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In vitro Protective Effects of an aqueous extract *of Clitoria ternatea* L. flower against hydrogen peroxide-induced cytotoxicity and UV-induced mtDNA damage in human keratinocytes

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Abstract

The traditional practice of eating the flowers of *Clitoria ternatea* L. or drinking their infusion as herbal tea in some of the Asian countries is believed to promote a younger skin complexion and defend against skin aging. This study was conducted to investigate the protective effect of *C. ternatea* flower water extract (CTW) against hydrogen peroxide-induced cytotoxicity and UV-induced mtDNA damage in human keratinocytes. The protective effect against hydrogen peroxide-induced cytotoxicity was determined by 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay and mtDNA damage induced by UV was determined by polymerase chain reaction (PCR). Pre-incubation of HaCaT with 100, 250 and 500 μg/mL CTW reduced cytotoxicity effects of H_2O_2 compared with control (H_2O_2 alone). CTW also significantly reduced mitochondrial DNA (mtDNA) damage in UV-exposed HaCaT ($p<0.05$). CTW was chemically-characterised using high resolution liquid chromatography-mass spectrometry (LC-MS). The main compounds detected were assigned as anthocyanins derived from delphinidin, including polyacylated ternatins, and flavonol glycosides derived from quercetin and kaempferol. These results demonstrated the protective effects of *C. ternatea* flower extracts that contain polyacylated anthocyanins and flavonol glycosides as major constituents, against H_2O_2 and UV-induced oxidative stress on skin cells, and may provide some explanation for the putative traditional and cosmetic uses of *C. ternatea* flower against skin aging.

Keywords: Oxidative stress, hydrogen peroxide, mitochondrial DNA, antioxidant, HaCaT, cytotoxicity, skin aging, *Clitoria ternatea*

INTRODUCTION

Clitoria ternatea L. (Leguminosae) or the butterfly pea originates from tropical Asia and was later distributed to South and Central America, East and West Indies, China and India (Zingare *et al*. 2013). There are at least 60 species in the genus *Clitoria*, and *C. ternatea* is the most frequently reported species (Zingare *et al*. 2013). The plant is a perennial and has conspicuous blue and white flowers resembling a conch-shell (Mukherjee *et al*. 2008).

The traditional uses of *C. ternatea* are extensive, ranging from medicinal to food dyes for its naturally vivid blue colour. There have been many pharmacological activities reported for the plant such as antimicrobial, anti-pyretic, anti-inflammatory, anticonvulsant, diuretic, anaesthetic, antidiabetic and insecticidal (Zingare *et al*. 2013; Mukherjee *et al*. 2008; Kosai *et al*. 2015). Previous research has focused on the roots, seeds and leaves of the plant for their uses as a laxative, brain and nerve tonic in the Ayurvedic medicinal system. The root juice in particular, was mixed with honey and ghee to improve mental health and memory in children or used as laxative when combined with ginger (Mukherjee *et al*. 2008). Root extracts improved memory in a number of *in vivo* studies, whilst an extract of leaves, stems, flowers and fruits was sedative *in vivo* (Howes and Houghton, 2003). However, there is a lack of research on the biological activities of the flowers to explain their traditional and potential uses. Apart for ornamental purposes, the flowers have been traditionally consumed in salads or drank as a herbal tonic to promote a younger skin complexion and defend against skin aging (Kaisoon *et al*. 2011; Lijon *et al*. 2017). Although there are some reports for the use of *C. ternatea* flower extract as a cosmetic ingredient (Kamkaen & Wilkinson 2009; Tantituvanont 2008; Lijon *et al*. 2017), there is a paucity of data on the application of *C. ternatea* flower extract in skin aging and scientific evidence to support this use is lacking.

