid), were filtered through a 0.22-mm Millipore filter and degassed before use. The partial purified indirubin obtained from silica gel column chromatography was dissolved in methanol (225 mg/ml), filtered and injected into a HPLC-C18 column with 90 mg/400 ml. The HPLC condition was used the gradient elution profile of 0-5-30 min/ 20-50-100%B at a flow rate of 2 ml/min. The eluent was monitored by measuring UV absorption at 552 nm.

2.8 Mass spectrometry

The active compound was analyzed by using a high-resolution ESI-TOF mass spectrometer (BioTOF III; Bruker Daltonics, Inc. Billerica, MA, USA).

2.9 Cell Cultures and Treatment

Human breast cancer epithelial cell line, MCF-7, was cultured in monolayer in DMEM medium supplemented with 10% fetal bovine serum, 1.5 g/l sodium bicarbonate, 0.1 mM MEM sodium pyruvate, streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml) at 37°C under a 5% CO_2 atmosphere. To perform cell attachment, MCF-7 cells were seeded at $5´10^4$ cells in a 96-well plate for 24 h. The cells were treated with different concentrations of 0.1, 1, 3, 10 and 30 µM indirubin. In addition, the cells without indirubin treatment were added in parallel with DMSO and used as negative control in our experiment. Triplicate

experiments were done for each concentration of indirubin and control. The change of cell morphology after indirubin treatment was visualized by microscopy with 40-fold magnitude.

2.10 Cell viability assay

The cell viability was determined by the trypan blue exclusion test. In this test, 100 µl of cell suspension was added to 100 µl of 0.5% trypan blue in PBS. The number of dead (blue) and living (white) cells was counted using hemacytometer. In addition, the cell proliferation was also determined by MTT assay method [12]. The cell viability was measured at 570 and 630 nm-wavelengths.

2.11 Statistical Analysis

Determination of differences among the groups was performed with one way ANOVA analysis. Data are expressed as mean ± S.E.M. and $p< 0.05$ was accepted as significant.

3. RESULTS AND DISCUSSION

3.1 Purification of Indirubin from *Indigofera tinctoria* Linn.

In our previous study, the TLC separation of kram powder using chloroform-hexane-methanol (7:4:1 v/v/v) as developing solvent gave two major pigments with blue and red colors. Both blue and red pigments were highly soluble in chloroform, but only red pigment was soluble in methanol. The red pigment could be separated from blue pigment by dissolving the kram powder in methanol [5]. Due to the impurity of indirubin and a long time of purification, the previous method was modified and used for purification of indirubin from kram powder. After methanol extraction of kram powders, the crude extracts were separated using a silica gel 60 column and the purity of partially purified indirubin was determined by TLC and HPLC. Comparing to the high impurity in crude extract, the partially purified indirubin was shown to have a trace of impurity (Figure 1). However, this method highly reduced the impurity elements from the indirubin and the indirubin could be easily fractionated by visualizing a red color of indirubin. Thereafter, the partially purified indirubin was purified by preparative HPLC using C18 column. The major peak obtained from HPLC was collected and the structural elucidation of purified indirubin was carried out by MS spectrometry. The mass spectra obtained for chromatographic peak contained a strong signal at m/z 263 $[M+H]^+$ as same as standard indirubin (Figure 2). The result was in agreement with the structural analysis of indirubin by UV-visible spectroscopy, MS and ^1H, ^{13}C-NMR spectrometry [5]. Therefore, our purification method of indirubin from natural kram powder is a simple and effective method to get the high amount of indirubin with high purity. Moreover, this strategy of indirubin production may be useful for industrial and pharmaceutical applications.

3.2 Effect of Pure Indirubin on Cell Proliferation of MCF-7 Cells

The inhibitory effect of indirubin on MCF-7 cells was examined by varying concentrations of indirubin and incubation times. The cell growth of MCF-7 cells treated with a high concentration of 30 µM indirubin decreased rapidly, whereas the cell growth of cells treated with the lower concentrations of 0.1 and 3 µM did not have any significant changes within 24 h. The concentrations of 1, 10 and 30 µM indirubin decreased the cell growth of MCF-7 cells significantly (Figure 3). A high concentration of 30 µM indirubin could inhibit the cell growth of MCF-7 cells about 42% within 24 h. In contrast to the treatment of indirubin after 72 h, the number of viable cells was slightly

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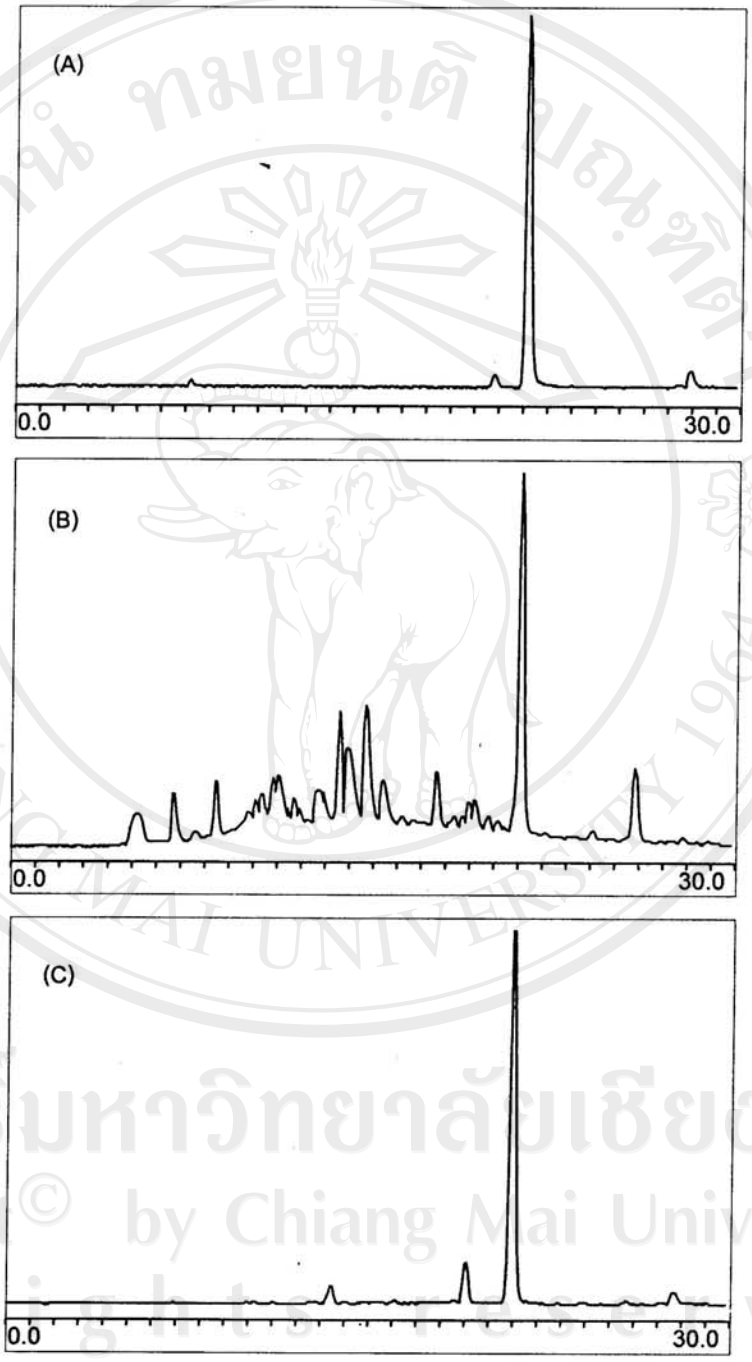

Figure 1. Purification of indirubin from *Indigofera tinctoria* Linn. by RP-HPLC. Labels: A, standard indirubin; B, crude indirubin; C, purified indirubin.

