Manuscript Details

Manuscript number	JEP_2018_508_R1
Title	Anti-aging properties of Curcuma heyneana Valeton & Zipj: A scientific approach to its use in Javanese tradition.
Article type	Research Paper

Abstract

Ethnopharmacological relevance: Temu giring, the rhizome of Curcuma heyneana Valeton & Zipj (C. heyneana), is native to tropical regions, especially in Indonesia. It is traditionally used as a skin care, for cosmetic and body freshness, in Javanese and Balinese women, and has many other bioactivities such as antioxidant, anticancer, antiinflammatory. Aim of the study: The purpose of this study was to determine the antiaging activity of C. heyneana to prove its traditional use. Materials and methods: The antioxidant activity was determined using the DPPH free radical method, and anti-aging activity was examined using in vitro assays such as tyrosinase inhibitor and collagenase inhibitor. In vivo tests were performed by observing histomorphologic changes in rat skin exposed to Ultraviolet (UV) rays. The total curcuminoid contents and chromatographic profiles were determined by Thin layer Chromatography (TLC) - densitometry. Results: In all in vitro assays, all of the extracts showed a dose-dependent manner in the final concentration range from 62.5–500 μg/mL for DPPH assay and 31.25–250 μg/mL for Tyrosinase inhibitor and collagenase inhibitor assay. Curcuminoid (CUR), the active principle of Curcuma genus shows antioxidant, tyrosinase inhibitor and collagenase inhibitor activity greater than all C. heyneana extracts. The in vivo assay results showed that the topical application of the crude extract of C. heyneana produced significant improvement effects on the UV-induced skin structure damage. The total CUR content was correlated with the anti-aging activity of Curcuma heyneana. Conclusions: The results show that C. heyneana contains antioxidant compounds and has potent anti-aging activity, indicating that it can be used as an anti-aging drug candidate or as a phyto-cosmeceutical.

Keywords	Curcuma; anti aging; antioxidants; collagenase inhibitor; tyrosinase inhibitor; rat skin histomorphologic changes
Taxonomy	Medicinal Use of Plants, Pharmacognosy, Herbal Remedies

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To
Editor in Chief
Journal of Ethnopharmacology

Subject: Submission of Revised Manuscript (JEP_2018_508)

Dear Editor,

Enclosed herewith please find the revised manuscript entitled "**Anti-aging properties of *Curcuma heyneana* Valeton & Zipj: A scientific approach to its use in Javanese tradition.**" (authors: Idha Kusumawati, Kresma Oky Kurniawan, Subhan Rullyansyah, Tri Anggono Prijo, Retno Widyowati, Juni Ekowati, Eka Pramyrtha Hestianah, Suprapto Maat, Katsuyoshi Matsunami) (JEP_2018_508).

I hope that the revised manuscript is now acceptable for publication in the Journal of Ethnopharmacology.

We are looking forward to hearing your response.

Sincerely yours,
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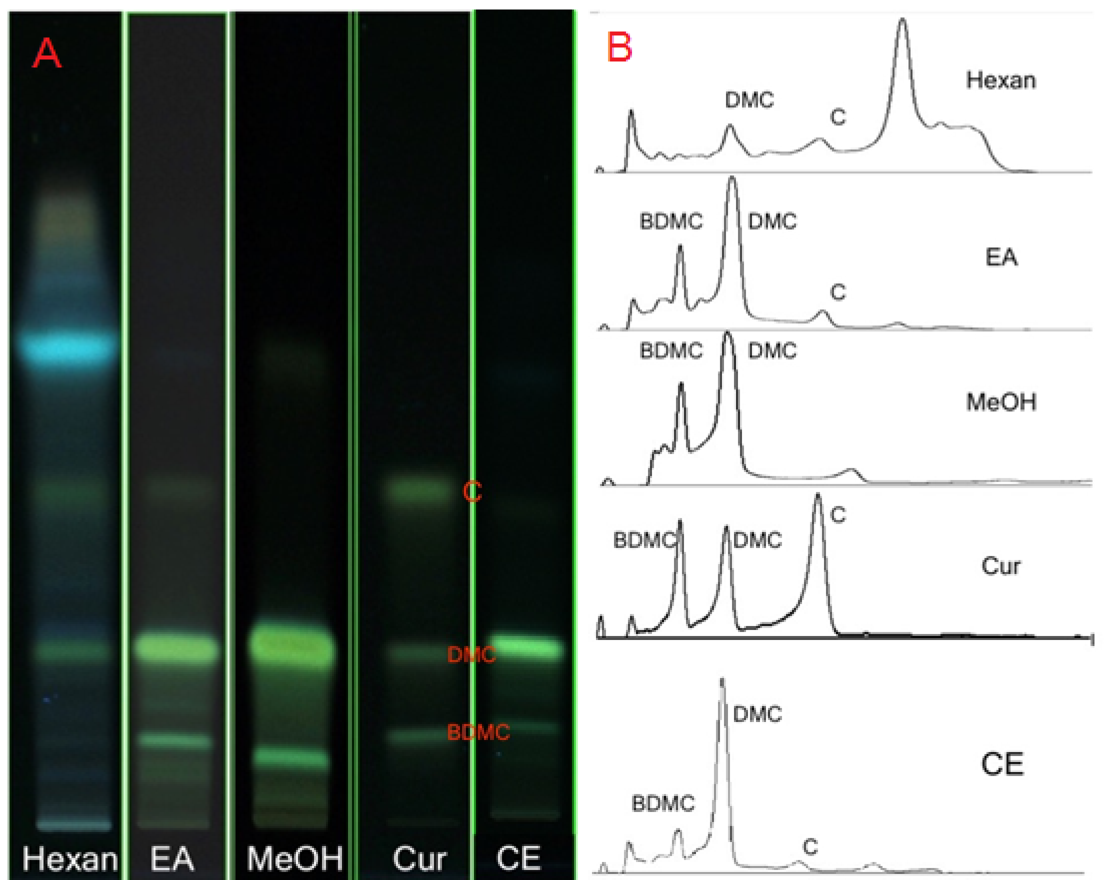
Reviewer 1

In this study, the antioxidant activity of Curcuma heyneana Valeton & Zipj were evaluated and the anti-aging activity was also been examined. This is a preliminary study.

1. TLC was used for the component analysis of Curcuminoids. The label for different loading strips is unclear. The difference of components from different fraction should be showed. Quantification needs to be compared. Additionally, the other methods such as HPLC and LC/MS/MS should be provided for enough components analysis.

    - Thank you for the valuable comment. Although we couldn't performed the analysis by LC-MS/MS because of the non availability of the instrument, we analysed again by TLC densitometry. I've changed the figure 1 that shows the photograph of TLC by adding a profile of chromatogram, so that differences in chemical content of various fractions can be seen clearly.



- Simultaneous quantitative determination of each component C, DMC and BDMC has also been performed and add the data according to the reviewer's comment as below.

Samples	Extraction Yield (g)	C (mg/g extract)	DMC (mg/g extract)	BDMC (mg/g extract)
CE	9.6900	3.11 ± 0.110a	15.97 ± 0.059a	3.25 ± 0.004a
HEX	6.4956	0.11 ± 0.091d	0.07 ± 0.011d	NA
EA	7.5991	0.73 ± 0.056b	3.62 ± 0.017b	2.40 ± 0.088b
MEOH	7.7003	0.27 ± 0.120c	1.77 ± 0.103c	0.93 ± 0.106c

-

2. DPPH assay is a method for anti-oxidant in vivo. For anti-aging, in vivo antioxidant activity should be considered
3. Other anti-aging animal experiments should be performed to confirm the anti-aging activity, including UV model.

