ORIGINAL ARTICLE

Clitorea ternatea flower extract induces platelet-derived growth factor (PDGF) and GPx gene overexpression in ultraviolet (UV) B irradiation-induced collagen loss

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ABSTRACT

Aim To determine the effect of *Clitorea ternatea* flower extract (CTFE) in the gel dosage form on the expression of GPx and platelet-derived growth factor (PDGF) in ultraviolet (UV) - B irradiation-induced collagen loss rat model.

Methods This is experimental research with post-test control group design. Twenty healthy male Wistar rats were divided into four treatment groups: a sham group, UVB control group, two treatment groups with gel of CTFE 5% and gel of CTFE 10%, respectively. Each group was treated with UVB at 302 nm with a MED of 160 mJ/cm2 for 5 days, whereas the sham group did not receive UVB. In the treatment groups CTFE 5% and CTFE 10% gel were given on the 6th to the 14th day. On day 14 all treatment groups were terminated, and GPx and PDGF gene expression were analysed using qRT-PCR.

Results In the group of gel of CTFE 10%, there was a significant increase in GPx gene expression (9.51±1.83) and PDGF (4.36±1.18) compared to the UVB control group which had GPx and PDGF gene expression of 4.90±1.64) and 0.032±0.01, respectively.

Conclusion The administration of CTFE gel showed an increase of the expression of GPx and PDGF genes on UVB irradiation-induced collagen loss rat model.

Key words: clitorea ternatea flower extract, collagen loss, GPx, PDGF

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INTRODUCTION

Collagen loss is initiated by the ultraviolet-B (UVB) radiation-induced overproduction of reactive oxygen species (ROS) (1) ROS overexpression induced diacylglycerol (DAG) and arachidonate acid secretion leading to activation of protein kinase C-3 (PKC3), thereby triggering proinflammatory cytokine and inhibiting growth factor including platelet-derived growth factor (PDGF) (2). On the other hand, inhibition of collagen synthesis is also affected by an oxidant/antioxidant imbalance such as glutathione peroxidase (GPx) obstruction (2–4). Recently, retinol is the first line therapy for collagen loss inhibition (5). However, retinol increases the risk of UV-induced skin damage including skin cancer (6). Studies showed that butterfly pea flower (*Clitoria ternatea*) is a source of anthocyanins and other flavonoids, which may have antioxidant activity (7,8). Unfortunately, no evidence supports a favourable role of *Clitorea ternatea* flower extract (CTFE) in the regulation of PDGF and GPx expression in the UVB irradiation-induced collagen loss.

Recent findings indicate that the induction of antioxidant enzyme such as GPx and improvement of PDGF level could be achieved by application of natural antioxidant (9–11). Flavonoid causes a reduction in postprandial inflammatory response such as high-sensitivity C-reactive protein and interleukin-6 (IL-6) with a concomitant increase in antioxidant capacity (12). Furthermore, flavonoid significantly induces several growth factors such as transforming growth factor-ß (TGF-ß) and PDGF through activation of p38 mitogen-activated protein kinases (MAPKs) pathway (13,14). Therefore, treatment with flavonoid may be a potential strategy for inhibiting collagen loss.

Clitorea ternatea flower contains several phytochemical compounds, such as flavonoids and phenolic acids (7,8). A previous study reported that *Clitorea ternatea* flower extract inhibited oxidative damage to bovine serum albumin in vitro (15). Anthocyanins in *Clitorea ternatea* inhibits the activity of proinflammatory cytokine including IL-6 and TNF-a by blocking ROS expression (16). The polyacrylate anthocyanins and flavanol glycosides as major constituents of CTFE inhibit UV-induced oxidative stress on skin cells (17). Furthermore, CTFE significantly inhibited the collagen density by inhibiting the MMP-1 expression.

Nevertheless, the effect of CTFE on the regulation of PDGF and GPx gene expression in the UVB irradiation-induced collagen loss has never been investigated.

The aim of this study was to evaluate the effect of CTFE on the regulation of PDGF and GPx gene expression in the UVB irradiation-induced collagen loss rat models.

MATERIAL AND METHODS

Material and study design

This post-test only control group study design was conducted in Stem Cell and Cancer Research (SCCR) Laboratory, Faculty of Medicine, Sultan Agung Islamic University, Semarang, Indonesia, from June – August 2022.

The study was approved by the Ethic Committee of Sultan Agung Islamic University (No. 306/VIII/2022/Komisi Bioetik).