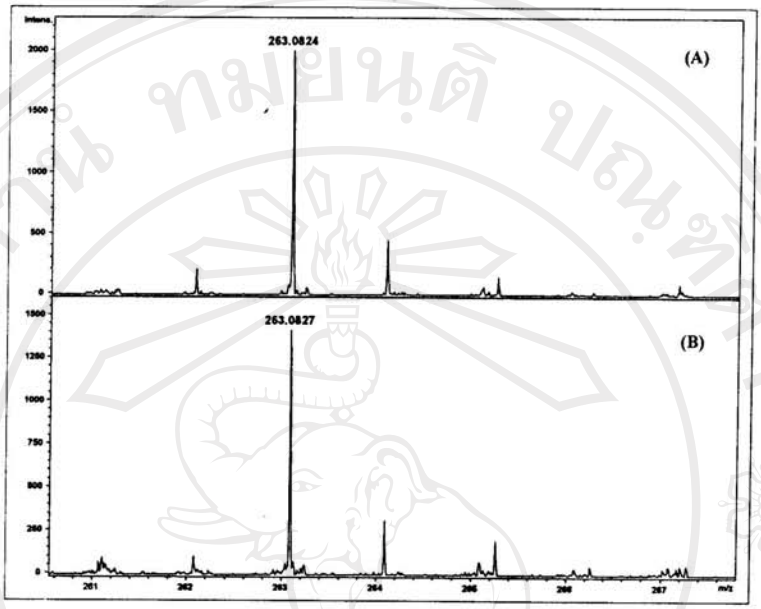

Figure 2. Mass spectra of purified indirubin and its standard. Labels: A, standard indirubin; B, pure indirubin.

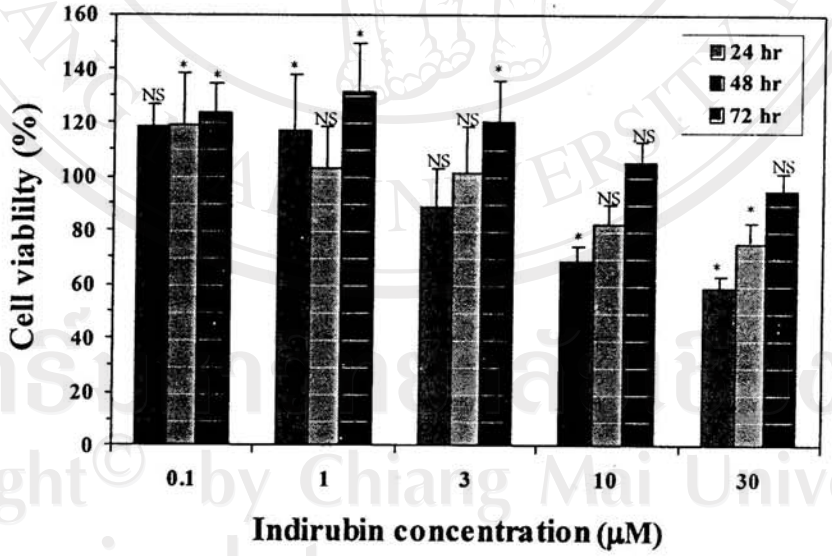

Figure 3. Cell viability of MCF-7 cells after treatment with indirubin at various concentrations (0.1, 1, 3, 10 and 30 μM). The cell number without treatment in each incubation time was used as control and set as 100% cell viability. Each bar represents mean ± S.E.M. of three experiments. Labels: NS, non significant; *, $p < 0.05$ versus control.

decreased more than the cell number at 24 and 48 h, respectively. At high concentration of 30 μM indirubin, an immediate toxic effect was possibly caused by an interference of cell membrane integrity or mitochrondrial function in the cells, and by inhibition of cell-cycle regulators, cyclin dependent kinase and glycogen synthase kinase-3β, at 5-100 nM concentrations [9]. Due to a limitation of indirubin on dissolving in methanol and DMSO, a high concentration of indirubin was shown to have co-crystallization in medium with slightly needle shape and might affect to the result of inhibitory of MCF-7 cells. Thus, we suggest that a longer incubation time and a higher concentration of indirubin could affect to the inhibitory effect of indirubin on MCF-7 cells, which caused by the degradation and crystallization of indirubin.

On the other hand, there are many reports about the limitation and problem of indirubin property, such as poor solubility, low absorption and genotoxicity [13-15]. Likewise, our study of indirubin in cell-based assay also observed the degradation and crystallization of indirubin that may be caused by the indirubin property, itself, and lead to reduce the cytotoxicity in cancer cells. The property of indirubin structure is not only the important factor in treatment, but the concentration of indirubin and the incubation times of treatment are also affected to the cytotoxicity in cancer cells. Due to its limitations, several indirubin analogs, such as 5-chloro-indirubin and indirubin-3´-monoxime, have been synthesized for better pharmacological properties and reduced toxicity [16]. In addition, some inhibitory effects of indirubin and its derivatives on proliferation of several cell lines have been studied; for example, indirubin derivatives have been used to treat chronic myelocytic leukemia and studied the antiproliferative effects [16-18]. These effects may be caused by inhibition of CDKs and GSK-3 and by interaction with the aryl hydrocarbon receptor (AhR), which could block cell proliferation of cancer cells [19-22]. It has also been reported that indirubins constituted a promising molecular scaffold from which rather selective molecules active on CDKs, GSK-3 and AhR were starting to be derived [23]. The crystallization of several indirubins with CDK2, CDK5 and GSK-3 provided a solid molecular model allowing one to pinpoint the specific interactions that contributed both to inhibitory efficacy and to kinase selectivity. This model will also be very useful in guiding the synthesis of more pharmacologically friendly indirubin with better properties, such as increased solubility, optimal cell permeability, most favorable tissue and intracellular distribution, while maintaining potency and selectivity. However, further chemical work and pharmacological evidences at molecular level are required to consider the above influence factors and to establish the possible correlation among the mentioned activities of the indirubin.

4. CONCLUSION

We successfully established a simple and effective purification method of indirubin extracted from *Indigofera tinctoria* Linn., by which only two steps of purification methos could obtain the high purity of indirubin. The purified indirubin showed a strong inhibition effect on the cell proliferation of MCF-7 breast cancer cells lines. In addition, the indirubin concentration and the incubation time were shown to be the essential factors on the cytotoxicity of indirubin on MCF-7 cells. Although the possible mechanism of indirubin on inhibition of MCF-7 cell growth is still not clear, our finding of indirubin purification method may be useful for industrial production and some preliminary data in anticancer action may be useful for

studying pharmacological characterizations against other cancer cells and further therapeutic application.

