Chiang Mai J. Sci. 2007; 34(3) : 329-337
www.science.cmu.ac.th/journal-science/josci.html
Contributed Paper

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

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Received: 20 November 2006
Accepted: 25 June 2007.

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.

Keywords: *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 dichloromethane: 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 μl. 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 ^{1}H, ^{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

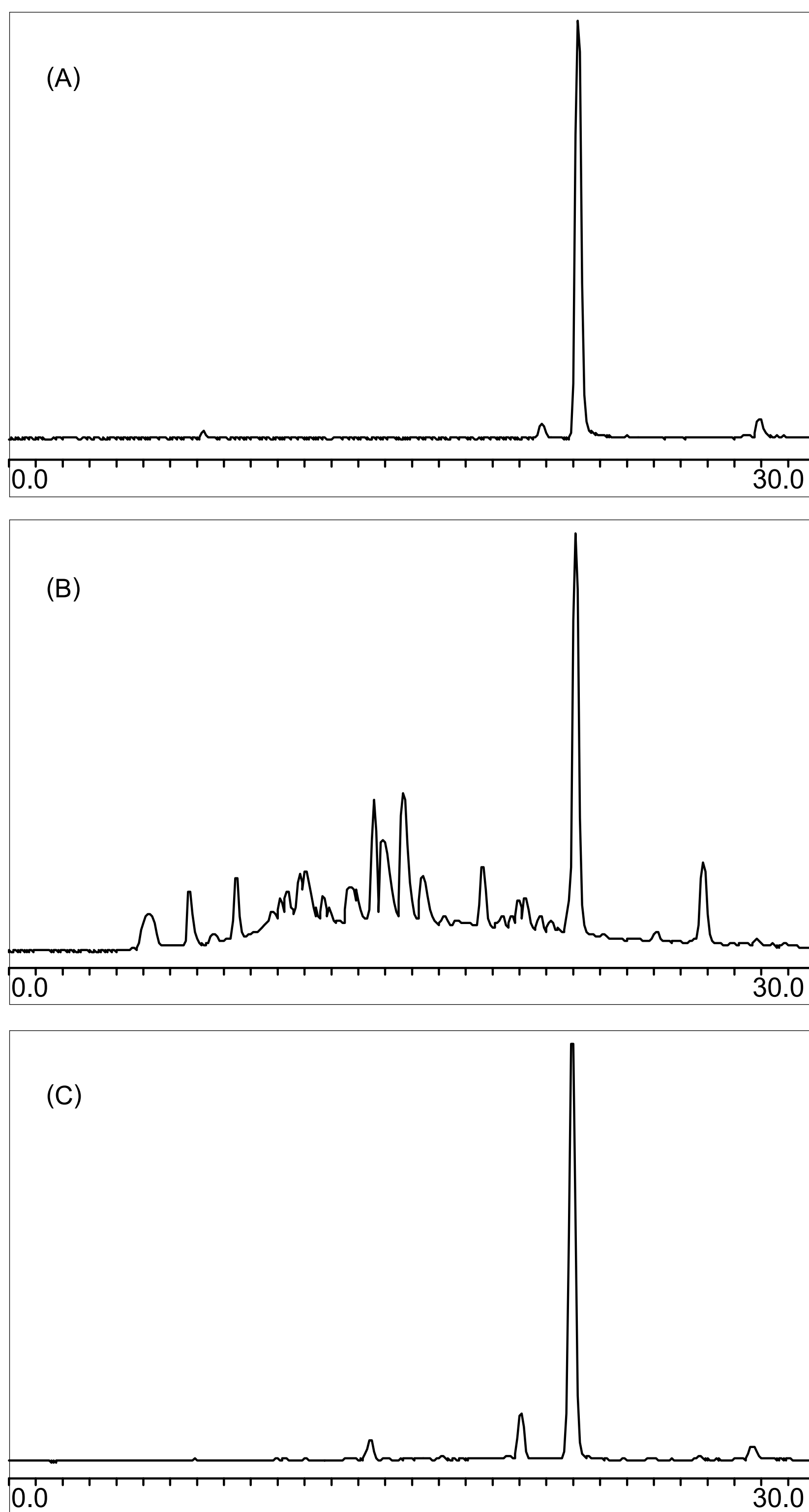

Figure 1. Purification of indirubin from *Indigofera tinctoria* Linn. by RP-HPLC. Labels: A, standard indirubin; B, crude indirubin; C, purified indirubin.