Methods

Extraction of *Clitorea ternatea* flower extract. *Clitorea ternatea* flowers were collected from Tawangmangu in Central Java Indonesia in May 2022 (Latitude -7.665158 and Longitude 111.129500). They were rinsed with tap water followed by distilled water to remove the dirt on the surface. The dried *Clitorea ternatea* flower was blended until small pieces and sieved with a mesh size of 120 mesh. 50 g of *Clitorea ternatea* flower were extracted in a maceration apparatus with 500 mL 98% ethanol for 24 h. The filtrate was then evaporated under rotary vacuum evaporator (IKA) and the crude extract west kept in refrigerator 4 (18,19). CTFE (5% and 10%) was dissolved in gel bases (Katechu, USA). The formulations were stored at 4 until further analysis.

Phytochemical screening of *Clitorea ternatea* flower extract. The crude CTFE was tested for the presence of flavonoids, alkaloids, tannins, steroids, terpenoids and saponins. The qualitative results are expressed as (+) for the presence and (−) for the absence of phytochemicals. The flavonoids were test using Wilstater's test according to Femanda et al. (20). Briefly, 2 mg of CTFE was mixed with HCl 500µL and 0.02 mg magnesium. The presence of flavonoids is characterized

by the occurrence of discoloration. The presence of alkaloids indicated with a brown coloured precipitate that were determined under Wagner's test, 15 mg of CTFE was stirred with 1% HCl (6 mL) on water bath for 5 minutes and filtered. The filtrate was added with a few drops of Wagner solution (2 grams of potassium iodide and 1.27 g of Iodine in 95 mL of distilled water) (21). Furthermore, tannins content was analysed in the CTFE with 1% ferric chloride, black or blue coloration was taken as a positive result of tannins (22). Liebermann-Burchard test was used to determine the presence of steroids and terpenoids, briefly 100 mg of CTFE was shaken with chloroform and a few drops of acetic anhydride were added to the test tube and boiled in a water bath and rapidly cooled in iced water. Concentrated H_2SO_4 (2 mL) was added alongside of the test tube. The formation of a brown ring at the junction of two layers and turning the upper layer to green shows the presence of steroids, while the formation of deep red colour indicates the presence of triterpenoids (23). The saponin presence was analysed under Forth's test, 500 mg of CTFE was shaken with 10 mL of distilled water. The formation of frothing, which persists on warming in a water bath for 5 min, shows the presence of saponins (23).

Total flavonoid content of *Clitorea ternatea* flower extract. Total flavonoid content was determined using the aluminum colorimetric method (24) with some modifications using gallic acid as a standard. A calibration curve of gallic acid was prepared in the range of 200 - 700µg/mL. Briefly, extract (0.5 mL) and standard (0.5 mL) were placed in different test tubes and to each 10% aluminum chloride (0.1 mL), 1 M potassium acetate (0.1 mL), 80% methanol (1.5 mL) and distilled water (2.8 mL) were added and mixed. A blank was prepared in the same manner where 0.5 mL of distilled water was used instead of the sample or standard, and the amount of aluminum chloride was also replaced by distilled water. All tubes were incubated at room temperature for 30 min. The absorbance was taken at 415 nm. The concentration of flavonoid was expressed as mg gallic acid equivalent (GE) per gram of extract.

Collagen loss animal model. Twenty healthy male Wistar rats (250±25 g) CV=10% were fed ad libitum and reared at 28 ºC and a photoperiod of 12 hours. After a week of acclimatization, rats were randomly divided into the following five groups: Sham/Untreated, UVB irradiation, UVB irradiation and 5% gel of CTFE and UVB irradiation and 10% CTFE gel. Each group consisted of five rats. This study used UVB light (broadband with peak emission at 302 nm CL-100M, UVP, USA). Rats were exposed to UVB light of 160 mJ/cm2 for 30 minutes for 5 consecutive days according to a previous study with a slight modification (25). The 200 mg of CTFE gels were administered topically on the dorsal rat skin daily for up to 14 days. UVB group rats did not receive any treatment. On day 15, all rats were terminated, and skin tissue was isolated for further analysis.

Collagen analysis. The tissue paraffin block was cut using a microtome to a thickness of 5 µm then stained with Masson Trichrome (Bio optica, catalog #04010802) and observed under the light microscope (Olympus CX21, Tokyo, Japan). The percentage of collagen density was calculated from the area of collagenous tissue formed on each slide using ImageJ.