ACKNOWLEDGEMENTS

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High-Performance Liquid Chromatograghic Method for the Analysis of Indigo Dye in *Indigofera tinctoria* Linn. (Karm) and *Baphicacanthus cusia* Brem.(Hom)

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Abstract

Indigofera tinctoria Linn. (Karm) and *Baphicacanthus cusia* Brem.(Hom) are the plants which have been used as natural source of indigo dye and are available in the upper north of Thailand. The major components of the indigo dye were found to be indigo and its derivative indirubin which gave maximum absorption at 600 and 552 nm, respectively. In this work, a HPLC method has been developed for the simultaneous characterization of indigo in the extract of *I. tinctoria* L. and *B.cusia* B. The indigo dye was separated on a Nucleosil 100-7 C18 column with a CH_3OH/ 0.2% TFA as eluent and measured with UV detection at 600 nm.for indigo. With this method the amount of indigo in the extracted could be analysed compare with standard indigo. In addition, we have shown that one alternative to increase the production of indigo dye can be done by plant cell culture. The fresh leaves of Karm were cut into 0.5 x 0.5 cm pieces. Sterilized leaves were grown on Murashige and Skoog agar medium (MS) supplemented with 5 mgl^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) and 30 gl^{-1} sucrose. The plant cell culture was cultured at 25^{o}C in the dark and light conditions for 2 months. It was found that the leaf cells could produce white callus with both condition but could not produce indigo dye. In future work, we will carry out to find proper inducer for the production of indigo dye by leaf cells culture and analyse by HPLC.

Key words: Indigo, *Indigofera tinctoria* Linn., *Baphicacanthus cusia* Brem., High-performance liquid chromatograghy

Introduction

The blue dye indigo is one of the oldest natural dyestuffs known to human beings (1). Indigo has, for centuries, been obtained from a variety of plant sources such as *Indigofera tinctoria* Linn.(Karm), *Polygonum tinctorium*, *Isatis tinctoria* (Woad) and *Baphicacanthus cusia* Brem.(Hom).

Indigofera plants and *Polygonum tinctorium* contain indoxyl β-D-glucoside (plant indican) which serves as starting material for indigo blue production. Indirubin, a pinky-red pigment similar to indigo blue in structure is produced from the *P. tinctorium* cell culture (2-3). Production of indirubin and indigo-related compounds by plant cell culture are important because intact plants produce the compounds in only small amounts over a 1-2 year growth period (4).

In the present work, a qualitative and quantitative HPLC methods is described for the identification and the separation of indigo pigments and compare with standard indigo. The method will be used for analysis of indigo produced by plant cell culture.

Materials and Methods

Plant Materials Fresh materials of *Indigofera tinctoria* Linn. (Karm) and *Baphica- canthus cusia* Brem.(Hom) were collected from the upper north of Thailand. Standard indigo was obtained from Fluka.

Indigo Determination Calibration curve was done by using various amount of standard indigo, obtained by dissolving 0.010 g of standard indigo in 10 ml of Dimethyl-sulphoxide (DMSO). The solution was then diluted to different concentration with the DMSO and measured the absorbance at 600 nm.

Separation and Identification of indigo pigments and standard indigo by HPLC This method was conducted using an HPLC system from Hewlett Packard (processor, pump, UV detector and injector model 1100). Analysis were carried out with a Nucleosil 100-7C18 column (4.6x250mm, 5 μm), with a flow rate of 1.0 ml/min using CH_3OH/ 0.2% trifluoroacetic acid (TFA) as eluent. Products were detected using a UV detector at 600 nm. Pigment concentrations and yields of indigo obtained from Karm paste were quantified by HPLC analysis with reference to a standard curve of indigo dye dissolved in DMSO.

Leaf cultures of *Indigofera tinctoria* Leaves of *I.tinctoria* L. (Karm) were washed with Lipon F and surface sterilized in 10% NaOCl (dil. 1:10 with distilled water) for 15-20 min. After washing with steriled H_2O, the leaves were cut into 0.5x0.5 cm. pieces. Sterilized leaves were grown on agar in Murashige-Skoog (MS medium) with 5 mgl^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) and 30 gl^{-1} sucrose. The cultures were grown in the dark and light condition at 25^{o}C.

Results and discussion

The reported investigations were carried out in three stages. Firstly, we carried out to find the optimum condition for effective separation of indigo pigment from standard indigo by using HPLC method. Then, the extract from *I. tinctoria* Linn. (Karm) paste was analysed on a Nucleosil 100-7 C18 column with a CH_3OH/ 0.2% TFA as eluent and measured with UV detection at 600 nm.for indigo. Finally, we have shown that one alternative to increase the production of indigo pigment can be done by leaf cells culture.

Pigment evaluation Standard indigo content was determined by an external calibration and a validated linear relationship between peak area and indigo contents (concentration at 0.1, 0.3, 0.5, 0.7 and 1.0 mg/ml)(Fig 1). The concentration of indigo

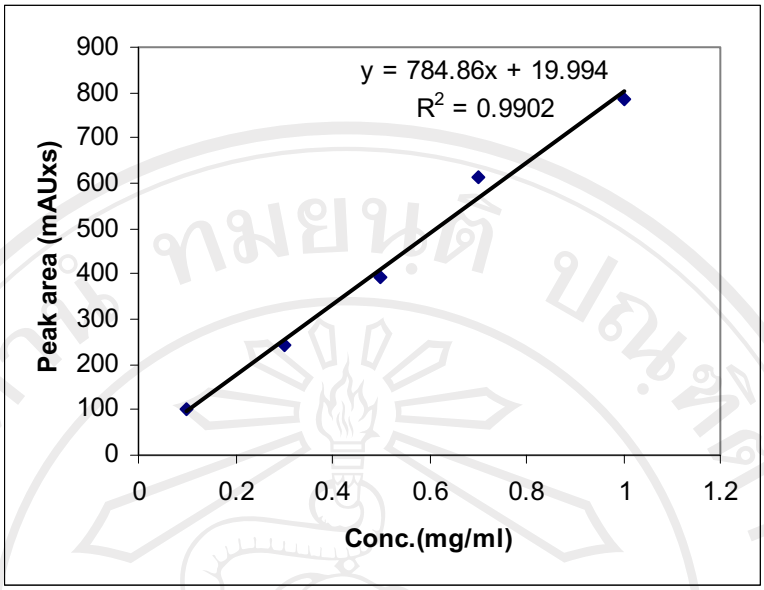

Fig. 1 Standard curve of indigo contents.

calculated from the following the regression equation : Y = 784.86X + 19.994
Y = peak area (mAUxs)
X = concentration of indigo (mg/ml)

HPLC of the extracts from Karm paste Dried and powdered Karm paste of *I.tinctoria* Linn. (0.010 g) was dissolved with 5 ml DMSO. All samples were filtered over a 0.45 μm. membrane filter and analyzed by HPLC. The UV 600 nm chromatogram was depicted in Fig.2. It can be seen that the total amount of indigo dye in Karm paste was 270 mgg^{-1} Karm paste. Chanayath et al.,(5) also found that the indigo from fresh *Indigofera tintoria* Linn.(Karm) and *Baphicacanthus cusia* Brem.(Hom) fermentation for 24 hrs gave the highest amount of indigo 3.27 and 4.72 mgg^{-1} fresh leaves, respectively. The comparision of indigo in the crude indigo paste from Karm and Hom revealed that Karm gave less indigo than Hom in the ratio of 3:4. The amounts of indigo yield were found depending on the origin and the age of plant (6).