For comments 2 and 3,

- Thank you for important comments. And we apologise to the reviewer for our compromising text.
- The in vitro antioxidant activity was evaluated by DPPH assay at first for judging the possibility of further study by using in vivo model (Figure 2, 3). As a result, the extract showed in vitro anti-oxidant activity (Figure 2). Therefore, we next performed UV induced in vivo skin damage model, because UV irradiation is one of the most plausible environmental factor for skin damage. The result was evaluated by histological analysis of rat skin (Figure 3). The topical skin aplication was choosed in our assay, not internal use, because of the traditional usage of Curcuma heyneana as for the treatment of skin (Ministry of Trade, 2009, and see also in our text).
-

Reviewer 2

The manuscript entitled "Anti-aging properties of Curcuma Heyneana Valeton and Zipj: A scientific approach to its use in Javanese tradition" study was investigated the antiaging properties of Curcuma Heyneana Valeton and Zipj in vitro and in vivo bioassays such as Antioxidant activity, tyrosinase inhibitor, collagenase inhibitor and the histomorphologic changes in rat skin exposed to Ultraviolet (UV).

Generally, there are two types of aging that exist may be due to genetic inheritance implicated in intrinsic (internal) involving cellular senescence and extrinsic (external) aging or photoaging. Therefore, the study performed by measuring the antioxidant activity and anti-aging activities in five increasing concentration of extracts in crude ethanolic extract, n - hexane extract, ethyl acetate extract and methanol extract groups. The histomorphologic

analysis of UV exposure on rat skin and changes after treatment with the samples (normal, vehicle, crude extract, and retinoic acid) were evaluated in rats.
Generally, manuscript is a clear and concise, it no needs correction. The İntroduction, methods, results and discussion are generally appropriate.

Specific comments follow.

Thank you for the valuable comments, we corrected according to the reviewer's comment as below.

Abstract
p.1, lines 4: the terms “anticancer, anti-inflammatory” must be “ anticancer and anti-inflammatory” ....corrected according to the comment.
p.1, lines 11: the terms "layer” must be “ Layer” .... corrected
p.1, lines 15: the terms “Tyrosinase” must be “ tyrosinase” .... corrected
Introduction
p. 4, lines 22: The term "aesthetic” must be "esthetic” .... corrected
Discussion
p. 17, line 1: The word " as" should be delete .... corrected
Conclusion
p. 17, line 1; The term "temu” must be "Temu” ..... corrected. Thank you for every comment

Reviewer 3
Thank you for the valuable comments, we corrected according to the reviewer's comment as below.

1. In abstract, Authors are advised to use the term ‘Tyrosinase inhibition and collagenase inhibition assays’ instead of inhibitor assays.
   .... corrected

2. In Materials and Methods Section No. 2.3, author has mentioned that, the total curcuminoid content of each extract was expressed in grams/100 grams of the extract. However, in Table No. 1, it was shown in mg/gm of the extract. Please clarify?
   The correct one is mg/g.........Sorry, it was our careless miss......... corrected

3. Authors are advised to describe in brief about the selection of mobile phases for chromatography estimation of total curcuminoid contents in each extract. Authors are advised to provide literature backups if any?
   The eluent used was obtained from the our optimization result, not from literature.
   We add a description in material & method section as follows; "The mobile phase condition was optimized to chloroform:methanol (40:1) through our study.

4. In Section No. 2.4, authors are advised to provide literature support for DPPH assay. In addition, authors are also advised to mention the concentration of DPPH in final reaction volume.
   The literature.was added................Thank you for the advise.
   Final concentration of DPPH is 250 µM...............added

5. In Section No. 2.5, authors are advised to provide literature support for Tyrosinase inhibition assay
   The literature..was added................. Thank you for the advise.

6. In Section No. 2.6, the final volume of the sample extracts in different concentrations are missing.
   Sorry for inconvinienice. ....added in the text
   Authors are advised to provide the sample volume added in the final reaction mixture.
   Sorry for inconvinienice. ....added in the text
   In this section, authors are advised to delete the institute name from where the substrate was obtained, as this information is already provided in sub-section 2.1.
   .Thank you for helpful suggestion.................done

7. In Section No. 2.8, authors are advised to provide literature support for this animal model.
   ..Thank you for helpful suggestion..................done

   In this, animal experiment, authors are advised to use the term ‘crude ethanolic extract’ instead of only crude extract.
   .Thank you for helpful suggestion... .done

   Authors are advised to explain briefly why ethanolic (crude) extract they have selected among all four extracts while all other extracts also have the potential in inhibiting Tyrosinase and Collagenase in a dose dependent manner.
   Thank you for valuable comment, we added the following text;

Crude ethanolic extracts was selected for further rat *in vivo* study, because the activity of antioxidant, tyrosinase inhibitor and collagenase inhibitor of CE was higher than the hexane, ethyl acetate and methanol fractions.

Authors are also advised to describe in brief rationale for selection of Retinoic acid as standard.

Thank you for important comment, we added a reference in material and method section as .... retinoic acid as positive controll (Kong et al., 2015).

Why was standard curcuminoids or Curcumin not used in this experiment for comparative analysis between crude ethanolic extracts and curcumin?

We focused on the crude extract, not purified curcuminoids and curcumin in this research. Because *C. heynana* has been traditionally used as a form of crude extract without fractionation and purification. In addition, the form of ethanolic crude extract is seems favorable for cosmeceutical products.

Anti-aging properties of *Curcuma heyneana* Valeton & Zipj: A scientific approach to its use in Javanese tradition.

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Abstract

Ethnopharmacological relevance: Temu giring, the rhizome of *Curcuma heyneana* Valeton & Zipj (*C. heyneana*), is native to tropical regions, especially in Indonesia. It is traditionally used as a skin care, for cosmetic and body freshness, in Javanese and Balinese women, and has many other bioactivities such as antioxidant, anticancer and antiinflammatory.

Aim of the study: The purpose of this study was to determine the antiaging activity of C. heyneana to prove its traditional use.

Materials and methods: The antioxidant activity was determined using the DPPH free radical method, and anti-aging activity was examined using *in vitro* assays such as tyrosinase inhibitor and collagenase inhibitor. *In vivo* tests were performed by observing histomorphologic changes in rat skin exposed to Ultraviolet (UV) rays. The total curcuminoid contents and chromatographic profiles were determined by Thin Layer Chromatography (TLC) - densitometry.

Results: In all in vitro assays, all of the extracts showed a dose-dependent manner in the final concentration range from 62.5–500 µg/mL for DPPH assay and 31.25–250 µg/mL for tyrosinase inhibition and collagenase inhibition assay. Curcuminoid (CUR), the active principle of *Curcuma* genus shows antioxidant, tyrosinase inhibitor and collagenase inhibitor activity greater than all *C. heyneana* extracts. The in vivo assay results showed that the topical application of the crude extract of *C. heyneana* produced significant improvement effects on the UV-induced skin structure damage. The total CUR content was correlated with the anti-aging activity of *Curcuma heyneana*.

Conclusions: The results show that *C. heyneana* contains antioxidant compounds and has potent anti-aging activity, indicating that it can be used as an anti-aging drug candidate or as a phyto-cosmeceutical.