C. ternatea flower has been reported to have antioxidant potential, mainly due to its anthocyanin content (Iamsaard *et al*. 2014; Kaisoon *et al*. 2011; Miguel 2011; Chayaratanasin *et al*. 2015; Kamkaen & Wilkinson 2009). Many anthocyanins are known to have antioxidant effects, which may have an important role in the prevention of many diseases such as cancer, cardiovascular diseases and skin aging; although there may be issues with their oral bioavailability (Miguel 2011; Howes and Simmonds 2014), thus topical use may be more appropriate for their relevance to dermatological conditions. Substantial evidence suggests oxidative damage from reactive oxygen species (ROS) that may originate from various factors

such as genetic, lifestyle, nutrition, chemicals and exposure to ultra-violet (UV), could lead to the deterioration of the skin surface leading to wrinkles, hyperpigmentation and sagging of the skin, which are aesthetically undesirable (Sjerobabski-Masnec & Situm 2010; Farage *et al*. 2010; Fisher *et al*. 2002; Gragnani *et al*. 2014). Many therapies and strategies such as chemical peeling, injection of rejuvenating agents, fillers or implants, surgery, nutraceuticals, or application of cosmetic products have been developed to target skin aging via different mechanisms (Ganceviciene *et al*. 2012). Of these approaches, anti-aging 'skin care' is highly sought after, particularly those formulated with organic and naturally-derived ingredients (Grand View Research Report 2016; US Deparment of Commerce 2015). Therefore, in this study, we investigated the potential anti-aging properties of *C. ternatea* flower water extract using a human keratinocytes cell line (HaCaT), assessing the protective effects against hydrogen peroxide-induced cytotoxicity and UV-induced mtDNA damage, to scientifically support the putative traditional benefits of *C. ternatea* flower extract for the skin, with potential development as an 'anti-aging' ingredient in cosmetic products.

MATERIALS AND METHODS

Chemicals, reagents and materials. The diphenyl-picryl hydrazine (DPPH) and 2, 2'-azino-bis (3- ethylbenzothiazoline-6-sulphonic acid (ABTS), hydrogen peroxide (18.2 M) were purchased from Sigma Aldrich, UK. Dulbecco Modified Eagle Medium (DMEM), Fetal Calf Serum (FCS) and trypsin were purchased from Lonza, UK. [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution was purchased from Promega, UK. QIAamp DNA Mini Kit for total DNA extraction was purchased from Qiagen, UK. The primers were designed and supplied by Eurofins, UK. 2x SensiMix Hi-ROX and MicroAmp Fast Optical 96-Well Reaction Plate were obtained from Bioline, UK and ThermoFisher Scientific, UK.

Plant material. Plant material was purchased from Siam Herbarium, Thailand. The voucher sample was deposited in the Herbarium of Medicinal Plant Research Group, Newcastle University (CT/NNAZ/001).

Extract preparation. Previous methods were followed with some modification (Thring *et al*. 2009; Islam *et al*. 2014). Briefly, powdered plant material was weighed (50 g) and extracted in 500 mL of freshly boiled de-ionised water for 30 minutes. The extract solution was allowed

to cool down with frequent shaking before sonication for 15 minutes. The extract was vacuumed-filtered for at least three times and the marc was discarded. Finally, the extract was freeze-dried and stored at -20 °C until used. Water was selected as solvent to reflect the traditional method for extract preparation.

Antioxidant assays. The diphenyl-picryl hydrazine (DPPH) and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assays were adapted from previous protocols (Chattuwatthana & Okello 2015).

Cell line and cell culture. Normal human keratinocytes (HaCaT) cell line was obtained from the Medical School of Newcastle University. The cells were maintained in complete DMEM (10% FCS and 1% pen-strep) with 5% CO_2 at 37 °C.