Production of the indigo pigment from plant cell culture The seeds of *I.tinctoria* Linn. were failed to get callus on Gamborg's B_5 medium dued to the sterilized seed were grown to plant. Therefore, we chose a new approach to culture plant cells from leaves of this plant on agar medium.

The sterilized leaves were cut into 0.5x0.5 cm. pieces and grown on agar MS medium in the dark and light at 25°C. After 2 months, it was found that the leaf cells could produce white callus in both condition (Fig. 3) but could not produce indigo pigments. Kim and Lee (7) were able to increase indirubin production by 72% more than the control under optimized conditions for elicitation of *Polygonum tinctorium* cells by chitosan. In future work, we will carry out to find suitable inducer for the production of indigo by leave cell culture and analyse by this chromatographic method.

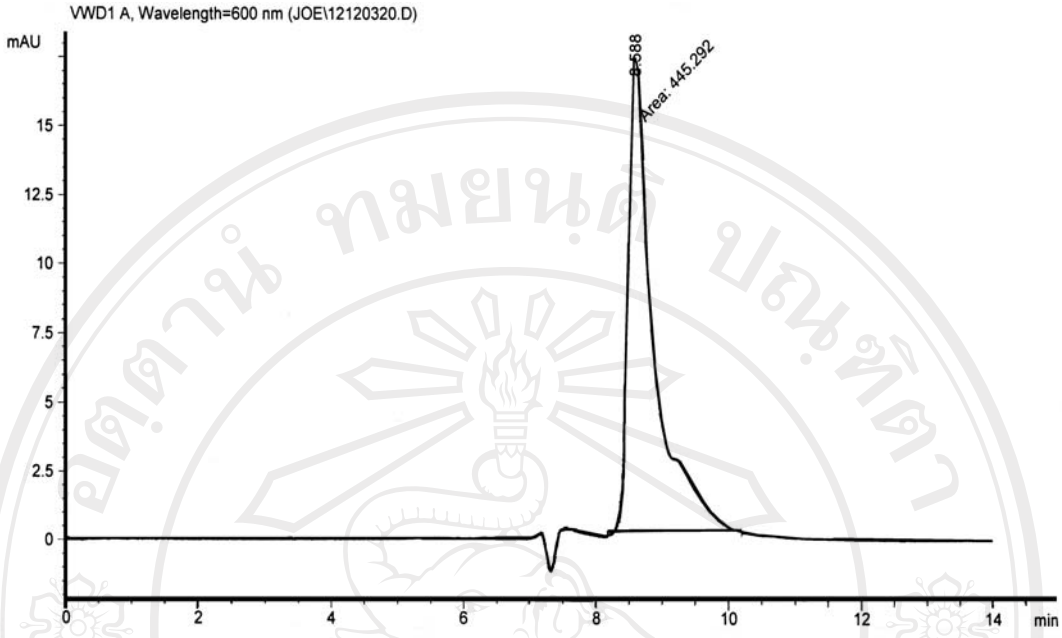

Fig. 2 HPLC analysis of Karm paste of *Indigofera tintoria* Linn.

Fig. 3 Plant cell culture from fresh leaves of *I.tinctoria* Linn. (Karm)

Conclusion

The HPLC method was developed to identify and quantify indigo pigment from Karm paste compare with indigo standard. The indigo content in Karm paste was 270 mgg^{-1} Karm paste. In addition, the leaf cells culture of *I.tinctoria* L. (Karm) could be done in both dark and light conditions. In future work, we will carry out to find proper elicitation on indigo production and analyse by HPLC method.

Acknowledgement

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Inhibitory Effect of Indirubin from *Indigofera tinctoria* Linn. on Human Breast Cancer (MCF-7) Cells

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Introduction

Indirubin and its derivatives have been reported to be the biologically actives, which are selective and potent inhibitors of cyclin-dependent kinases). CDK1/cyclin B kinase isolated from starfish oocytes was inhibited by indirubin with IC_{50} value of 10 μM. Indirubin-3'-monoxime exhibited a higher inhibitory potency with an IC_{50} value of 0.18 μM. The water-soluble derivative indirubin-5'-sulfonate was much more potent, exhibiting an IC_{50} value as low as 0.055 μM (Hoessel et al., 1999). Indirubin has also been shown to have an anti-inflammatory action by inhibiting the production of interferon-gamma, a well known inflammatory cytokine (Kunihata et al., 2000). Indirubin are also potent ATP competitive inhibitors of glycogen synthase-3 (Leclerc et al., 2001). Testing of a series of indoles and bis-indoles against GSK-3β, CDK1/cyclin B and CDK5/P25 showed that only indirubin could inhibit these kinases. Indirubin have been discovered as potent ligands of the aryl hydrocarbon receptor (AhR), also known as the dioxine receptor (Adachi et al., 2001). Indirubin act as inducers for cytochrome P450 1A1/2 mediated by AhR in mammals in vivo (Sugihara et al., 2004). Indirubin was found to reduce both the expression and production of RANTES in influenza A/NWS/33-infected H292 cells. Indirubin was one of the components with immunomodulatory activity on the expression of RANTES (Mak et al., 2004).

Indirubin, a naturally occurring pigment, was found in many indigo-producing plants, such as *Indigofera tinctoria* Linn. (Kram in Thai name), *Polygonum tinctorium* and *Isatis tinctoria* (Woad). Indirubin was a dark red isomer of the blue indigo. The objectives of this work were to establish a simple HPLC method for the separation, identification and purification of the indirubin in the pigment extracted from *I. tinctoria* (kram powder) and study the cellular response of pure indirubin with human cell lines.

Materials and methods

Chemical material

Indirubin, indirubin-3'-monoxime were obtained from ALEXIS Biochemicals. Human breast cancer epithelial cell line (MCF-7, ATCC#CRL1721) was purchased from ATCC. DMEM and MEM Sodium pyruvate were purchased from Atlanta Biologies, GA, USA. Antibiotic-Antimycocytic was obtained from biowest, Sodium bicarbonate from Sigma-aldrich, St Louis, MO, USA. and Fetal bovine serum from Gibco BRL, Grand Island, NY,USA

Preparation of kram powder

Fresh plant materials of *Indigofera tinctoria* Linn. were cut to small pieses and put into cotton bag before soaking in water for 24 h. The bag was taken off and then added twice in volume of Ca $(OH)_2$ solution (pH ~11), blew the air for 15 mins to precipitate pigments. The upper solution were discarded and the kram paste was passed through drum dryer to get kram powder.