Keywords: *Curcuma*, anti aging, antioxidants, collagenase inhibitor, tyrosinase inhibitor, rat skin histomorphologic changes

1. Introduction

Anti-aging medicine includes observation of visual appearance. Morphological changes to the physical constitution, appearance, and skin are simple indexes to measure aging. Old age is characterized by changes such as decreases in height, increases in the number of wrinkles, and decreases in the size of the face. Study of the mechanisms of aging and anti-aging medicine, in terms of the appearance, involves conducting research on the visual characteristics influenced by the aging process and by anti-aging medicine (Yamada, 2012; Fore 2006; Fisher et al. 2008)

Physical appearance is strongly influenced by aging of the skin. Changes in skin appearance provide visible signs of aging, including wrinkles, irregular pigmentation, sagging, and elastosis. Skin appearance changes have an important impact on self-esteem and social welfare. Physical appearance can be an indication of health condition, and may also be correlated with the risk of death (Cosgrove et al. 2007; Fore 2006; Sies & Stahl 2004). There are two types of skin aging. Intrinsic aging is a normal aging process. On the other hand, extrinsic aging or premature aging, also known as photoaging, is an aging process caused by environmental influences, particularly by UV radiation (Kusumawati & Indrayanto 2013; Saric & Sivamani 2016). UV exposure is essential for vitamin D activation, but on the skin, UV rays can cause serious problems, such as dermal degradation of collagen and elastic fibers, and can even cause skin cancer (Tu and Tawata 2015; Sies & Stahl 2004; Verschooten et al. 2006). UV radiation stimulates melanin production by human epidermal melanocytes in the skin. The content of melanin in the skin determines the color of the skin. Melanogenesis is a process by which the skin produces melanin. Melanin has a photoprotective function; however, accumulation in large quantities reduces the esthetic appearance. Melanogenesis is catalyzed by tyrosinase. Inhibition of tyrosinase inhibits the process of melanogenesis and is

expected to prevent abnormal pigmentation of the skin (Lin et al. 2011; Saewan & Jimtaisong 2013).

Traditionally, the people of Java and Bali use lulur or body scrub to assist in the prevention of the aging process. Lulur is part of beauty care and body freshness in Javanese royal tradition. Since the 17th century, the use of lulur has also been a part of the daily ritual for people of Java and Bali. Lulur has a remarkable softening effect on the skin, making the skin supple and glowing. One of the most well-known ingredients used for lulur in Java and Bali is temu giring (*C. heyneana*). Traditionally, lulur temu giring is used by brides to rejuvenate and soften the skin and eliminate bad odors on the body. Lulur temu giring is usually mixed with other ingredients such as rice flour, turmeric, pandan leaves, and lime peel, to form a natural scrub, and is applied to the skin every day until the wedding day (Ministry of Trade, 2009).

Curcuma (Zingiberaceae) is a rhizomatous species that has various ethnomedicinal uses. There are about 50 *Curcuma* species in the tropics, the most studied being turmeric (*C. longa)* (Dutta, 2015; Srivastava et al., 2009). Chemical content that has certain pharmacological activity is the key of a medicinal plant. These compounds become an important basis that guides its potential as a source of new drugs. The major bioactive compounds in the *Curcuma* genus is CUR that consisted curcumin (C) and its derivatives, demethoxy curcumin (DMC), and bis-demethoxy curcumin (BDMC); these have been shown to have a broad spectrum of biological action (Dutta 2015; Jayaprakasha et al. 2006). C has strong antioxidant properties, and thus, it is a choice for cancer prevention, liver protection, and premature aging (Anand et al., 2008; Gul and Basheer, 2016; Jayaprakasha et al. 2006; Mohiuddin et al. 2010).

C. heyneana, one of the species of the *Curcuma* genus, is a plant that is widely found in Java, Indonesia. It is used traditionally for the treatment of diseases such as for cancer and inflammatory conditions, and also for skin appearance. The chemical content of *C heyneana*

is known to include germacrone, dehydrocurdione, isocurcumenol, curcumenol, curcumanolides A and B, zerumbone, oxycurcumenol, and zedoarondiol (Cho et al., 2009). Based on its CUR content and antioxidant activity, in the current research, we performed *in vitro* and *in vivo* bioassays to investigate the anti-aging properties of this plant, in order to provide a scientific basis for its development as an anti-aging medicine or as a phytocosmeceutical.

2. Methods and Materials

2.1. Chemicals and solvents

The following reagents were analytical quality: curcumin (Sigma-Aldrich, Singapore), mushroom tyrosinase, a collagenase from ChC, DPPH (Sigma-Aldrich, Japan), and ((7-methoxycoumarin-4-yl) acetyl-L-prolyl-L-leucylglycyl-L-leucyl-[Nβ -(2,4- dinitrophenyl)-L-2,3-diaminopropionyl]-L-alanyl-L-arginine amide) (Peptide Institute, Osaka, Japan). Water was purified in a Millipore Milli-Q system (Bedford, MA). Solvents and reagents were purchased from TCI (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and Nacalai Tesque, Merck (Indonesia), unless otherwise specified. All chemicals and solvents were of analytical grade.

2.2. Plant material and extract preparation

C. heyneana rhizomes were obtained from a cultivated area in Pucangan hill, Jombang, East Java, Indonesia, in March 2015 (coordinates 112° 17’ 7’’ E and 7° 23’ 10’’ S). A voucher specimen was identified and deposited in the Herbarium of Airlangga University (KLN CH032015). The rhizomes were washed, cut into pieces, and dried using an oven at 40°C for three days. Dry rhizomes were ground into powder and stored in a sealed container until use. Ethanolic extract was obtained by extracting the plant material (100 g of rhizomes) using

70% ethanol (plant:solvent, 1:10, w/v), in a microwave (30% generator power), for one min. Dry rhizome powder (100 g) was also extracted, separately, with the same conditions, using n-hexane, ethyl acetate, and methanol, respectively. The extracts were dried by evaporating the solvent under reduced pressure to obtain the crude ethanol extract (CE), n-hexane extract (HEX), ethyl acetate extract (EA), and methanol extract (MEOH).

2.3. Simultaneus determination of C, DMC and BDMC content

Curcuminoid standard was dissolved in methanol at 1.0 mg/mL to obtain a stock solution. Five concentrations were derived from the stock solution in order to determine the calibration curve of Curcuminoid, and were applied on the TLC plate to obtain the final amounts of 65-130 ng/spot. The mobile phase condition was optimized to chloroform:methanol (40:1) through our study.

Each sample was prepared in methanol at 2.5 mg/mL, and was spotted 1.0 µL with a 7.0 mm bandwidth on an aluminum TLC plate 60F254 (20 × 10 cm; E. Merck, Germany) using a Camag Linomat 5 syringe, in triplicate, under the following conditions: application rate, 150 nL/s; space between each band, 7.0 mm; slit dimension, 5.00 mm × 0.45 mm; and scanning speed, 20 mm/s. The TLC plate was eluted for 8 cm using chloroform:methanol (40:1) as mobile phase, in the automated developing chamber version 2 (Camag, Muttenz, Switzerland). The plates were inspected using a CAMAG visualizer under 365 nm, and were photographed and uploaded to the computer software (WinCats). Densitometric scanning was carried out and operated by Camag Videoscan software. The C, DMC and BDMC content in the sample was determined by the peak area using linear regression, and was expressed in mg/g of the extract.

2.4. Antioxidant assay

The antioxidant activity was determined using a DPPH assay (Matsunami et al., 2006). The extract solution was prepared in DMSO at 10 mg/mL, and serially diluted into different concentrations. One microliter of each different concentrations of extract solution, in triplicate, was mixed with 100 µL freshly prepared DPPH methanolic solution (250mM), on a 96-well microplate; and the final concentrations of the extracts were 100, 50, 25, 12.5, and 6.25 ug/mL. After incubation in the dark for 30 min, at room temperature, DPPH level of each well was evaluated by detection of the absorbance at 515 nm, using a Multiscan Go Thermo Scientific microplate reader. DMSO was used as a negative control and trolox as a positive control. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

Inhibition ratio (%) = $[(A_{control} - A_{test}) / A_{test}] \times 100$

where $A_{control}$ is the absorbance of the control (DMSO) and A_{test} is the absorbance of the extracts. The IC50 of the inhibition ratio was determined graphically.