Cytotoxicity assay: 10, 000 cells/well were plated into a 96-well plate overnight at 37 °C. The next day, the medium was aspirated, cells were rinsed with PBS (100 µL) and fresh DMEM was added (100 µL). HaCaT was treated with varying concentrations (50-2000 µg/ mL) of the plant extracts for 24 h to determine the nontoxic concentrations. The number of viable cells after treatments was measured using MTS assay, expressed as the percentage of viable cell to untreated cells. The MTS assay was performed as described by the manufacturer (Promega, UK). Briefly, the medium was aspirated and cells, were rinsed with PBS (100 µL) and fresh DMEM (100 µL) was added. The MTS solution (20 µL) was added into each well and the plate was incubated for 4 h at 37 °C before reading at 490 nm with Spectromax Plus 384® and viewed with Softmax Pro V5 software.

Hydrogen peroxide induced-cytotoxicity assay. 10, 000 cells/well were plated in a 96-well plate overnight at 37 °C. HaCaT cells were pre-incubated with the plant extracts at different concentrations for 24 h, prior to exposure to H_2O_2 (200 µM, negative control (NC)) from a freshly prepared 1000 µM stock solution. Positive control (PC) was the untreated HaCaT. Cell viability was determined by MTS assay as described above after 24 h.

UV-induced mtDNA damage assay: 2.5 x10^5 cells/ 3 mL of HaCaT in complete DMEM were seeded in 35-mm culture dishes and allowed to settle overnight at 37 °C. The used medium was removed and cells were rinsed with 1 mL PBS and subsequently incubated with CTW diluted in complete DMEM to a total volume of 3 mL for 24 h at 37 °C. After incubation, the medium was removed, washed with 1 mL of PBS and 3 mL of phenol-free DMEM with 1% pen-

strep was added to each dish. Later, the dish was placed under the UV lamps (seven 6 foot (ft) iSOLde Cleo performance 100 W-R lamps (Cleo) (iSOLde, Germany) fitted into a custom built irradiation unit for ~ 40 minutes, equivalent to 4.0 standard erythemal damage (SED). The UV source delivered a flux of 40 mJ/cm^2 between 300 and 400 nm wavelength and the dose received by the samples was measured with a DMc150 Monochromator (Bentham Instruments Ltd., UK). After exposure, the cells were scrapped in a circular motion and observed under the microscope to ensure all cells were collected prior to DNA extraction.

DNA extraction: The protocol for total DNA extraction was followed as recommended by the manufacturer using a QIAamp DNA Mini Kit (Qiagen 2016). Briefly, the collected cell pellets were suspended in PBS (200 µL), added with Proteinase K (20 µL) and lysis buffer (200 µL). The mixture was vortexed (15 s) and incubated (56 °C) for 10 minutes, before adding absolute ethanol (200 µL). The mixture was further vortexed (15 s) and added to QIAmp Spin Column for centrifugation (8000 rpm for 1 minute). The flow-through solution was discarded and the column was washed with washing buffer 1 (500 µL) and centrifuged (8000 rpm for 1 minute). The flow-through was discarded, washed with washing buffer 2 (500 µL) and centrifuged (14 000 rpm for 3 minutes). The flow-through was discarded and the column was spun (14 000 rpm for 1 minute). Finally, DNA was eluted with an elution buffer (60 µL) and stored at 4 °C for one day prior to use.

qPCR procedures: DNA samples were thawed and vortexed (15 s) to mix. DNA samples were diluted with RNase/DNase free water to obtain the required concentration (6ng/µL). The protocol was performed on ice. The forward (AL4: CTGTTCTTTCATGGGGAAGC) and reverse primers (AS1: AAAGTGCATACCGCCAAAAG) were diluted with water to obtain a 10 µM of forward and reverse primers working solutions. Later, the PCR mastermix, 2x SensiMix Hi-ROX and primers were assembled on ice. Then, 18 µL of the mastermix working solution was added into each well of a MicroAmp Fast Optical 96-Well Reaction Plate and 2 µL of DNA samples were added and thoroughly mixed. The final concentrations for 2x SensiMix Hi-ROX, each primer and samples were 1x, 0.25 µM and 1.2 ng/µL respectively. The PCR was run using StepOnePlus Real Time PCR system (Applied Biosystem, UK). The PCR conditions comprised of pre-incubation phase of 10 minutes at 95 °C followed by 30 cycles of; 98°C for 15 seconds, 60°C for 15 seconds, and 72°C for 55 seconds; with a final stage of 72°C for 7 minutes. A threshold in the linear range of the amplification plot was set. The number of cycles at which

the florescence crossed the threshold (Ct) were used to compare the mtDNA damage between samples. DNA sample with a low Ct value indicates low damage (high copy number) and a high Ct value indicates high damage (low copy number).

