Fungal Infection Enhances Anthocyanin Production in Blue Flowers of *Clitoria ternatea*

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\textbf{Authors’ contributions}

This work was carried out in collaboration between all authors. Author AP designed the chemical study, searched related literatures, analyze statistical data and wrote the manuscript. Author SB designed and evaluated the microbiological study and wrote the manuscript. Author NY managed the microbiological study and related literature searches. Author BK managed the chemical study and related literature searches. All authors read and approved the final manuscript.

\textbf{ABSTRACT}

\textbf{Aims:} A white fungus which commonly infects blue petals of *Clitoria ternatea* L. (CT) was identified and total anthocyanin content and antioxidant activity of CT extracts from infected and uninfected CT were compared.

\textbf{Study Design:} Experimental research.

\textbf{Place and Duration of Study:} Samples of infected and non-infected CT were collected from Muang, Khon Kaen and chemical analysis was undertaken at Khon Kaen University between July and December 2012. Fungus was identified at Department of Microbiology in April 2013.

\textbf{Methodology:} Samples of dried CT, with and without white fungus infection, were collected. The CT-infected fungus was isolated for microscopic characterization and DNA isolated for amplifying of its ITS rDNA, and analyzing their sequences with neighbor joining phylogenetic tree. Total anthocyanin content by pH differential method and antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay were used to compare infected and non-infected CT extracts in comparison to trolox. Both extracts
were analyzed by HPLC in comparison to cyanidin.

**Results:** Morphology and DNA of the infected fungus was characterized and confirmed to be *Penicillium citrinum* KKS-Rx1. In comparison to aqueous extracts of non-infected CT, infected CT gave about 1.5 times higher total anthocyanin content (*P*<.002) and cyanidin (*P* = .009). Trolox equivalent antioxidant capacity of infected-CT extracts was 1, while that of non-infected CT extracts was 0.7 (*P*<.001).

**Conclusion:** *Penicillium citrinum* was identified from white fungus infection on deep blue flowers of *Clitoria ternatea* and shown to enhance the anthocyanin content and antioxidant activity of the CT extracts.

**Keywords:** Fungal infection; anthocyanin; cyanidin; *Clitoria ternatea*; *Penicillium citrinum*.

**ABBREVIATIONS**

CT - *Clitoria ternatea* L.; HPLC - High performance liquid chromatography; DPPH - 1, 1-diphenyl-2-picrylhydrazyl radical.

**1. INTRODUCTION**

*Clitoria ternatea* Linns. (CT) is commonly known as butterfly pea. It is widely distributed in Thailand, and elsewhere in tropical Asia. *C. ternatea* is well-known in traditional medicine [1] and anthocyanins are the main chemicals of interest [2]. Anthocyanins are the largest group of water-soluble pigments in the plant kingdom. Anthocyanins are particularly abundant in berries and other fruits with red, blue, or purple color, and in red wines [3] and are responsible for most of the red, blue, and purple colors of fruits, vegetables, flowers, and other plant tissues. Cyanidin has been reported to be a pharmacologically active anthocyanin with anti-oxidant [3-4], anti-microbial [4] and anti-inflammatory activity [5-7]. Anthocyanin production can be enhanced by specific fungal elicitors [8].

This study aims to identify a white fungus which infects blue flowers of CT and to compare total anthocyanin and cyanidin content, and antioxidant activity, of the extract from both infected and non-infected CT.

**2. MATERIALS AND METHODS**

Blue petals of CT flowers, with and without natural fungal infection, were collected from Muang, Khon Kaen, in August 2012. The petals were isolated, dried and ground to produce CT powder. About 50 mg of each of the CT powder samples was thoroughly mixed with 100 ml of water at 50°C for 3h. The supernatant was separated for immediate analysis.