2.5. Tyrosinase inhibitor

Tyrosinase inhibitory activity was determined using the dopachrome method with L-DOPA as the substrate (Widyowati et al., 2016). The sample solution (10 µL) was mixed with 40 µL of 2.5 mM L-tyrosine solution in 0.05 M phosphate buffer (pH 6.80), and 50 µL of mushroom tyrosinase (100 U/mL) in the same buffer, in a 96-well microplate, and the final concentrations of the extracts were 100, 50, 25, 12.5, and 6.25 µg/mL, in triplicate. After incubation at 25°C for 5 min, the amount of dopachrome was determined by measuring the optical density (OD) at 475 nm using a Multiscan Go Thermo Scientific microplate reader. Arbutin was used as a positive control. The inhibition of tyrosinase was determined using the following formula:

Inhibition ratio (%) = $[1 - (A_{sample} - A_{blank}) / (A_{control} - A_{blank})] \times 100$

Where $A_{control}$ is without the test sample and A_{blank} is without both the test sample and tyrosinase. The IC50 of the inhibition ratio was determined graphically.

2.6. Collagenase inhibitor

Collagenase inhibitory activity was measured using the modified method described by Teramachi et al. (Teramachi et al., 2005). The sample mixture (10 µL), 10 µg/mL of the enzyme (collagenase derived from *Clostridium histolyticum*) and 50 mM Tricine buffer (pH 7.5), was added to a 96-well microtiter plate, the final concentrations of the extracts were 100, 50, 25, 12.5, and 6.25 µg/mL in triplicate. After 10 min incubation at 37°C, the substrate solution ((7-methoxycoumarin-4-yl) acetyl-L-prolyl-L-leucylglycyl-L-leucyl-[Nβ -(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-alanyl-L-arginine amide) ~~(Peptide Institute, Osaka, Japan)~~ was added, to obtain a final concentration of 10 µM, and was allowed to react. The fluorescence values were measured after 0 and 30 min incubation at 37°C, at an excitation of 320 nm and an emission of 405 nm, using a fluorescence plate reader (EnSpire, Perkin Elmer, Inc., Japan). Caffeic acid was used as a positive control, and the IC50 of the activity was determined graphically

2.7. Animals and histomorphologic analysis of UV exposure on rat skin

The research was designed based on the ethical standards for animal use, and was approved by the Airlangga University Ethical Committee of Animal Experimentation (protocol number 1146/10). UV model of animal experiment was done using the modified method described by Saric and Sivamani (Saric and Sivamani, 2016). Forty male rats (ICR), aged 3 months and weighing 150-200 g, were used in this study. They were obtained from the laboratory animal center.

The back of each rat was shaved every three days; the area shaved measured 3 x 3 cm. Rats were randomly grouped into four groups: normal, vehicle, crude extract, and retinoic acid as positive controll (Kong et al., 2015).

The observation time was six weeks. In the normal group (n = 10), animals did not receive UV ray exposure. In the vehicle group (n = 10), all animals received UV ray exposure for 21 days, followed by application of the vehicle (Natrium CMC 0.05%) topically for a 21 day period. In the crude ethanolic extract group (n = 10), all animals received UV ray exposure for 21 days, followed by application of the crude ethanolic extract (10% in the vehicle) topically for a 21 day period. In the retinoic acid group (n = 10), all animals received UV ray exposure for 21 days, followed by application of retinoic acid (0.05% in the vehicle) topically for a 21 day period.

2.7.1. Ultraviolet radiation (UVR)

UV light exposure was performed using a wooden box, which at the top of the box was installed a TL20W / 12RS UV lamp. Animals are placed inside the wooden box, where the distance of the UV lamp is 20 cm from the back of the animals. All animals from all groups, except animal of control group, were exposed for 60 seconds every day (exposure was increased 60 seconds every two days) for 21 days. The exposure time was optimized based on the strength of the lamp, according to the dose of erythematous on the skin of the animal.

2.7.2. Biopsy procedures and histologic analysis

Rat skin histology samples were obtained by fixing rat skin biopsies into 10% formalin solution, and were then embedded into paraffin blocks for sectioning and staining using a combination of hematoxylin–eosin. All of the sample histology slides were observed by one pathologist using an optical microscope (Olympus BX50, USA), and were captured by Software Cell D (Olympus).

2.8. Statistical analysis

The results are expressed as means ± SEM (standart error of the mean. Statistical differences between groups were estimated using one-way analysis of variance (ANOVA) with Duncan's test, and were considered statistically significant at $p < 0.05$.

3. Results

The extraction yield of CE obtained with the extraction process using 70% ethanol was 9.69 g. The extraction processes using n-hexane, ethyl acetate, and methanol, respectively, produced 6.4956 g HEX, 7.5991 g EA, and 7.7003 g MEOH (Table 1).

Table 1. The extraction yield of extract and the C, DMC and BDMC content in extracts of *C. heyneana* rhizome

Samples	Extraction Yield (g)	C (mg/g extract)	DMC (mg/g extract)	BDMC (mg/g extract)
CE	9.6900	3.11 ± 0.110a	15.97 ± 0.059a	3.25 ± 0.004a
HEX	6.4956	0.11 ± 0.091d	0.07 ± 0.011d	NA
EA	7.5991	0.73 ± 0.056b	3.62 ± 0.017b	2.40 ± 0.088b
MEOH	7.7003	0.27 ± 0.120c	1.77 ± 0.103c	0.93 ± 0.106c

Values are expressed as mean ± SEM (n = 3); means in the same column followed by different letters are significantly different at $p < 0.05$ using Duncan's tests. CE, crude ethanolic extract; HEX, n-hexane extract; EA, ethyl acetate extract; MEOH, methanol extract; C, Curcumin; DMC, demethoxy curcumin ; BDMC, bis-demethoxy curcumin.

3.1. Determination of C, DMC and BDMC content

CUR, consisting of C, DMC, and BDMC, is the principal active compound that contributes to the activity of plants in the genus *Curcuma* (Anand et al., 2008; Dutta, 2015; Gonçalves et al., 2014; Gul and Basheer, 2016; Jadoon et al., 2015; Sarvalkar et al., 2011). In figure 1A shows that DMC has a larger spot than C and BDMC in all extracts.