Extraction of indirubin from kram powder

I. tinctoria L. (Kram in Thai name) powder was suspended in methanol. After filtration, methanol was evaporated under reduced pressure. The residue was dissolved in small volume of methanol and analysed by TLC.

TLC analysis

Crude indirubin were dissolved in methanol and spotted on silica gel 60 F plates (Merck). Elution was carried out with the mixture : dichloromethane : hexane : methanol (7:4:0.3, v/v/v). The results were compared with indirubin standard.

Separation of crude indirubin from methanol extraction by column chromatography

A glass column 54x2.5 cm containing 120 g of silica gel 60 was used to separate the crude indirubin and eluted with dichloromethane : hexane : methanol (7:4:0.3, v/v/v). The eluates were concentrated and the purity of indirubin was then checked by TLC and HPLC.

Purification and identification of indirubin by HPLC

Purified indirubin were performed using a reverse-phase HPLC column (9.4x250 mm, ZORBAX ODS) attached to a L-4250 UV-VIS detector and an L-6200A pump (all from Hitachi, Tokyo, Japan) with a 2 mL sample injection loop. Two mobile phases, A (0.1% v/v TFA, 5% v/v ACN) and B (0.1% v/v TFA, 95% v/v ACN), were used for all samples. All mobile phases were filtered (45 μm filter for organic solvent; Millipore) and degassed by sonication (Bransonic 52; Branson, Shelton, CT, USA). All solvents were HPLC grade. Purified indirubin from silica gel column chromatography were dissolved with methanol, filtered and injected into the HPLC column with 400 μL. The gradient elution profile was : 0-5-30 min / 20-50-100 %B respectively at a flow rate of 2 mL/min. Product was monitored by measuring UV absorption at 552 nm.

Cell cultures

Human breast cancer epithelial cell line was cultured in monolayer in DMEM medium supplemented with 10% fetal bovine serum, 1.5 g/L sodium bicarbonate , 0.1 mM MEM sodium pyruvate, streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL). The cancer cell lines were incubated at 37^{o}C under a 5% CO_2 atmosphere.

Cell based assay by treatment with indirubin and cell viability test

MCF-7 cells were seeded with $5x10^4$ cells in 96 well plates and were attached on well overnight before treatment. Cells were treated with pure indirubin with the different concentrations of 0.1, 1, 3, 10 and 30 μM. The change of cell morphology after indirubin treatments was visualized by microscopy with 40-fold magnitude. The cell proliferation of cell lines was determined by using MTT assay methods. This assay was based on the cellular cleavage of the tetrazolium salt, MTT, into a formazan that is soluble in cell culture medium and was measured at 570 and 630 nm directly in 96-well assay plates using microplate reader. Absorbance was directly proportional to the number of living cells in culture.

Results and discussion

***Purification and identification of indirubin from Indigofera tinctoria* Linn.**

In the previous study, the separation of kram powder by TLC using chloroform-hexane-methanol (7:4:1 v/v/v) as developing solvent gave two major components of blue and red colour. Both blue and red pigments were highly soluble in chloroform but only red pigment was soluble in methanol. The red pigment could be separated from blue pigment by dissolving the kram powder in methanol (Chanayath et al., 2002). The same method has been used for extraction of indirubin from kram powder.

Dried Kram powder were extracted with methanol. After filtration, the precipitate were extracted with methanol for 6 times. The red dye were pooled and evaporated to dryness. The crude indirubin were then separated using silica gel $60F_{254}$ column and fractions of red pigment were pooled and evaporated to dryness. The crude extract and partially purified indirubin were analysed by HPLC compared with standard indirubin. It was found that crude indirubin had a lot of impurities and the partially purified indirubin from the column still had

trace of some impurity (Fig.1). Therefore, the partially purified indirubin were further purified by preparative HPLC. The major peak obtained from HPLC were collected. The structural elucidation of isolated compounds was carried out by MS spectrometry.

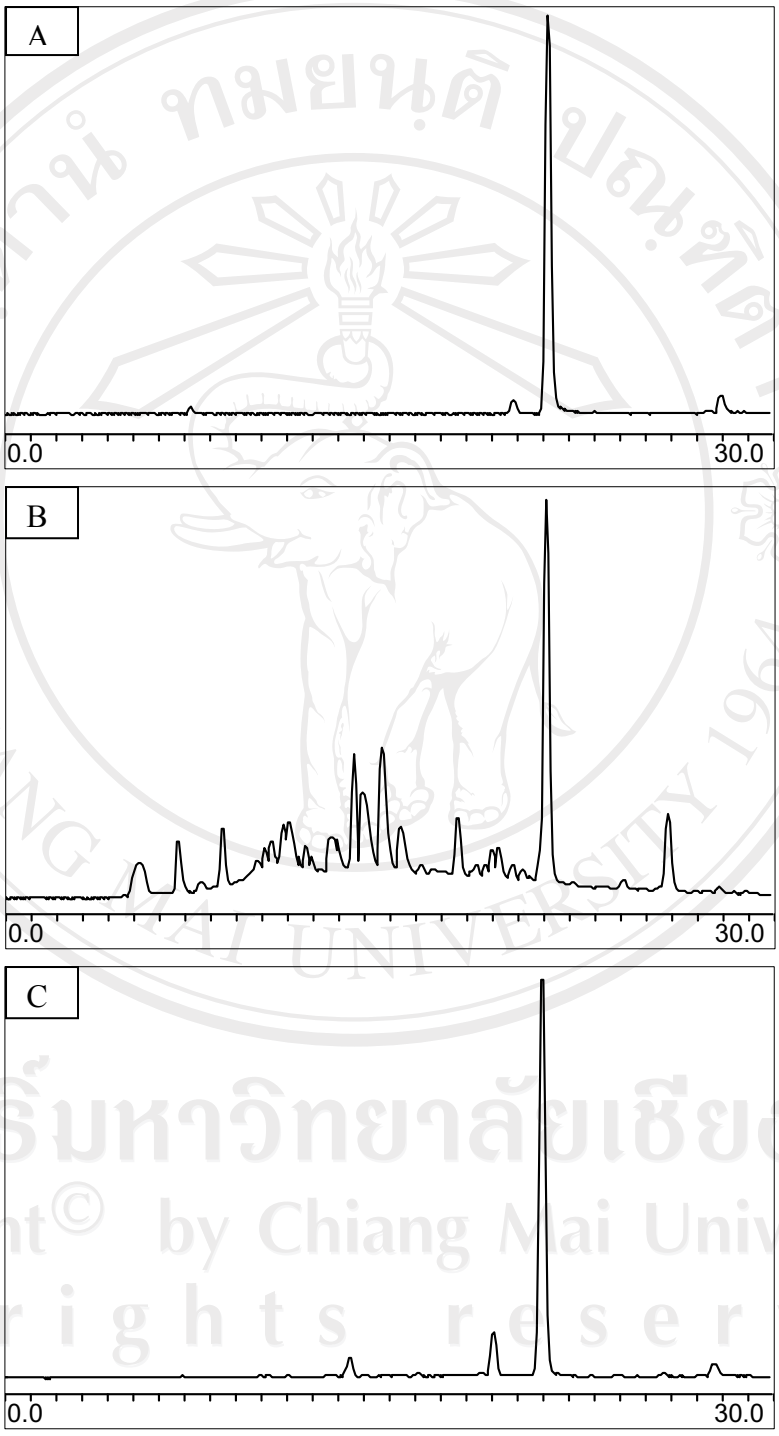

Fig. 1 HPLC chromatograms of (A) standard indirubin, (B) crude indirubin and (C) pure indirubin.