GPx and PDGF gene expression by qRT-PCR. Total RNA from rat skin tissue was extracted with TRIzol (Invitrogen, Shanghai, China) according to the manufacturer's protocol. Briefly, first-stranded cDNA was synthesized with 1 g of total RNA using Super-Script II (Invitrogen, Massachusetts, USA). SYBR No ROX Green I dye (SMOBIO Technology Inc, Hsinchu, Taiwan) was used for reverse-transcription in a real-time PCR instrument (PCR max Eco 48), and mRNA levels of the PDGF and GPx genes were measured using the respective primers (Table 1). The used thermocycler conditions were as follows: initial step at 95°C for 10 minutes, followed by 50 cycles at 95°C for 15 seconds, and 60°C for 1 minute. The gene expression was recorded as the Cycles threshold (Ct). Data were obtained using Eco Softwa-

Table 1. Primer sequences for GPx, PDGF and GAPDH genes

Gene symbol	Primer sequence 5'→ 3'
GPx	Forward GPx 5' - AGTTCGGACATCAGGAGAATGGCA-3' Reverse GPx 5' - TCACCATTCACCTCGCACTTCTCA -3'
PDGF	Forward PDGF 5' - GAGCTAGCG AGA ATC CCA AAA GCC TCA A-3' Reverse PDGF 5' - CTCTCGAGG CGG GGT TGC AGA AGT GT -3'
GAPDH	Forward GAPDH 5' - GTCTCCTCTGACTTCAACAGCG-3' Reverse GAPDH 5' - ACCACCCTGTTGCTGTAGCCAA-3'

re v5.0 (Illumina Inc, San Diego, CA, USA). All reactions were performed in triplicate, and data analysis used the $2^{-\Delta\Delta}$ Ct method (Livak method).

Statistical analysis

All data are presented as mean±standard deviation (SD). Data analysis used one-way ANOVA and continued with the Least Significant Difference (LSD) test with $p<0.05$.

RESULTS

The phytochemical screening of crude ethanolic extract of *Clitorea ternatea* flower revealed the presence of some secondary metabolites such as alkaloid, saponin, tannin, and flavonoid (Table 2). The total flavonoid content of CTFE was 682.02±9.48 mg GE/g. These values are higher than total flavonoid content of the same family with methanol solvent (26).

Table 2. Phytochemical screening of secondary metabolites from *Clitorea ternatea* flower extract

Chemical component	Name of the test	CTFE
Alkaloids	Wagner test	++
Flavonoids	Wilstater test	+++
Tannins	Braemer's test	+
Saponins	Forth test	+
Steroids	Lieberman Burchardt test	-
Terpenoids	Lieberman Burchardt test	+

CTFE, *Clitorea ternatea* flower extract

In this study, we validate the collagen loss induced-UVB under Masson Trichome staining. Based on the results of histological observations, the collagen content in the dermal tissue induced by UVB significantly decreased, as indicated by the turquoise colour on the Masson Trichome staining (Figure 1). These data indicated that the UVB irradiation successfully inhibit collagen synthesis leading to collagen loss condition.

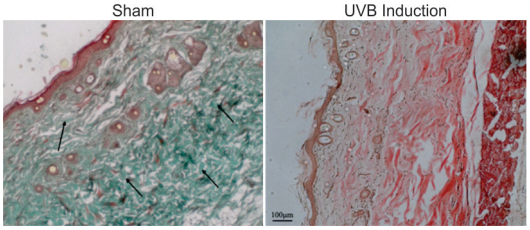

Figure 1. Collagen expression in all study groups, sham and UVB induction group. Histological view of skin tissue with specific Masson Trichrome staining that is sensitive to collagen in green (100x magnification)

A significant increase of CTFE gels of GPx gene expression in doses-dependent manner was found (Figure 2). In the control group, naturally induced GPx gene expression was 4.90±1.64- fold greater than in the sham group. The 5% and 10% of CTFE groups had significantly increased GPX gene expression of 6.01±1.25 and 9.51±1.83, respectively, - fold greater than the sham group.

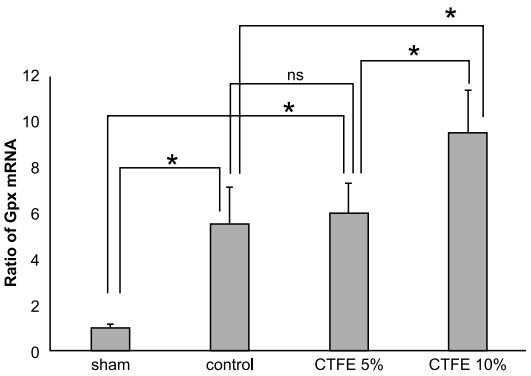

Figure 2. The effect of *Clitorea ternatea* flower extract (CFTE) on GPx expression on UVB irradiation-induced collagen loss rat models. Data are presented as fold change in gene expression relative to UVB unexposed group
*p< 0.05; ns, non-significant

CTFE gels significantly increased PDGF gene expression in doses-dependent manner (Figure 3). In the control group, PDGF gene expression was depleted until 0.032±0.01-fold greater than the sham group. The 5% and 10% of CTFE groups had significantly increased PDGF gene expression 0.44±0.15 and 4.36±1.18, respectively, - fold greater than the sham group.