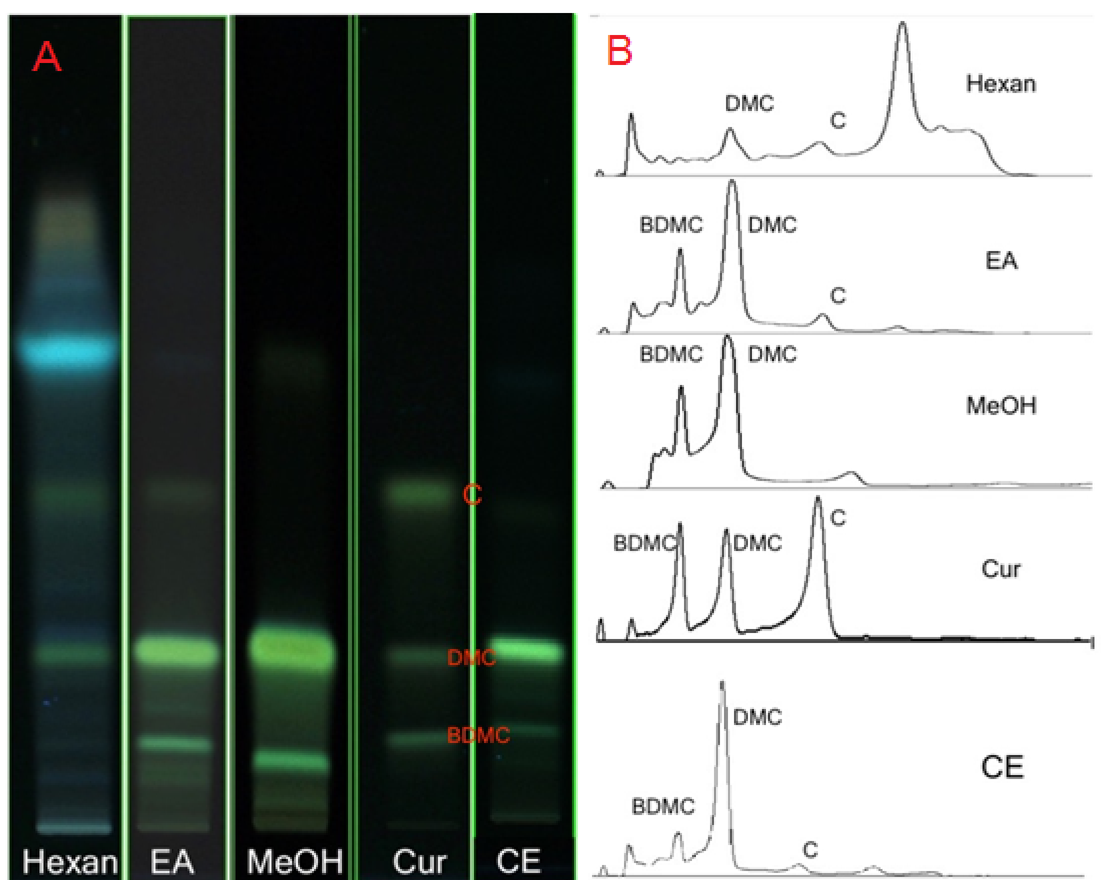


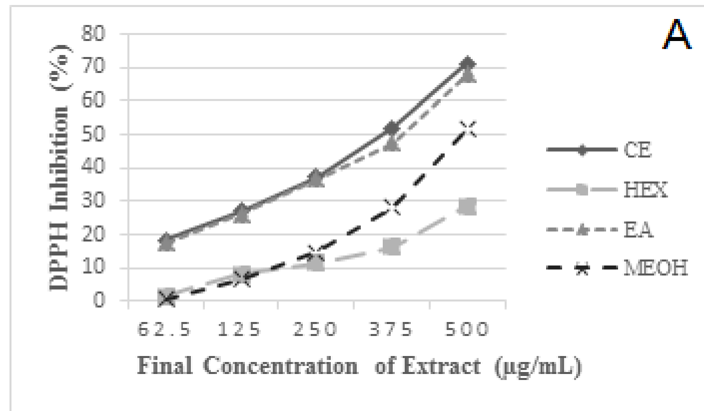
Figure 1. TLC profiles of HEX, EA, MEOH, CE and CUR (color online only).

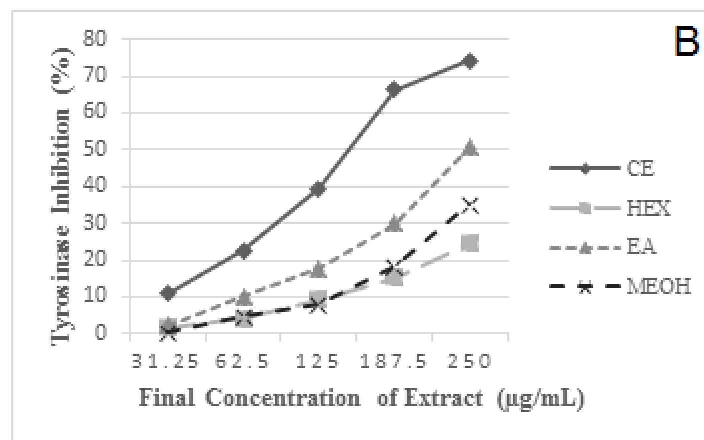
TLC plate was developed in chloroform:methanol (40:1) and was visualized as the spots with fluoresence color under 365 nm.

C, DMC and BDMC content was quantified using the TLC densitometric method, (Figure 1B). The results showed that the DMC has a larger peak area and content than C and BDMC in all extracts (Table 1).

3.2. Antioxidant, tyrosinase inhibitor, and collagenase inhibitor activity

In all assays, all of the extracts showed dose-dependent activity in the final concentration range from 62.5–500 μg/mL for the DPPH assay and 31.25–250 μg/mL for the tyrosinase inhibitor and collagenase inhibitor assays (Figure 2).





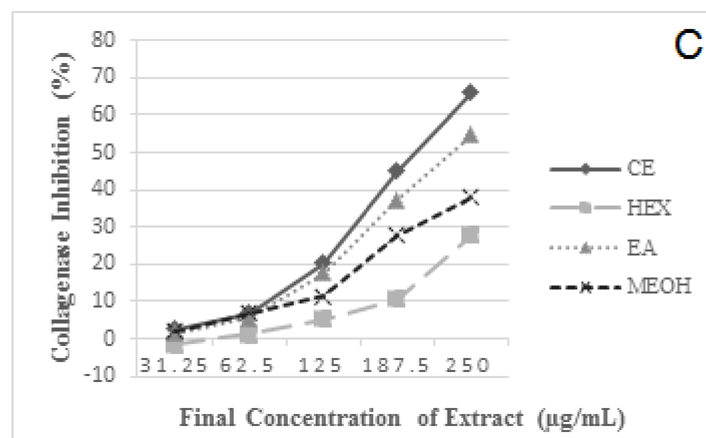


Figure 2. Dose-dependent antioxidant (A), tyrosinase inhibitor (B), and collagenase inhibitor (C) activity evaluated with *in vitro* assays. The tests were performed in triplicate. CE, crude ethanolic extract; HEX, n-hexane extract; EA, ethyl acetate extract; MEOH, methanol extract.

The IC50 calculation for each assay used the curves for each standard and the samples of *C. heyneana*. The curves were calculated using five concentration in the ranges described in Figure 2 and showed the linear relationships and strong correlation coefficients (r > 0.95). CUR, the principal active constituent of the *Curcuma* genus, showed antioxidant, tyrosinase inhibitor, and collagenase inhibitor activity greater than all *C. heyneana* extracts. All of the extracts had an IC50 greater than 300 µg/mL, and n-hexane and methanol extract had an IC50 greater than 500 µg/mL. The IC50 of tyrosinase inhibitor activity for all the extracts exhibited a wide range, from 155.50 to 403.99 µg/mL, while the collagenase inhibitor activity IC50 ranged from 204.80 to 454.75 µg/mL (Table 2).

Table 2. IC50 values of antioxidant, tyrosinase inhibitor, and collagenase inhibitor activity, for all extracts

Samples	IC50 (µg/mL)		
	Antioxidant	Tyrosinase Inhibitor	Collagenase Inhibitor
CUR	60.08 ± 1.17a	59.65 ± 1.19b	140.33 ± 2.01b
CE	338.18 ± 8.17b	155.50 ± 3.99c	204.80 ± 2.12c
HEX	more than 500	403.99 ± 0.41f	454.75 ± 5.52f
EA	363.26 ± 7.21b	265.44 ± 1.70d	238.58 ± 1.07d
MEOH	more than 500	359.04 ± 12.15e	323.44 ± 5.61e
T	28.97 ± 0.41a	-	-
AR	-	38.02 ± 0.25a	-
CA	-	-	30.16 ± 0.55a

Values are expressed as mean ± SEM (n = 3); means in the same column followed by different letters are significantly different at $p < 0.05$ using Duncan's tests. CUR, curcuminoid; CE, crude ethanolic extract; HEX, n-hexane extract; EA, ethyl acetate extract; MEOH, methanol extract; T, trolox; AR, arbutin; CA, caffeic acid.

Linear range (µg/mL) for antioxidant assay: T = 6.25–50; C = 12.50–100; samples = 62.50–500.

Linear range (µg/mL) for tyrosinase inhibitor assay: AR = 6.25–50; C = 12.5–100; samples = 62.5–500.

Linear range (µg/mL) for collagenase inhibitor assay: CA = 6.25–50; C = 31.25–250; samples = 62.5–500 .