The mass spectra obtained for chromatographic peak contains a strong signal at m/z 263 $[M+H]^+$ related to standard indirubin (Fig.2). The result corresponded to the work of Chanayath et al., 2002 who reported that the red pigment extracted from *I. tinctoria* (kram) was purified and analysed by UV-visible spectroscopy, mass, ^{1}H, ^{13}C-NMR spectrometry and showed that it had chemical structure as indirubin.

Effect of pure indirubin on cell proliferation

The effect of indirubin on MCF-7 cell lines was studied by measuring cell numbers by Coulter counting and relative numbers of viable cells using MTT assay after treatment of cancer cell lines with pure indirubin for various time periods. The ability of pure indirubin to induce cell death was estimated by analyzing its effect on cell morphology (Fig.3). The observation of MCF-7 cells under a microscope showed that at a high concentration (30 µM) the number of viable cells decreased rapidly whereas, at lower concentrations (0.1, 1 µM), the cell numbers started to decrease markedly within 24 h (Fig.4). In contrast, the treatment of indirubin after 72 h showed that the number of viable cells decreased slightly more than the treated cells after 48 and 24 h, respectively.

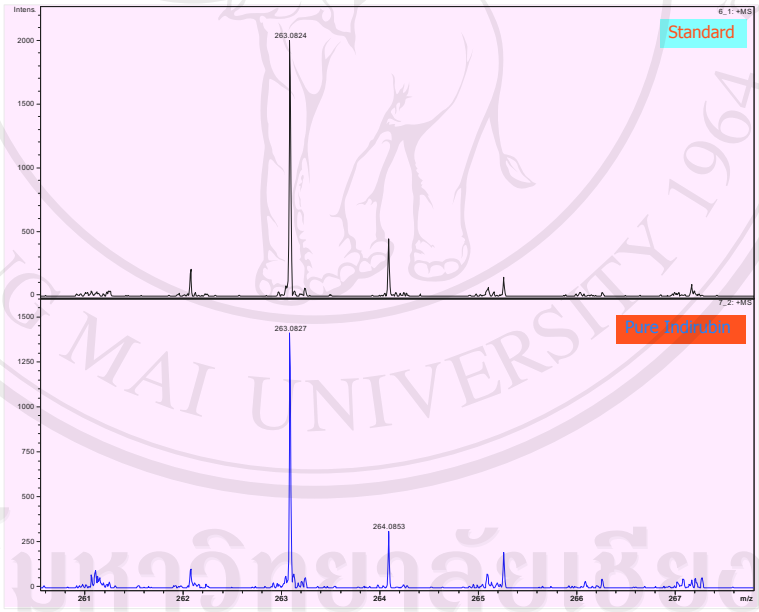

Fig. 2 Mass spectra of pure indirubin.

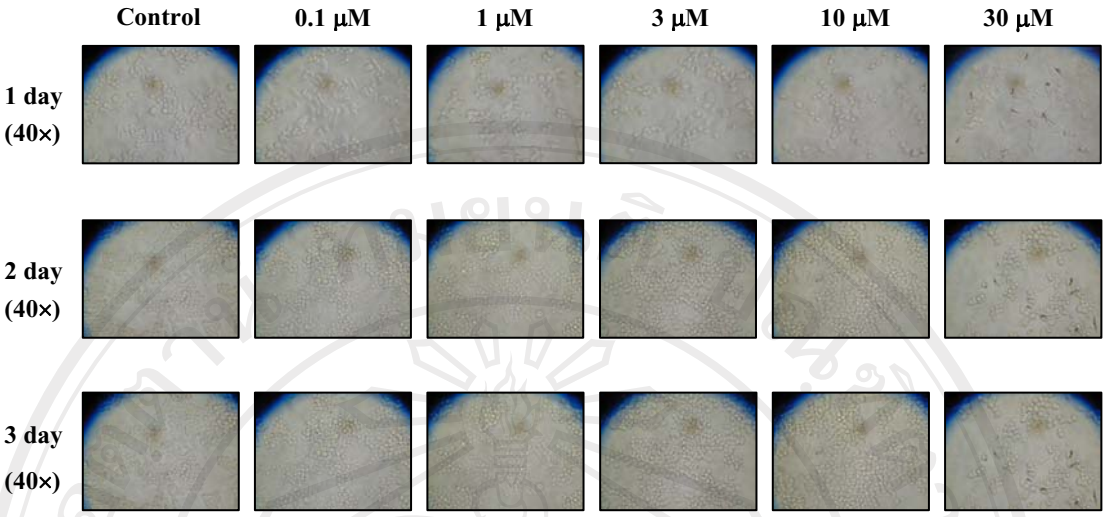

Fig. 3 Cell morphology of MCF-7 cells after treatment with different concentrations of indirubin for 24, 48 and 72 h.

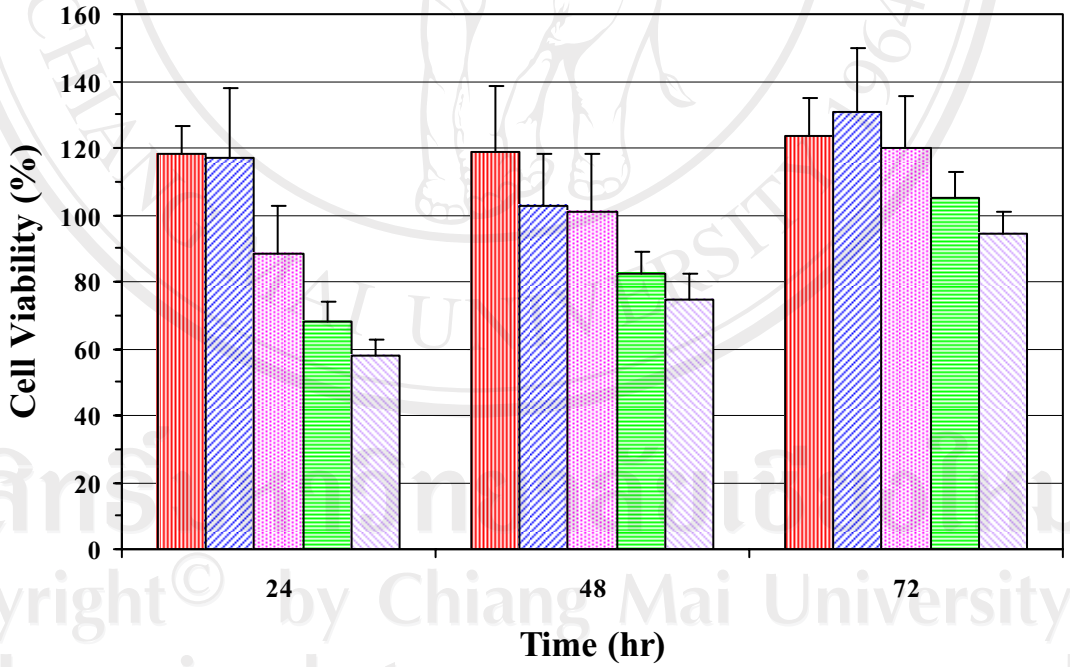

Fig. 4 Effect of cell viability and time after treated indirubin as various concentrations (0.1µM, 1µM, 3µM, 10µM, and 30µM).