UV absorption: 3 mL of the plant samples were pipetted into a cuvette and the absorption spectrum from 190-800 nm was determined using a Cary Spectrophotometer™. Blank (water) subtracted from the background reading.

Phytochemical analysis: Previous methods were followed for qualitative analysis (Khanam *et al*. 2014), total phenolic content (TPC) (Chattuwatthana & Okello 2015) and total anthocyanin content (Lee *et al*. 2005).

Liquid chromatography–serial mass spectrometry (LC-MS/MS): The extract was reconstituted at 10mg/ml prior to chemical characterisation using LC–UV–MS/MS analysis. Analyses were performed on a Thermo Scientific system consisting of an 'Accela' U-HPLC unit with a photodiode array detector and an 'LTQ Orbitrap XL' mass spectrometer fitted with an electrospray source (Thermo Scientific, Waltham, MA, USA). Chromatography was performed on 5 µl sample injections onto a 150-mm x 3 mm, 3 µm Luna C-18 column (Phenomenex, Torrance, CA, USA) using the following 400µl/min mobile phase gradient of H_2O/CH_3OH/CH_3CN +1% HCOOH: 90:0:10 (0 min), 90:0:10 (5 min), 0:90:10 (60 min), 0:90:10 (65 min), 90:0:10 (67 min), 90:0:10 (70 min) followed by return to start conditions and equilibration in start conditions for 5 min before the next injection. The ESI source was operated with polarity switching and the mass spectrometer was set to record high resolution (30 k resolution) MS1 spectra (*m/z* 125– 2000) in positive mode using the orbitrap and low resolution MS1 spectra (*m/z* 125– 2000) in negative mode and data dependent MS2 and MS3 spectra in both modes using the linear ion trap. Detected compounds were assigned by comparison of accurate mass data (based on ppm), and by available MS/MS data, with reference to the published compound assignment system (Schymanski *et al*., 2014) and with supportive UV spectra.

Statistical analysis: ANOVA (Dunnet's test) was used to compare differences between test groups and the negative control (H_2O_2 only) using Graphpad Prism Software.

RESULTS

Antioxidant activities. The antioxidant activities for *C. ternatea* water extract (CTW) against DPPH and ABTS free radicals showed concentration-dependent effects (Figure 1). Both assays showed that CTW extract can scavenge free radicals, although the potency is lower than the positive control (Trolox) (Table 1). The DPPH and ABTS assays are based on the ability of the extract constituents to donate an electron to the pre-formed radical resulting in decreasing absorbance (Badarinath *et al*. 2010; Tirzitis and Bartosz, 2010). Two assays for evaluation were used to ensure consistency of the activity (Moon and Shinamoto, 2009).

Table 1: IC_{50} values for *C. ternatea* water extract (CTW) and Trolox standard in DPPH and ABTS assays. IC_{50} value for all assays were obtained from the best fit line constructed from the dose-response graphs at which 50% inhibition was observed. A lower IC_{50} value indicates higher antioxidant potency.