3.3. Histomorphologic effects of UV exposure on rat skin

Crude ethanolic extracts was selected for further rat *in vivo* study, because the activity of antioxidant, tyrosinase inhibitor and collagenase inhibitor of CE was higher than the hexane, ethyl acetate and methanol fractions. Histology analysis showed changes caused by UV exposure and changes after treatment with the samples. In Figure 3, after UV exposure epidermal thickness (ET) of vehicle group increased three-fold from 4.07 ± 0.27 µm (normal skin) to 12.21 ± 0.41 µm. Topical application of the extract for 21 days decreased the ET to 8.09 ± 0.10 µm, while topical application of retinoic acid as a reference drug decreased the ET to 4.14 ± 0.27 µm, which was not significantly different to the normal group. UV ray exposure caused pyknotic nuclei of epidermal cells, considered to be sunburn cells (SBCs). SBCs incidence was calculated per linear millimeter of the epidermis for each animal. There was only 4.40 ± 0.28 SBC/mm2 in the epidermis of normal rats. UV exposure increased the SBCs in vehicle group to 60.80 ± 2.56 SBC/mm2. Topical application of the extract decreased six-fold in the number of SBC. Normal dermis showed orderly arranged collagen,

abundant elastic fibers, and extrafibrillar matrix. In vehicle group, UV exposure damaged the arrangement of collagen so that the distance between collagen increased by approximately five times, as compared to normal rat skin. Topical application of the extract decreased the distance between collagen by 22% compared to normal group, while application of retinoic acid regenerated the distance between collagen to a normal group.

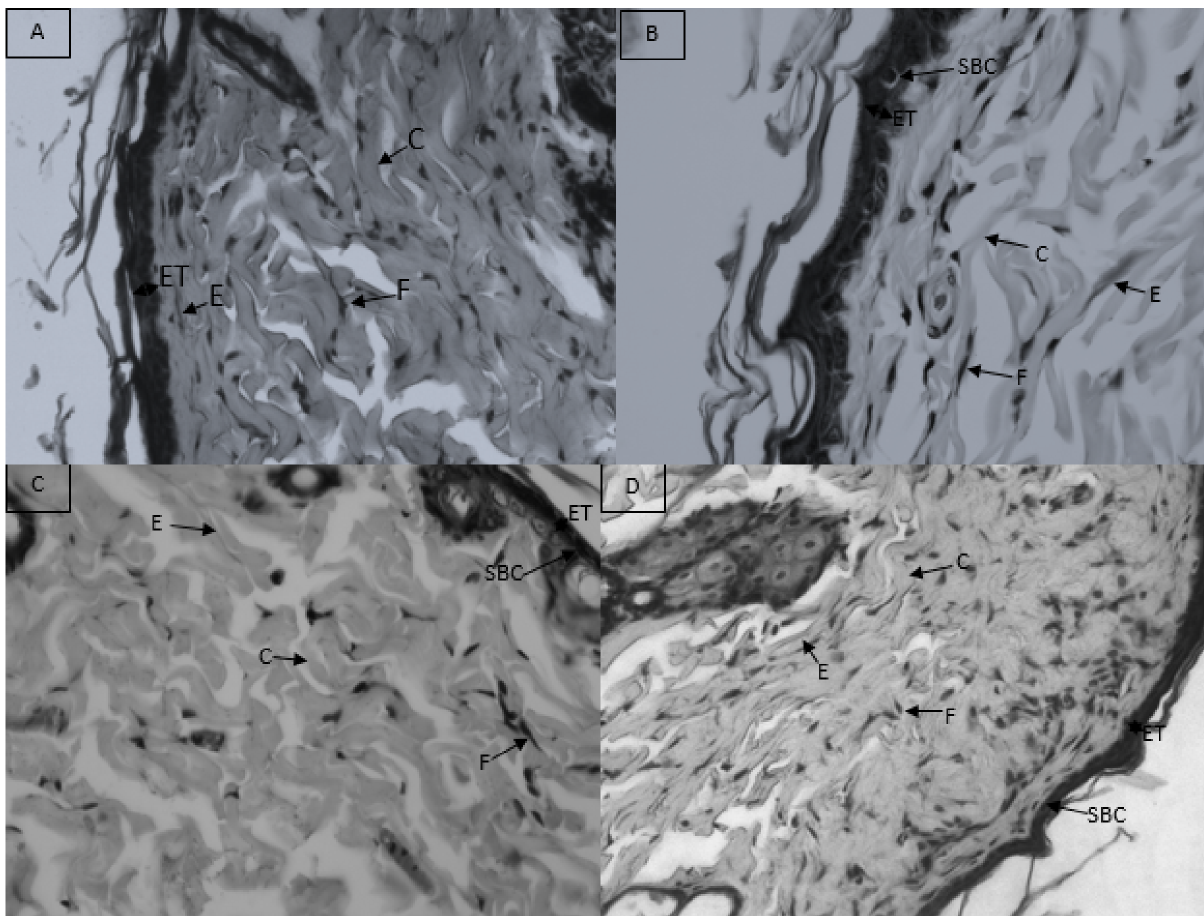


Figure 3. Histologic analysis of rat skin specimen.

Histologic rat skin specimens was performed using H & E staining. (A) Normal group, no UV exposure + no treatment; (B) vehicle group, UV exposure + vehicle treatment; (C) crude extract group, UV exposure + crude extract treatment; (D) retinoic acid group, UV exposure + retinoic acid treatment. Cellular morphology was viewed at 40x magnification. ET, epidermal thickness; SBC, sunburn cell; C, collagen fiber; F, fibroblast; E, elastic fiber.

Normal skin had 41.53 ± 0.60 fibroblasts/10,000 μm^2. In vehicle group, after UV exposure, the number of fibroblast decreased to 9.74 ± 0.26 fibroblasts/10,000 μm^2. After topical application of the crude extract, the number of fibroblast increased to 16.60 ± 0.33 fibroblasts/10,000 μm^2; but the application of retinoid acid increased the number of fibroblast larger. In addition, in the normal dermal layer, the elastic fibers were neatly arranged, but in the vehicle group, after UV exposure, the number of elastic fibers reduced, and the structure became irregular. Topical application of crude extract increased the number of elastic fibers so that the arrangement was tightly and regularly (Table 3).

Table 3. Histologic analysis of UV exposed rat skin specimens

Groups	Epidermal Thickness (μm)	Number of SBC/mm^2 in Epidermis	Number of Fibroblasts/10,000 μm^2	Distance Between Collagen Fibers (μm)	Elastic Fibers
Normal	4.07 ± 0.27b	4.40 ± 0.28b	41.53 ± 0.60b	3.09 ± 0.10b	++++
Vehicle	12.21 ± 0.41a	60.80 ± 2.56a	9.74 ± 0.26a	14.70 ± 0.40a	+
CE	8.09 ± 0.10a,b	11.80 ± 0.95ab	16.60 ± 0.33a,b	11.60 ± 0.42ab	++
RA	4.14 ± 0.27b	28.00 ± 1.76ab	40.86 ± 0.30b	3.02 ± 0.16ab	++++

Values are expressed as mean ± SEM (n = 10); means in the same column followed by different letters are significantly different at $p < 0.05$ using Duncan's tests. (a) Significantly different from the normal group at $p < 0.05$. (b) Significantly different from the vehicle group at $p < 0.05$. Normal group, no UV exposure + no treatment; vehicle group, UV exposure + vehicle treatment; CE group, UV exposure + crude extract treatment; RA group, UV exposure + retinoic acid treatment.

The results showed that the topical application of the crude extract of *C. heyneana* produced significant improvement in the UV-induced damage to the skin structure.