The inhibitory effect of indirubin on MCF-7 cells showed that 30 µM indirubin could inhibit the MCF-7 cells about 42% inhibition within 24 h (Fig. 4). Longer time of treatment (incubation time) and high concentration of indirubin could influent to the degradation of indirubin including crystallization. At high concentration (30 µM) indirubin had an immediate toxic effect possibly caused by an interference of cell membrane integrity or mitochrondrial

function in the cells. Due to the limitation of indirubin on dissolving in methanol and DMSO, at high concentration of indirubin showed to have co-crystallization in medium with slightly needle shape and might affect to the result of inhibitory of MCF-7 cells. Marko et al. (2001) reported that indirubin was shown to inhibit proliferation of several cell lines, including MCF-7 , at micromolar concentrations. It is worth noting that several derivatives of indirubin have been shown to directly inhibit the cell-cycle regulators, cyclin dependent kinase and glycogen synthase kinase-3β, at 5-100 nM concentrations (Leclerc et al. 2001). Furthermore, Mak et al. (2004) have recently reported that indirubin reduced both the expression and production of RANTES in influenza A/NWS/33-infected H 292 cells with a dose-dependent 50-200 μM.

Conclusion

The RP-HPLC and MS studies of a methanol extract of *indigofera tinctoria* Linn. indicated indirubin as the major compound. The influence of purified indirubin has been tested on the growth of a human breast cancer cell lines, MCF-7. The result showed that at the concentration of 30 μM, indirubin inhibited cell growth about 42% within 24 h.

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II-P-18

ANTIBACTERIAL ACTIVITY OF METHANOL EXTRACTS FROM *COSCINIUM FENESTRATUM* (GAERTN) COLEBR.

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The objective of this research was to study the antibacterial activity of *Coscinium fenestratum* (Gaertn) Colebr. Menispermaceae. The methanol extract was used in the antibacterial test by disk diffusion method. Eight kinds of bacteria including *Escherichia coli, Enterococcus faecalis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Staphylococcus epidermidis* and *Vibrio cholerae* were used. It was found that the methanol extracts from *Coscinium fenestratum* (Gaertn) Colebr. Menispermaceae gave high antibacterial activity against *S. epidemidis*, *S. aureus*, *E. faecalis* and *E. coli*, respectively. The major component of the methanol extracts from *C. fenestratum* separated by thin layer chromatography (TLC) was tested with Dragendorff's and Mayer's reagent as alkaloid. Results from the antibacterial tests of the major component against *S. epidemidis* demonstrated that the minimum inhibitory concentrations (MIC) of the methanol extract was 300 µg/mL.

Keywords: *Coscinium fenestratum*, antibacterial activity, disc diffusion method, *Staphylococcus epidermidis*

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Introduction : *Coscinium fenestratum* (Gaertn) Colebr., belonging to the family Menispermaceae is also known in Thai common name as Ham. The stem of this menispermaceous plant has long been used in Ceylon and Southern India as a yellow dye and bitter tonic. The stem yields a yellow dye which is used either alone or in combination with turmeric and other colouring materials. The root of *C. fenestratum* contains alkaloids berlambine, dihydroberlambine, 12,13-dihydro-8-oxo berberine, tetrahydroberberine, oxyberberine and noorxy hydrastinine (Datta et al, 1988; Malhotra et al, 1989). It has been reported that the stem extract was used for the treatment of diabetes mellitus, fever reduction, suppression of inflammation, anticancer, antidiarrhea and antibacterial (Shirwaikar et al, 2005). With this in mind, the present study aimed to investigate the antibacterial activity of methanol extracts from the stem bark of *C. fenestratum.*

Methods: Dried powder from stem of *C. fenestratum* was extracted by soxhlet extraction with methanol for 72 h. The methanol extract was filtered and evaporated to dryness. This sample was used for subsequent studies. The methanolic extract was subjected to TLC analysis. Silica gel plates spotted with crude extract and developed with dichloromethane-methanol (8:2). The fluorescent band which detected under UV lamp were collected. The antibacterial activity of the extracts from *C. fenestratum* stem bark was determined by

i) Disk Diffusion Technique : To screen the antibacterial activity, Whatman's filter paper No. 1 disks of 6 mm diameter were used. Each disk containing 0.25-2 mg of the methanolic extract was placed on a lawn culture of battery of microorganisms. The plates were incubated at 37°C overnight and examined for zones of inhibition.

ii) Determination of Minimum Inhibitory Concentrations (MIC) : Stock solution of the methanol extract (1000 µg/mL) was added to Nutrient Agar (NA) medium in the a sterile Petri disk and mixed well. The final concentrations of the plant extract ranged from 1.6-1000 µg/mL at 12 different dilutions. The *Staphylococcus epidermidis* was grown in Nutrient Broth (NB) medium at 37°C for 24 h. Each Petri disk was added with 100 µL of the tested bacterium which spread well and incubated overnight. One control plate was also incubated without incorporation of any plant preparation. The MIC of the respective

preparation was the lowest concentration in the medium that completely inhibited the visible growth.

Results and Discussion : Crude methanolic extract of *C. fenestratum* stem bark exhibited a zone of inhibition by disk diffusion method against 4 out of 8 microorganism tested. The Gram-positive bacteria *S. epidermidis* and *S. aureus* were observed to be sensitive to the extract at the concentration of 25-200 mg/mL and *E. faecalis* was also observed at 150-200 mg/mL. *S. epidemidis*, however, had a considerably large clear zone of inhibition (2.71 cm). Among the Gram-negative bacteria, *E. coli* exhibited the lowest zone of inhibition 0.67 cm in diameter (Table1). The antibacterial test showed that the MIC was 300 µg/mL for *S. epidemidis* (Table2). However, *E. faecalis* was found to be inhibited by alcoholic extract from stem bark of *Berberis asiatica* (MIC 9.76 µg/mL). In addition, the alcoholic extract from stem bark of *B. asiatica* was found to be the best for the lone fungal test organism, i.e., *Candida albicans* (MIC 9.76 µg/mL) (Bhandari et al, 2000).

Table 1. Antibacterial activity of methanol extract from *C. fenestratum* at different concentrations (mg/mL). Each assay was done in three replicates.