**2.1 Quantitative Analysis**

**2.1.1 Total anthocyanins**

Total anthocyanin content of CT extracts was analyzed by pH differential method (AOAC method 2005.2) [9-10]. In brief, the extract was diluted with 25mM potassium chloride buffer at pH 1.0 and 0.4M acetate buffer at pH 4.5, and the absorbance of each extract was recorded (at 520 nm and 700 nm). The total anthocyanin content was calculated as follows:


\[
\text{total anthocyanin content (mg/L)} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times L}
\]

Where \( A = (A_{520} - A_{700})_{\text{pH}1.0} - (A_{520} - A_{700})_{\text{pH}4.5} \), \( A_{520} \) and \( A_{700} \) = absorbance at 520 and 700 nm, respectively, \( MW = \) molecular weight of 449.2 g/mol for cyanidin-3-glucoside, \( DF = \) dilution factor, \( \varepsilon = 26,900 \) L/mol for molar extinction coefficient of cyaniding-3-glucoside, \( L = \) path length in cm, and 1000 = conversion factor from g to mg.

2.1.2 Cyanidin

HPLC was used to determine the cyanidin content of CT extracts. The extracts were diluted with water at appropriate dilution before injection onto a C-18 column (HiQ Sil, 5μm, 250 × 4.6 mm, U.S.A.) with the column temperature controlled at 40°C. A gradient flow of the mobile phase of 0%, 12% to 100% of part A (0.1% formic acid in 50% acetonitrile) and 100%, 88% to 0% of part B (0.1% formic acid in water) at 10, 20 and 29 min were used [11-13]. The chromatograms were recorded at 520nm using diode array detector software (Agilent, U.S.A.). Standard graphs of cyanidin (Sigma-Aldrich, U.S.A.) were used to determine the cyanadin content of the extracts.

2.1.3 Anti-oxidative activity

An anti-oxidative activity test with 2,2-diphenyl-1-picrylhydrazyl radical (DPPH, Sigma, U.S.A.) [14] was used to compare the extracts from fungal infected and non-infected CT. Fresh CT extracts in ethanol (about 0.1 mg/ml) were sonicated and serially diluted, then reacted with 0.1 mM DPPH in methanol for 30 min in the dark. The absorbance at 517nm of each extract was recorded with a microplate reader (Biorad, Japan) and calculated for anti-oxidant activity and IC\(_{50}\), using 2mM Trolox (Sigma, U.S.A.) as the standard.

2.2 Microbial Assay

2.2.1 Sample preparation

The CT-infected fungi were isolated and identified. Dried samples of the infected CT were mixed with sterile distilled water to make a ten-fold serial dilution, i.e. \(10^{-1} - 10^{-5}\). 0.1ml of each diluent was spread on potato dextrose agar (PDA) containing 100 ppm of oxytetracycline (ANB Laboratories, Thailand) then incubated at 25°C for 3-7 days. After that, a colony of the fungi grown on the PDA was sampled for isolation by the cross streak technique. Samples of single isolated colonies, which represented the majority, were cultured in PDA slant and designated as strain KKU-Rx1 for identification.

2.2.2 Morphology

Colonies of the strain KKU-Rx1 were morphologically observed after the isolates were cultured on Czapek’s agar medium containing 30% sucrose (Himedia Laboratories, India). Slide culture was carried out to observe micromorphological characteristics such as conidia, phialides, vesicles, asci and ascospores under a bright field microscope (Nikon Eclipse 50i, Japan), as previously described for manual identification of fungi [9-13]. Analysis of nucleotide sequences of DNA extraction was carried out from 4-day-old mycelia of strain KKU-Rx1 which were picked up from PDA plates and transferred into 1.5ml microcentrifuge tubes. Then, DNA extraction buffer (480µl of 20% sodium dodecyl sulfate: 20µl mercapto
(both Bio Basic Inc., Canada)) was added, pulverized, vortexed and soaked in dry ice. After that, an equal volume of a mixture of phenol: chloroform: isoamyl alcohol (25:24:1) was added, thoroughly mixed, and centrifuged at 12,000 rpm for 5 min. The aqueous phase was transferred into fresh microcentrifuge tubes and the DNA precipitated by adding cold 3M sodium acetate (Bio Basic Inc., Canada) and two volumes of 99.9% cold ethanol, centrifuged and air-dried for about 10-15 min. The DNA pellet was dissolved in TE buffer to which was added RNase enzyme (United States Biological, U.S.A.) at a volume of 1/10, and then incubated overnight at 37ºC. After incubation, DNA extraction was repeated with the same procedure twice to obtain DNA in TE buffer stored at 4ºC. The DNA was quantified by agarose gel electrophoresis (Seakem LE agarose, Cambrex Bio Science Rockland Inc., U.S.A.) and observed under UV light (ECX-F15M, Vilbier Lournat, France) with absorbance at 280 nm.