4. Discussion

The chromatographic profile of the extract indicates the pattern of biological activity or the chemical content present in the extract (Bhise and Salunkhe, 2009; Sanja et al., 2009). All compounds, not only active compounds, has a contribution to pharmacological activity of the extract so that chromatographic profile analysis is a comprehensive approach to the quality consistency and stability of the extract. In addition, other approaches are also made by selecting one or more compounds used for herbal product quality assurance. This selected compound is usually known as an active principle of a medicinal plant (Xie et al., 2006). In the *Curcuma* genus, CUR have been known as the principal active constituents that play a role in the various biological activities; these consist of C, DMC, and BDMC (Amalraj et al., 2017; Sasikumar, 2005). The total curcuminoid content found in the *C. heyneana* samples was lower than other *Curcuma* species reported in the literature (Dutta, 2015; Nahak and Rajani, 2011). The CUR content in turmeric comprises ≈77% C, ≈17% DMC, and ≈3% BDMC (Goel and Aggarwal, 2010). All extracts of *C. heyneana* exhibited higher DMC than C (Figure 1). These differences result in lower antioxidant activity of the *C. heyneana* extract, compared to C. CE had higher total CUR content than HEX, EA and MEOH. CE also exhibited higher antioxidant activity, tyrosinase inhibitor activity and collagenase inhibitor activity than HEX, EA and MEOH. This suggests that differences in total CUR content in the extracts affect their biological activity.

Photoaging is caused by chronic exposure to UV light. This leads to incomplete repair of damage, which ultimately results in abnormal structures and functions; this is termed premature aging (Chiu et al., 2017; Fore, 2006). UV exposure on rat skin for 21 days,

according to erythema dose, caused changes in the structure and affected the function of the skin. Epidermal thickness increased three-fold compared to normal conditions. This is due to the regeneration of cells in basal keratinocytes. Mature keratinocytes develop into stratum spinosum, stratum granulosum, and stratum corneum. This is reflected by an increase in epidermal thickness, acting as a protective function of the epidermis against UV exposure (Fore, 2006). The thickness of rat skin epidermis treated with topical CE and retinoic acid was lower than that treated with the vehicle only. Retinoic acid has been used for skin aging therapy. The use of topical retinoic acid shows histologic improvements such as increased collagen synthesis through blocking collagenase activity which is the basis of skin aging therapy (Kong et al., 2015). The ability of retinoic acid to suppress the increase in ET was greater than that of the CE. Both retinoic acid and the CE induced intrinsic skin protection, in order to restore normal skin conditions.

The histological appearance of UV exposed rat skin revealed a large number of SBCs. UV exposure is believed to cause direct injury to nuclear DNA in keratinocytes, thus stimulating the formation of SBCs. SBCs are easily recognized by their pyknotic nuclei and eosinophilic cytoplasm. Ultrastructurally, SBCs show the characteristics of apoptotic keratinocytes, with the appearance of nuclear chromatin condensation and dark cytoplasm tonofilament due to densely packed bundles. The decrease in the number of SBCs suggests a mechanism of defense or repair of DNA damage so there is no stimulation of apoptotic keratinocyte (Okada et al., 2003).

UV exposure causes dermal changes in both cellular and cell matrix components. The dermal layer contains connective tissue composed of various types of collagens, elastin, fibronectin, proteoglycan, and other extracellular matrix molecules. UV exposure causes an imbalance between the synthesis and degradation of the extracellular components. This imbalance caused the amount of fibroblasts decreases and thus affects its functional ability. The

synthesis of new collagen decreases, collagen fibers become messy, also collagen fragmentation that causes disorganization of the whole skin. Structure and composition Elastic fibers undergo irreversible changes so elastin becomes amorphous and functional activity is poor. The structure and composition of elastic fibers changes, causing fibers with amorphous elastin, which have poor functional activity. Collagen fibers become disorganized, randomly oriented, and fragmented (Ghersetich et al., 1994; Hwang et al., 2012; Pasquali-Ronchetti and Baccaeani-Contri, 1997; Zouboulis and Boschnakow, 2001).

Chronic UV skin exposure causes photoaging which is characterized by thickening, roughness, coarse wrinkles, mottled pigmentation, and histologic changes, including impairment of collagen fibers, extreme accumulation of abnormal elastic fibers, and an enhancement of glycosaminoglycans (Serafini et al., 2014). Synthetic or natural substances that can inhibit, delay, or repair damage caused by UV exposure are referred to as photo-chemoprevention.

The results of this study indicated that the antioxidant activity of crude extract *C. heyneana* was smaller than curcuminoid. This was related to the content of demethoxy curcumin greater than curcumin in *C. heyneana*. This research also showed that curcuminoid levels in crude *C. heyneana* extract plays a significant role in antioxidant activity. Crude extract C. heyneana also had activity of tyrosinase inhibitor and collagenase inhibitor. This corresponded to improvements in the appearance of UV induced-rat skin histomorphometry with parameters such as epidermal thickness, number of sunburn cells, collagen tissue, fibroblasts and elastin.

5. Conclusions

C. heyneana known ~~as~~ Temu giring has been used traditionally as skin care. The present study showed that crude extract of *C.heyneana* has the potential to be used as an anti-aging compound, and may be developed into phytocosmeceutical products.

Glossary

AR : Arbutin

BDMC : Bis-demethoxy Curcumin

C : Cucumin

CA : Caffeic Acid

CE : Crude Ethanol Extract

CUR : Curcuminoid

DMC : Demethoxy Curcumin

EA : Ethyl Acetate Extract

ET : Epidermis Thickness

HEX : n-Hexane Extract

MEOH : Methano Extract

RA : Retinoic Acid

SBC : Sunburn Cell

SEM : Standart Error of Mean

T : Trolox

TLC : Thin Layer Chromatography

UV : Ultra Violet

Acknowledgments

This work was supported by an International Research Collaboration and Scientific Publication grant from The Ministries Of Research, Technology, and Higher Education, Republic of Indonesia (Grant no. 597/UN3.14/LT/2017). Thanks to PT. Rembaka who has given the drug reference used in the in vivo test.

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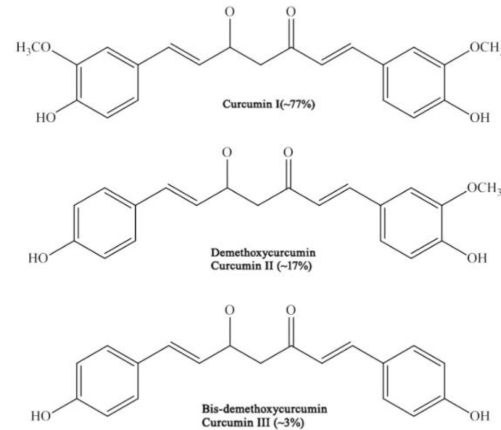
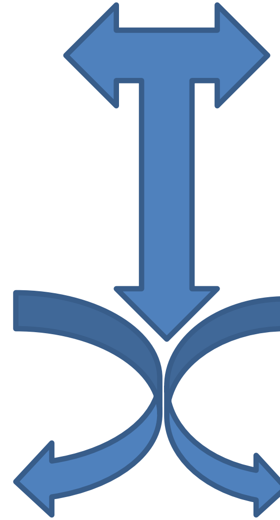
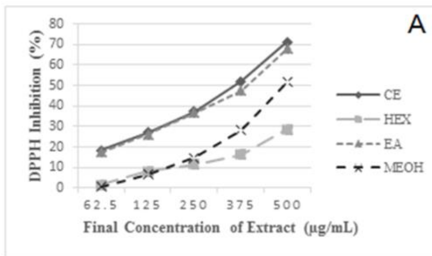
Traditionally uses *C. heyneana* as skin care (body scrub & face mask)