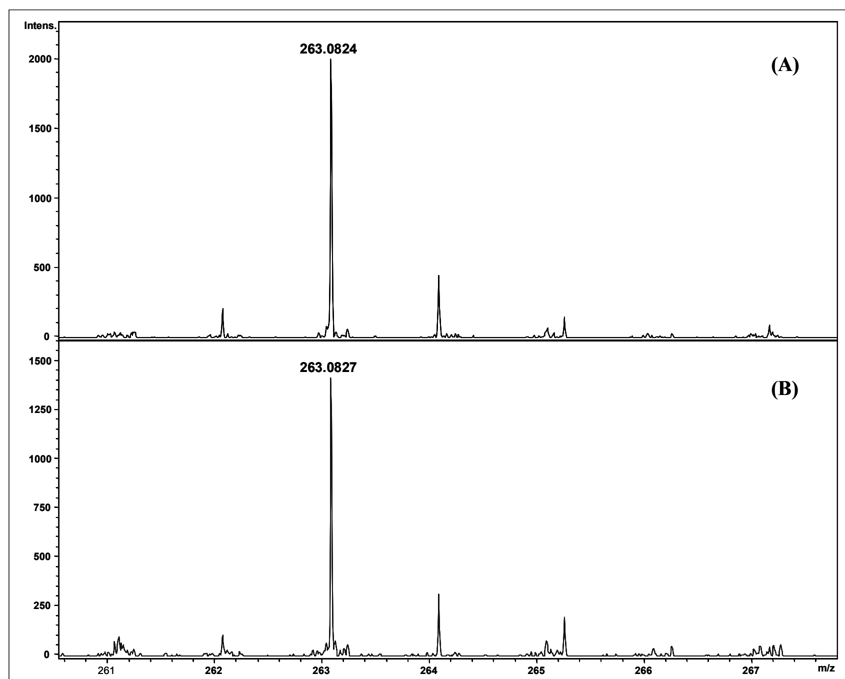

Figure 2. Mass spectra of purified indirubin and its standard. Labels: A, standard indirubin; B, pure indirubin.

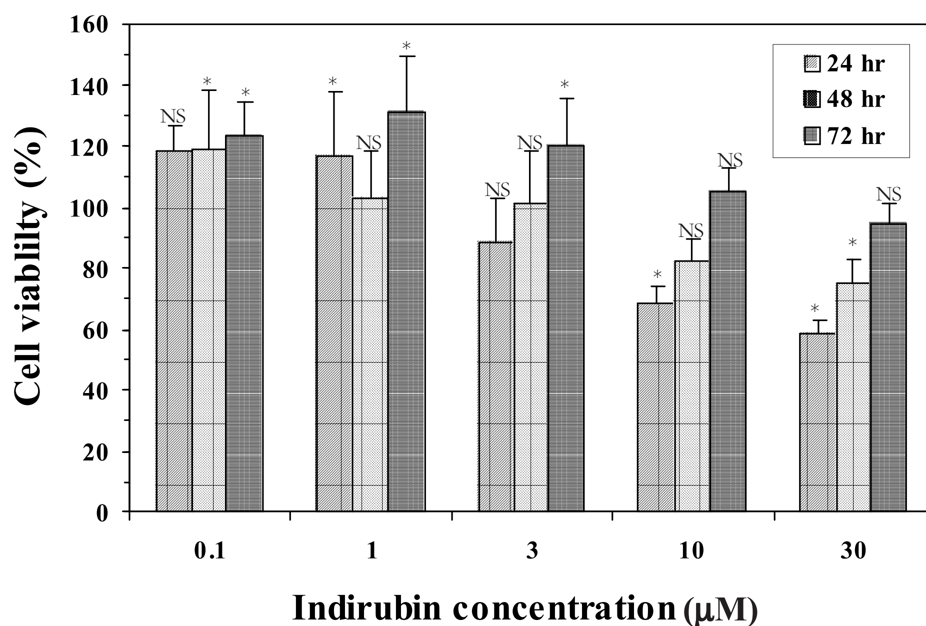

Figure 3. Cell viability of MCF-7 cells after treatment with indirubin at various concentrations (0.1, 1, 3, 10 and 30 μM). The cell number without treatment in each incubation time was used as control and set as 100% cell viability. Each bar represents mean ± S.E.M. of three experiments. Labels: NS,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

We gratefully acknowledge the financial support from the Systems Biology Laboratory, Institute of Biological Chemistry, Academia Sinica, Taiwan and Nanotechnology Pilot Plant Project of Faculty of Science, Chiang Mai University, Thailand.

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