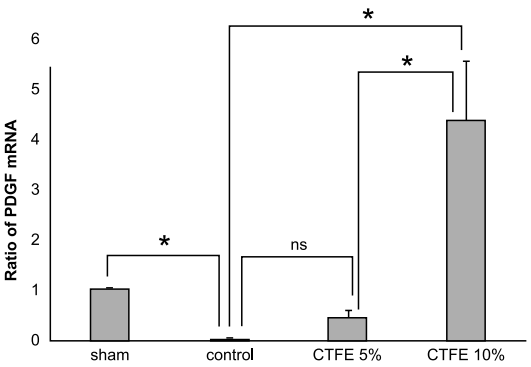

Figure 3. The effect of *Clitorea ternatea* flower extract (CFTE) on platelet-derived growth factor (PDGF) expression on UVB irradiation-induced collagen loss rat models. Data are presented as fold change in gene expression relative to UVB unexposed group
*p> 0.05

DISCUSSION

In this study the detected phytochemical compounds are known to have medicinal importance including antioxidant, anticancer, antiinflammation, and antibacterial activity. A previous study

also reported that flavonoids compound has antioxidant functions and inhibiting a high scavenging activity of harmful ROS, including UVB irradiation (27). Furthermore, in this study we evaluated the effect of CTFE on the GPx and PDGF gene expression on the UVB irradiation-induced collagen loss rat models.

The key point for a limiting oxidative stress and preventing UVB-induced damage to the skin is a removal or detoxification of reactive oxygen species (ROS). This is accomplished in large part by antioxidant enzymes. A number of enzymes have been identified that are involved in this process including the ROS scavengers including glutathione peroxidase (GPx) (28). UVB-induced oxidative stress has been reported to be associated with transient decreases in GPx activity in mouse skin (27-28). Reduced expression of SOD and GPx activity has also been described in human keratinocytes treated with UVB *in vitro* (29-31). Flavonoids scavenge free radicals effectively by forming semiquinone radicals, which bind to free radicals to form a stable quinone structure (32). In addition, previous study supported the previous study that flavonoid compounds such as quercetin form hydrogen bonds with Ser212 through the 3'-OH group, thereby inhibiting the activity of kinase protein (MEK1) (33). Quercetin also inhibits the activation of phosphoinositide 3-kinase (PI3K) and activates protein kinase (MAPK) to induce the expression of antioxidant enzymes (29). The ability of flavonoid compounds as antioxidants has been shown to reduce oxidative stress conditions by increasing the GPx enzyme (27,31,34). In this study we also evaluated the effect of CTFE on the PDGF gene expression.

UVB irradiation suppressed PDGF expression leading to inhibition of TGF-β expression on the collagen synthesis pathway (35,36). TGF-β is a prototypical fibrogenic cytokine, which increases the expression of extracellular matrix (ECM) genes and regulates the downregulation of matrix-degrading enzymes through the SMAD pathway, mainly associated with collagen synthesis (37–39).

Previous studies reported that the C-6 structure of flavonoids inhibited the expression of the TGF-ß/Smad and PI3K/mTOR signalling pathways. This structure also inhibited the a-SMA expression and collagen synthesis (40). Flavonoid also induced the expression of PDGF through MAPK pathway (41). The flavonoid chrysoeriol induced PDGF–induced ERK1/2 activation (42). A previous study reported that PDGF mediated collagen synthesis through the stimulation of type III collagen production (43). The B ring structure of flavonoids also acts as a scavenger of hydroxyl free radicals, so the ROS produced due to UVB rays can be suppressed (44). If ROS overexpression is not established, the growth factor activation pathway is unaffected, and collagen synthesis can occur normally (45).

In conclusion, the CPTE may inhibit UVB irradiation-induced collagen loss through elevation GPx and PDGF gene expression. CPTE might accelerate the restoration of UVB irradiation-induced collagen loss.

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TRANSPARENCY DECLARATION

Conflict of interest: None to declare

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