Assays	CTW (µg/mL) IC_{50})	Trolox standard (µg/mL) IC_{50}
DPPH	195.5	3.32
ABTS	42.9	6.51

Effect of H_2O_2 on HaCaT cell viability induced after 24 h, 48 h and 72 h proliferation. Incubation of HaCaT with H_2O_2 showed a concentration-dependent and time-dependent cytotoxicity. As expected, the higher H_2O_2 concentration introduced to HaCaT, the more potent the cytotoxic effect. However, the inverse correlation observed between H_2O_2 concentrations and the time point of when H_2O_2 was exposed to HaCaT suggests that the cytotoxic effect is dependent on cell concentration. Longer incubation of HaCaT allowed the cells to proliferate and therefore, reducing the cytotoxic effect of H_2O_2 (Ohguru *et al*. 1999). Significant decreased in cell viability was observed at 100 µM (30%), 200 µM (70%), 250 µM (80%) and 300 µM (90%) with H_2O_2 induced after 24 h of proliferation. After 48 h (Figure 2 B), significant reduction in cell viability was observed at 150 µM (20%), 200 µM (30%), 250 µM (55%), and 300 µM (70%). After 72 h (Figure 2 C), significant reduction in cell viability was observed at 200 µM (30%), 250 µM (50%) and 300 µM (70%). Considering the experimental design, where the cells were allowed to acclimatize overnight and treated with plant extracts for 24 h before H_2O_2 exposure, a concentration of 200 µM H_2O_2 induced after 48 h of

proliferation was selected as an appropriate concentration to induce toxicity, to assess the protective effect of the plant extracts against H_2O_2.

***In-vitro* protective effects against 200 µM H_2O_2.** The protective effect of CTW against H_2O_2 induced toxicity on HaCaT was evaluated at concentrations ranging from 50-2000 µg/mL. CTW concentrations of 50, 100, 250 and 500 were non-toxic to HaCaT as indicated in Figure 3. However, CTW concentrations of 1000, 1500 and 2000 µg/mL were toxic to HaCaT. Pre-incubation of HaCaT with CTW with 100, 250, 500 µg/mL reduced the toxic effect of H_2O_2 on HaCaT as indicated by the significantly higher percentage cell viability (10-20 %) at the specified concentrations, compared with negative control (NC), $p>0.05$. A lower CTW concentration (50 µg/mL) did not show any significant protective effect on HaCaT.

***In-vitro* protective effect against UV-induced mtDNA damage.** CTW concentration of 250 µg/ mL was selected for UV assay based on the activity in the H_2O_2 assay. The positive control (protected HaCaT from UV) resulted in significantly lower mtDNA damage compared with the negative control (exposed HaCaT). Similarly, pre-treated HaCaT with CTW showed significantly lower mitochondrial DNA (mtDNA) damage compared with the negative control, $p< 0.05$ (Figure 4). Analysis of CTW spectra absorption showed high absorption in the UVB and UVC range (Table 2). Two peaks were also identified in the visible light wavelength range.

Table 2: *C. ternatea* water extract (CTW) absorption spectra. The absorption spectra for CTW. Peaks were observed in the visible light, UVB and UVC range.

Spectra (nm)	**Peak observed (nm) and Optical Density (OD)**
UVC (200-290)	266 (3.767), 289 (3.264),
UVB (290-320)	294 (3.261)
UVA (320-400)	-
Visible light (400-800)	573 (0.884), 619 (0.875)

Phytochemical analysis. Qualitative and quantitative analysis of phytochemicals indicated the presence of saponins and phenolics, including flavonoids, with TPC and TAC values of 38.5 ± 1.21 and 3.61 ± 0.71 mg/ mL respectively (Table 3).

Table 3: The qualitative analysis of phytochemical, total phenolic content (TPC) and total anthocyanin content (TAC). The presence or absence of compound denoted with (+) or (-). Data for TPC and TAC were mean ± SD (n=4).