### 2.2.3 DNA analysis

The internal transcribed spacer (ITS) rDNA was amplified by polymerase chain reaction (PCR) using 2 universal primers ITS1 (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS4 (5’-TCCTCCGCTTATTGATATCG–3’). In brief, PCR was conducted with initial denaturation (94ºC, 1 min) and 30 cycles of denaturation (94ºC, 30 sec), annealing (50ºC, 30 sec) and extensions (72ºC, 45 sec), and final extension at 72ºC for 10 min. The PCR products were then purified using a PCR purification kit (Gene JETTM, Fermentas, Germany). The purified PCR product was observed with agarose gel electrophoresis before analyzing the nucleotide sequence by DNA sequencer (1st Base DNA, Malaysia) and comparing it to the international nucleotide sequence database (GenBank®, National Center of Biotechnology Information, National Library of Medicine, U.S.A.). Nucleotide sequences of the samples were aligned with those of other fungi using multiple alignment of nucleic acid and protein sequences software, ClustalX2 (Conway Institute UCD Dublin, Science Foundation Ireland, Ireland), inferred phylogenies with maximum likelihood methods being performed using Phylip 3.6a programs (Institut Pasteur, Paris, France) and diagrammatically drawn using TreeView 32 (Taxonomy and Systematics at Glasgow, U.K.).

### 2.3 Statistical Analysis

Data were expressed as means ± standard deviations (SD). Student’s t-test was used to compare the results from fungal-infected and non-infected CT and significance was considered at 0.05.

### 3. RESULTS AND DISCUSSION

The fungal strain KKU-Rx1 isolated from the CT sample was restrictedly grown to exhibit furrows which radiated towards the central area in a crateriform pattern of about 20 cm in diameter on Czapek’s agar. The conidia area of each colony was pale blue-green which turned green with white edges and leathery fibers upon maturation (Fig. 1A). Pale yellow smooth-walled conidiophores of the underside of the colony (Fig. 1B) were composed of substrata or aerial hyphae (about 50-200×2.5-3 microns), phialides (about 13-17×3-3.5 microns) (Fig. 1C) and smooth oval or spherical conidia (about 3-3.8 microns), as shown in Fig. 1D. The isolated colonies, thus, most likely resemble *Penicillium citrinum* as previously described [15].
Fig. 2 shows the PCR products of amplified ITS rRNA gene from strain KKU-Rx1. Lanes 1 and 6 were molecular weight markers (1.5kb) while lanes 2 – 5 were the purified PCR products of ITS rDNA from the strain KKU-Rx1 with a molecular size of about 600bp. The phylogentic tree of ITS DNA sequence showed the strains KKU-Rx1 were most related to *Penicillium citrinum* strain SCSAAF0015 with maximum similarity at 100% (Fig. 3).