Bacterias	Clear zone (cm)				
	25	50	100	150	200
S.epidemidis	1.64± 0.04	2.10± 0.08	2.29± 0.06	2.58± 0.01	2.71± 0.01
S.aureus	0.78± 0.10	1.17± 0.03	1.50± 0.09	1.64± 0.12	1.91± 0.08
E.faecalis	-	-	-	0.66± 0.04	0.72± 0.05
E.coli	-	-	-	0.64± 0.03	0.67± 0.03
K.pneumoniae	-	-	-	-	-
Ps.aeruginosa	-	-	-	-	-
S.typhi	-	-	-	-	-
V.cholerae	-	-	-	-	-

- : Negative

Table 2. Effect of methanol extract from *C. fenestratum* at different concentrations on *S. epidermidis* growth.

Crude extract (µg/mL)	Growth of *S. epidermidis*
1000	-
800	-
600	-
500	-
400	-
350	-
300	-
250	+
200	+
40	+
8	+
1.6	+
0	+

+ : Sensitive
- : Resistant

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A HPLC Method to Quantify Indican from Callus of *Indigofera tinctoria* Linn. (Kharm)

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Abstract

A method to quantify the indigo precursor indican (indoxyl-β-D-glucoside) from callus of *Indigofera tinctoria* Linn. (Kharm) leaf has been developed. Callus was extracted in methanol and indican was identified and quantified using high performance liquid chromatography (HPLC). The indican was separated on a Nucleosil 100-7 C18 column with a methanol containing 0.2% trifluoroacetic acid (TFA) as eluent and measured with UV detection at 280 nm. The callus was prepared from the fresh leaves of Kharm which were cut into 0.5 x 0.5 cm pieces. Sterilized leaves were grown on modified Murashige and Skoog agar medium (MMS) supplemented with 4 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin, 125 mgl^{-1} tyrosine and 30 gl^{-1} sucrose. The plant cell was cultured at 25°C in the dark condition for three weeks. Using the HPLC technique, callus indican content was quantified by comparing with standard indican. Results showed that with this method it is possible to measure indican content in a short time. Indican is naturally hydrolyzed by native β-glucosidase to glucose and indoxyl which will change to indigo.

Introduction

Indigo is considered to be the oldest dye with uses known in acient times. The dye was generally extracted from various species of plants such as *Indigofera tinctoria* Linn.(Kharm), *Polygonum tinctorium*, *Isatis tinctoria* (Woad) and *Baphicacanthus cusia* Brem.(Hom) [1]. *Indigofera* plants contain indican (indoxyl β-D-glucoside) which is a precursor of the blue dye indigo and is present in large amounts in *I. tinctoria* L. leaves. When leaves are broken, indican is hydrolyzed to indoxyl and glucose by the native β-glucosidase located in chloroplasts of mesophylls cells [2].

In previous studies we have shown that the leaf cells culture of *Indigofera tinctoria* Linn. (Kharm) could produce callus when cultured on modified Murashige and Skoog (MMS) medium at 25°C for three weeks.

Due to HPLC has become an important method for rapid identification of compounds in plant extracts and small amount can be use. Thus, HPLC remains the method of choice for quantitative analysis of indigo precursors. In the present work, a HPLC method was studied to identify and quantify the indican in *I. tinctoria* Linn.(Kharm) in comparison with standard indican. The method will be used to follow the callus indican content in the optimization of indican production from Kharm leaf cells culture.

Materials and Methods

Callus Induction

Separation and Identification of Indican by HPLC

Results and Discussion

In the production of indican from leaf cells culture, the sterilized leaves of *I.tinctoria* Linn. (Kharm) were cut into 0.5 x 0.5 cm and grown on modified Murashige and Skoog (MMS) medium agar [3] in the dark at 25°C. After three weeks, it was found that the leaf cells could produce white callus (2.1821 grams wet weight per 10 ml culture medium).

For extraction and isolation of the indican, the leaf cells culture (2.1821 g) was suspended in 33 ml of methanol and sonicated in a Branson Sonifer 450 for 30 min. After filtration, the methanol was evaporated under reduced pressure. The residue was dissolved in 1 ml of methanol and 0.3 µl of the extract was analyzed by HPLC.

There were two sharp peaks in HPLC profile and the indican peak was the second one with a retention time (t_R) of 2.9 min. The peak at t_R 2.5 was not identified (Fig.1). The indican content of callus culture was 0.1070 mg/g of fresh weight of callus. Results showed that this method measured the indican content in a short time and small amount of the sample could be detected. The indigo precursors from *Polygonum tinctorium* had been identified by HPLC and it was found that the indican peak showed the t_R at 7.6 min [1] with different condition, which used the longer time for identification of indican peak. Since Marero *et al.* [4] reported that the indigo plant callus had another red pigment called indirubin which would be produced from indoxyl in a process similar to that of indigo. Further characterization of the peak at t_R 2.5 min would be achieved by HPLC analyse, using purified indirubin as standard.

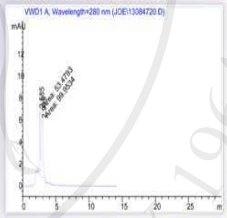

Fig.1 HPLC analysis of callus culture from *I.tinctoria* L. leaves.

Conclusions

A HPLC method to determine indican content in *I.tinctoria* Linn.(Kharm) callus culture was performed. Indigo precursor content was 0.1070 mg/g of wet weight by comparison with standard indican. Results confirmed that with this method it is possible to measure indican content in a short time and small amount of sample could be identified. By this method the indican content will be able to follow in the optimized production of indican by the leaf cells culture.

Acknowledgement

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Poster Presentation

1. February 3-6, 2004 **"High-performance liquid chromatographic method for the analysis of indigo dye in *Indigofera tinctoria* Linn. (Karm) and *Baphicacanthus cusia Brem*. (Hom)"** The 15th Annual Meeting of the Thai Society for Biotechnology Sustainable Development of SMEs Through Biotechnology and The JSPS-NRCT Symposium on The Forefront of Bioinformatics Application, Pang Suan Kaew Hotel, Chiang Mai, Thailand.
2. November 22-26, 2004 **"A HPLC method to quantify indican from *Indigofera tinctoria* Linn. (KHARM)"** The 17th FAOBMB Symposium/ 2nd IUBMB Special Meeting/ 7th A-IMBN Conference on "Genomics and Health in the 21st Century" Bangkok, Thailand.

3. November 2-3, 2006 **"Antibacterial activity of metranol extracts from *Coscinium fenestratum* (Gaertn) Colebr"** The 18th Annual meeting of the Thai Society for Biotechnology "Biotechnology : Benefits & Bioethics" The Montien Riverside Hotel, Bangkok, Thailand.

Oral Presentation

November 2-5, 2005 **"Inhibitory effect of indirubin from *Indigofera tinctoria* Linn. on human breast cancer (MCF-7) cells"** BioThailand 2005 "Challenges in the 21st Century", Queen Sirikit National Convention Center, Bangkok, Thailand.

Publication

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Scholarship

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