TEST COMPOUNDS	CTW sample
1. Alkaloids (Dragendorff's)	(-)
2. Saponins (Froth)	(+)
3. Terpenoids (Salwoski)	(-)
4. Phenolics (Ferric chloride)	(+)
5. Flavonoids (Alkaline reagent)	(+)
6. Aldehyde (Schiff)	(-)
7. TPC as mg gallic acid equivalent /g dried extract	38.5 ± 1.21
8. TAC as mg cyanidin-3-glucoside equivalent /g dried extract	3.61 ± 0.71

Liquid chromatography–serial mass spectrometry (LC-MS/MS). The assigned compounds detected in the *C. ternatea* water extract (CTW) using high resolution LC-MS/MS are presented in Table 4. The main constituents detected included flavonol glycosides which were assigned from their observed $[M+H]^+$ ions and supportive UV spectra and MS/MS interpretation. The detected flavonol glycosides consisted of the aglycones of quercetin, myricetin and kaempferol, and included three malonylglycosides of kaempferol eluting with retention times of 22.4 min, 22.8 min and at 25.6 min. The detection of glycosides and malonylglycosides of kaempferol, quercetin and myricetin in the CTW investigated in this study is in agreement with the known constituents of *C. ternatea* flowers (Kazuma *et al*. 2003a; 2003b). Other main constituents detected in CTW were assigned as acylated delphinidin glycosides from their observed M^+ ions, with supportive UV spectra and MS/MS interpretation. These included a series of ternatins, which are known to occur in the flowers of *C. ternatea* (Kazuma *et al*. 2003b), thus supports the chemical authentication of the plant material investigated in this study. Other detected constituents in CTW included coumaroyl-sucrose and -glucose, observed from their $[M + NH_4]^+$ ions; and the amino acids phenylalanine and tryptophan, observed from their $[M + H]^+$ ions, as indicated in Table 4.

Table 4: Compounds assigned from LC-MS/MS analysis of the *Clitoria ternatea* flower water extract.

Assigned compound[#] (or isomer)		Retention time (min)	Molecular formula	(*m/z*)	Ion	ppm[#]
1.	Phenylalanine	2.9	$C_9H_{11}NO_2$	166.086	$[M + H]^+$	0.752
2.	Coumaroylsucrose	3.5	$C_{21}H_{28}O_{13}$	506.188	$[M + NH_4]^+$	2.259
3.	Tryptophan	4.4	$C_{11}H_{12}N_2O_2$	205.097	$[M + H]^+$	0.418
4.	Coumaroylglucose	7.7	$C_{15}H_{18}O_8$	344.134	$[M + NH_4]^+$	0.921
5.	Ternatin A3 or C2	9.6	$C_{66}H_{75}O_{39}$	1491.391	M+	2.006
6.	Myricetin rhamnosyl-rhamnosyl-glucoside	13.5	$C_{33}H_{40}O_{21}$	773.214	$[M + H]^+$	1.132
7.	Ternatin B4 or C1	14.3	$C_{60}H_{65}O_{34}$	1329.337	M^+	1.862
8.	Myricetin neohesperidoside	16.0	$C_{27}H_{30}O_{17}$	627.157	$[M + H]^+$	2.143
9.	Quercetin hexosyl-rhamnosyl-rhamnoside	16.4	$C_{33}H_{40}O_{20}$	757.222	$[M + H]^+$	4.028
10.	Quercetin hexosyl-rhamnoside	17.6	$C_{27}H_{30}O_{16}$	611.163	$[M + H]^+$	3.074
11.	Ternatin B2 or B3	18.1	$C_{75}H_{81}O_{41}$	1637.429	M^+	2.328
12.	Kaempferol hexosyl-rhamnosyl-rhamnoside	18.8	$C_{33}H_{40}O_{19}$	741.225	$[M + H]^+$	2.435
13.	Delphinidin 3-*O*-[4-hydroxycinnamoyl-rhamnosyl-glucoside], 5-*O*-(6-*O*-malonyl-glucoside), bis-*O*-[3,4-dihydroxycinnamoyl-glucoside]	19.2	$C_{75}H_{81}O_{42}$	1653.422	M^+	1.692
14.	Quercetin hexosyl-rhamnoside	19.8	$C_{27}H_{30}O_{16}$	611.162	$[M + H]^+$	1.765
15.	Kaempferol hexosyl-rhamnoside	20.1	$C_{27}H_{30}O_{15}$	595.167	$[M + H]^+$	2.089
16.	Kaempferol hexosyl-rhamnoside	20.2	$C_{27}H_{30}O_{15}$	595.167	$[M + H]^+$	3.316
17.	Quercetin hexoside	20.4	$C_{21}H_{20}O_{12}$	465.104	$[M + H]^+$	2.747
18.	Ternatin B2 or B3	21.3	$C_{75}H_{81}O_{41}$	1637.429	M^+	2.847
19.	Ternatin B4 or C1	21.7	$C_{60}H_{65}O_{34}$	1329.339	M+	2.689
20.	Ternatin B1	22.2	$C_{90}H_{97}O_{48}$	1945.52	M^+	3.022
21.	Kaempferol rhamnosyl-malonyl-glucoside	22.4	$C_{30}H_{32}O_{18}$	681.168	$[M + H]^+$	2.686
22.	Kaempferol rhamnosyl-malonyl-glucoside	22.8	$C_{30}H_{32}O_{18}$	681.168	$[M + H]^+$	3.405
23.	Kaempferol hexosyl-rhamnoside	23.3	$C_{27}H_{30}O_{15}$	595.167	$[M + H]^+$	2.812
24.	Kaempferol hexoside	23.6	$C_{21}H_{20}O_{11}$	449.109	$[M + H]^+$	2.677