![Fig. 1](image1)

**Fig. 1.** Microscopic observations of KKU-Rx1 strain isolated from a sample of dried butterfly bean incubated 14 days at room temperature. (A) Colony on Czapek’s agar, (B) Colony viewed from the underside of Czapek’s agar, (C) Cylindrical-shaped conidiophores, and (D) Chain oval-to-round shaped conidia (3-3.8 µm each)

Chemical analyses of anthocyanins from the extracts were compared. Measurement of anthocyanin content has long been of interest to the fruit juice and wine industries as an indicator of product quality, particularly the expectation that the anthocyanins will have beneficial effects on human health. The pH differential method (AOAC method 2005.2) employs structural changes detectable by UV absorbance of anthocyanin chromophores between pH 1.0 and 4.5 at 2 wavelengths and calculates total anthocyanin content by using the molecular extinction coefficient of the major anthocyanin such as cyanidin-3-glucoside [9], one of the most active anthocyanin species [16]. Total anthocyanin extracted from fungal-infected CT (1.8mg/g dried weight) was about 1.5 times higher than that from non-infected CT (1.2 mg/g dried weight), as shown in Fig. 4 (a). The pH differential method was shown to correlate with HPLC analysis of fruit juice samples on the determination of cyanidin-3-glucoside [10]. However, the effect of fungal infection on anthocyanins of CT was not known. HPLC was thus adopted [11-13] for quantification of cyanidin. Chromatograms in Fig. 4 (d) and (e) showed that fungal infected or non-infected CT gave 2 separate peaks at the same retention times, of about 15 and 24 min, but larger peak areas and heights were obtained from infected CT.
Fig. 2. Agarose gel electrophoresis of PCR products from KKU-Rx1 with primer ITS1 and ITS4. Lanes 1 and 6, 1500bp molecular weight marker; lanes 2-5, KKU-Rx1 strain.

Fig. 3. Neighbor joining phylogenetic tree based on ITS rDNA sequence of KKU-Rx1 and related strains.

The retention times of the extracts with and without fungal infection were not significantly different from the relevance (p<0.01) and those of the peaks at about 15 min were not significantly different from that of standard cyanidin (p<0.05). By calculation, 1.1mg of cyanidin was extracted from 1 g of dried petals of infected CT whereas 0.8 mg was extracted.
from those of non-infected CT. Thus, results from pH differential and HPLC methods confirmed that *P. citrinum*-infected CT petals significantly enhanced total anthocyanin content (*P*=.002) and cyanidin (*P*=.009).

![Graph showing comparison of anthocyanin content between infected and non-infected CT petals.](image)

**Fig. 4.** Effect of *P. citrinum* infection on (a) average total anthocyanin contents, (b) average cyanidin content of *C. ternatea* aqueous extracts (CT extract) derived from HPLC analysis, the chromatograms of (c) standard cyanidin, (d) infected CT extract and (e) non-infected CT extract (*P* = 0.002, **P** = 0.008, *n* = 4 and error bars = standard deviations)

DPPH is a stable free radical with a spare electron which delocalizes over the molecule and absorbs light at 520 nm. Following oxidative reaction, it loses its light absorption which can be used to assay oxidative reactions of substances [14]. From DPPH assay, the IC$_{50}$ of *P. citrinum*-infected and non-infected CT extracts were estimated to be 0.43 and 0.66 µg equivalent to cyanidin-3-glucoside/ml, suggesting about 1.5 times higher antioxidant activity with fungal infection (data not shown). In this test, Trolox was used as the positive control and gave IC$_{50}$ of 0.45µg/ml. Thus, the antioxidative activities of both CT extracts are proportional to their anthocyanin content.
Infection of CT with Penicillium citrinum enhanced anthocyanin productivity, resulting in increased anthocyanin, particularly cyanidin, content and increased antioxidant activity. Penicillium citrinum can be found in soil, cereals, spices and indoor environments throughout the tropics, and may be one of the most commonly occurring eukaryotic life forms on earth [17]. It was reported as an unusually opportunistic microorganism in one immune-compromised patient [18].

4. CONCLUSION

Blue petals of C. ternatea which were found to be infected with observable white fungus were microbiologically and chemically investigated. The fungus was identified to be Penicillium citrinum. Fresh aqueous extracts of the infected CT petals showed significantly higher total anthocyanin and higher cyanidin content compared to non infected petals. Antioxidative activities of the extracts were in line with the chemical analysis. Thus, an increase in the anthocyanin content on CT flowers infected by P. citrinum was conclusive.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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