25. Ternatin D1	24.9	$C_{84}H_{87}O_{43}$	1783.468	M^+	3.826
26. 6''-Malonylastragalin	25.6	$C_{24}H_{22}O_{14}$	535.109	$[M + H]^+$	1.567

[#]All compounds assigned by comparison of accurate mass data (based on ppm), and by interpretation of available MS/MS and/or UV spectra.

DISCUSSION

The use of plants as cosmetics has been known since time immemorial, with evidence provided from cosmetic recipes recorded on the Egyptian's Smith and Ebers papyri (Leake 1952). Many plants, including herbs, fruits and vegetables, contain polyphenols, which are widely documented as potent antioxidants that can scavenge free radicals. *C. ternatea* flower extract has been reported to be used as a source of useful cosmetic ingredients, however, there is a paucity of data on the application of *C. ternatea* flower extract in skin aging (Kamkaen and Wilkinson, 2009; Tantituvanont *et al*. 2008; Lijon *et al*. 2017), whilst scientific evidence to support or understand its use in cosmetic products is also lacking.

The present study demonstrated that pre-treatments of human keratinocytes (HaCaT) with an aqueous *C. ternatea* flower extract significantly protected HaCaT cells against H_2O_2-induced cytotoxicity and UV-induced mitochondrial DNA (mtDNA) damage. H_2O_2 can generate the hydroxyl radical, a highly reactive free radical, in the presence of transition metals such as iron and copper through a reaction known as the Fenton reaction (El-Bahr 2013; Winterbourn 1995). Exposure to UV radiation has been shown to be one of the most damaging factors to the skin, as it can cause direct damage to cells, protein and DNA or may induce various signalling transduction pathways to mediate the damage (Fisher *et al*. 2002). It was suggested that UV initiates the molecular responses in human skin through the generation of ROS, where ROS generated from UV could oxidize and cause the activation of cell surface receptors triggering the downstream signal transduction pathways (Fisher *et al*. 2002). UV also activates NADPH oxidase, which generates H_2O_2 , leading to further increase in oxidative stress (Fisher *et al*. 2002). It should also be considered that exposure to UV radiation is also associated with the development of melanomas (Shain and Bastian, 2016) in addition to the impact on skin aging.

In this study, the major constituents detected in the *C. ternatea* flower extract (Table 4) were assigned as kaempferol, quercetin and myricetin glycosides and acylated anthocyanins derived from delphinidin. Flavonols and their glycosides, and anthocyanins, are widely documented to have potential benefits to human health which may be mechanistically associated with antioxidant effects (Sivaprabha *et al*. 2008; Soto-Vaca *et al*. 2012; Howes and Simmonds, 2014). It may therefore be hypothesised that the ability of the *C. ternatea* flower extract to scavenge free radicals (Figure 1) is due to the presence of flavonol glycosides and anthocyanins, which reduced the oxidative stress arising from H_2O_2 and UV exposure, thus explaining the reported protective effects against UV-induced skin damage. It is therefore emphasised that extracts of *C. ternatea* flower should be chemically-characterised (as in the present study) when subjected to further pharmacological tests relevant to cosmetic and therapeutic use, and when investigated in clinical studies, such that the constituents can be appropriately associated with the effects observed.

There is a paucity of data on the potential protective effects of *C. ternatea* flower extract against skin aging. However, other botanical extracts rich in anthocyanins or anthocyanidins have been reported to provide defence against UV-induced oxidative stress in human keratinocytes, including black soybean seed, orange extracts and pomegranate, and the constituents delphinidin and cyanidin 3-*O*-glucoside (Tsoyi *et al*. 2008; Cimino *et al*. 2006; Cimino *et al*. 2007; Svobodov *et al*. 2008). For example, pre-treatment and post-treatment with an anthocyanin rich extract (from bilberry) prevented UV damage by reducing ROS production, membrane lipid peroxidation and depletion of intracellular glutathione in HaCaT (Svobodov et al. 2008). The same study also showed similar suppression of H_2O_2-induced damage as reported here. In a different study using the RAW 264.7 macrophage cell line, the total *C. ternatea* extract and its flavonol fraction were able to suppress ROS generated by NADPH oxidase and mitochondria, whilst the anthocyanin fraction inhibited NO production (Nair *et al*. 2015). This further supports the protective effect of *C. ternatea* against skin aging.

We assessed the UV-induced mtDNA damage, as mtDNA is more susceptible to oxidative damage because of its lack of protective histones and close location to the ROS producing system (electron transport chain) (Birch-Machin *et al*. 2013; Krutmann & Schroeder 2009).UV-induced mtDNA damage has been measured in a previous study using qPCR, which is based on the observation that UV-induced damage can cause strand breaks in the mtDNA

(Kalinowski *et al*. 1992). The principle of the qPCR mtDNA damage assay is that any kind of DNA lesion can slow or block the progression of DNA polymerase, in which DNA samples with fewer lesions will be amplified to a greater extent, compared with DNA sample with more extensive damage (Furda *et al*. 2014). A study by Rothfuss *et al*. (2009) showed that the D-loop region of the mtDNA is more vulnerable to ROS-induced mtDNA damage, thus was the primer selected in this study to target the D-loop.

C. ternatea flower extract significantly reduced mtDNA damage caused by UV radiation, suggesting potential prevention of oxidative stress arising from mitochondrial dysfunction. It also has the ability to absorb UVB and UVC (Table 2) that may play a role in modulating the observed protective effects. The ability to act as a UV filter suggests further evaluation for its potential addition to sun protection topical products would be warranted. A cosmetic formulation containing an anthocyanin extract of purple sweet potato improved UV absorption, so reduced the amount of UV reaching the epidermis (Chan *et al*. 2010), further indicating the relevance of anthocyanins for their protective role against UV radiation. The observed results from this study provide the first scientific evidence and rationale for the potential anti-aging and skin-protective properties of a chemically-characterised *C. ternatea* flower extract.

CONCLUSION

This study demonstrated that an aqueous extract of *C. ternatea* flowers that contains flavonol glycosides and acylated anthocyanins as major constituents, was able to protect human keratinocytes from H_2O_2-induced cytotoxicity and UV-induced mtDNA damage, providing scientific evidence for its traditional and potential uses as a defence against skin aging. The results justify the further evaluation of *C. ternatea* flowers and constituents for their ability to protect skin against the effects of UV exposure, from both the cosmetic and therapeutic perspectives.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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