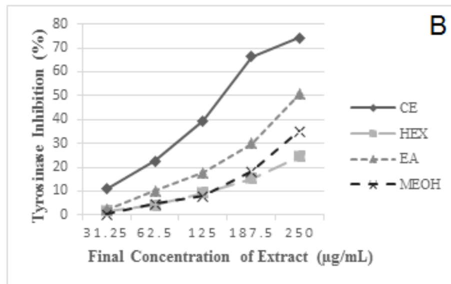


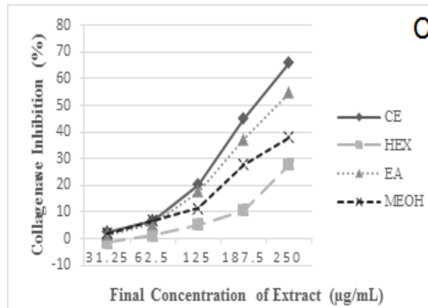
The active principle substances in *C. heyneana*



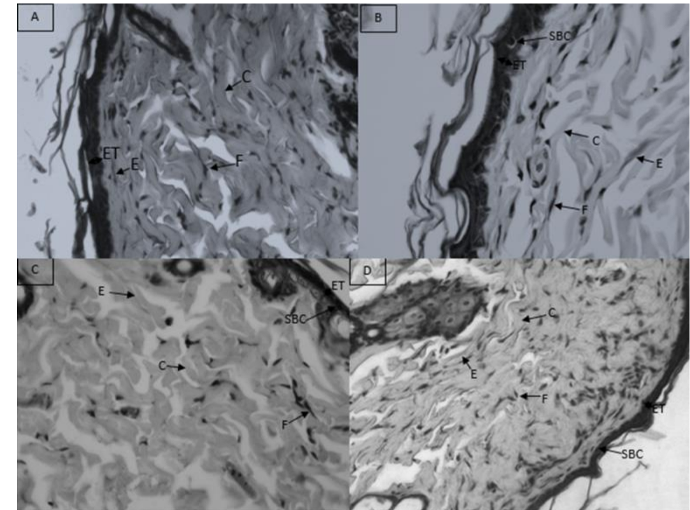
Anti aging in vitro assay







Anti aging in vivo assay



Anti-aging properties of *Curcuma heyneana* Valeton & Zipj: A scientific approach to its use in Javanese tradition.

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Abstract

Ethnopharmacological relevance: Temu giring, the rhizome of *Curcuma heyneana* Valeton & Zipj (*C. heyneana*), is native to tropical regions, especially in Indonesia. It is traditionally used as a skin care, for cosmetic and body freshness, in Javanese and Balinese women, and has many other bioactivities such as antioxidant, anticancer and antiinflammatory.

Aim of the study: The purpose of this study was to determine the antiaging activity of C. heyneana to prove its traditional use.

Materials and methods: The antioxidant activity was determined using the DPPH free radical method, and anti-aging activity was examined using *in vitro* assays such as tyrosinase inhibitor and collagenase inhibitor. *In vivo* tests were performed by observing histomorphologic changes in rat skin exposed to Ultraviolet (UV) rays. The total curcuminoid contents and chromatographic profiles were determined by Thin Layer Chromatography (TLC) - densitometry.

Results: In all in vitro assays, all of the extracts showed a dose-dependent manner in the final concentration range from 62.5–500 μg/mL for DPPH assay and 31.25–250 μg/mL for tyrosinase inhibition and collagenase inhibition assay. Curcuminoid (CUR), the active principle of *Curcuma* genus shows antioxidant, tyrosinase inhibitor and collagenase inhibitor activity greater than all *C. heyneana* extracts. The in vivo assay results showed that the topical application of the crude extract of *C. heyneana* produced significant improvement effects on the UV-induced skin structure damage. The total CUR content was correlated with the anti-aging activity of *Curcuma heyneana*.

Conclusions: The results show that *C. heyneana* contains antioxidant compounds and has potent anti-aging activity, indicating that it can be used as an anti-aging drug candidate or as a phyto-cosmeceutical.

Keywords: *Curcuma*, anti aging, antioxidants, collagenase inhibitor, tyrosinase inhibitor, rat skin histomorphologic changes

1. Introduction

Anti-aging medicine includes observation of visual appearance. Morphological changes to the physical constitution, appearance, and skin are simple indexes to measure aging. Old age is characterized by changes such as decreases in height, increases in the number of wrinkles, and decreases in the size of the face. Study of the mechanisms of aging and anti-aging medicine, in terms of the appearance, involves conducting research on the visual characteristics influenced by the aging process and by anti-aging medicine (Yamada, 2012; Fore 2006; Fisher et al. 2008)

Physical appearance is strongly influenced by aging of the skin. Changes in skin appearance provide visible signs of aging, including wrinkles, irregular pigmentation, sagging, and elastosis. Skin appearance changes have an important impact on self-esteem and social welfare. Physical appearance can be an indication of health condition, and may also be correlated with the risk of death (Cosgrove et al. 2007; Fore 2006; Sies & Stahl 2004). There are two types of skin aging. Intrinsic aging is a normal aging process. On the other hand, extrinsic aging or premature aging, also known as photoaging, is an aging process caused by environmental influences, particularly by UV radiation (Kusumawati & Indrayanto 2013; Saric & Sivamani 2016). UV exposure is essential for vitamin D activation, but on the skin, UV rays can cause serious problems, such as dermal degradation of collagen and elastic fibers, and can even cause skin cancer (Tu and Tawata 2015; Sies & Stahl 2004; Verschooten et al. 2006). UV radiation stimulates melanin production by human epidermal melanocytes in the skin. The content of melanin in the skin determines the color of the skin. Melanogenesis is a process by which the skin produces melanin. Melanin has a photoprotective function; however, accumulation in large quantities reduces the esthetic appearance. Melanogenesis is catalyzed by tyrosinase. Inhibition of tyrosinase inhibits the process of melanogenesis and is

expected to prevent abnormal pigmentation of the skin (Lin et al. 2011; Saewan & Jimtaisong 2013).

Traditionally, the people of Java and Bali use lulur or body scrub to assist in the prevention of the aging process. Lulur is part of beauty care and body freshness in Javanese royal tradition. Since the 17th century, the use of lulur has also been a part of the daily ritual for people of Java and Bali. Lulur has a remarkable softening effect on the skin, making the skin supple and glowing. One of the most well-known ingredients used for lulur in Java and Bali is temu giring (*C. heyneana*). Traditionally, lulur temu giring is used by brides to rejuvenate and soften the skin and eliminate bad odors on the body. Lulur temu giring is usually mixed with other ingredients such as rice flour, turmeric, pandan leaves, and lime peel, to form a natural scrub, and is applied to the skin every day until the wedding day (Ministry of Trade, 2009).

Curcuma (Zingiberaceae) is a rhizomatous species that has various ethnomedicinal uses. There are about 50 *Curcuma* species in the tropics, the most studied being turmeric (*C. longa)* (Dutta, 2015; Srivastava et al., 2009). Chemical content that has certain pharmacological activity is the key of a medicinal plant. These compounds become an important basis that guides its potential as a source of new drugs. The major bioactive compounds in the *Curcuma* genus is CUR that consisted curcumin (C) and its derivatives, demethoxy curcumin (DMC), and bis-demethoxy curcumin (BDMC); these have been shown to have a broad spectrum of biological action (Dutta 2015; Jayaprakasha et al. 2006). C has strong antioxidant properties, and thus, it is a choice for cancer prevention, liver protection, and premature aging (Anand et al., 2008; Gul and Basheer, 2016; Jayaprakasha et al. 2006; Mohiuddin et al. 2010).

C. heyneana, one of the species of the *Curcuma* genus, is a plant that is widely found in Java, Indonesia. It is used traditionally for the treatment of diseases such as for cancer and inflammatory conditions, and also for skin appearance. The chemical content of *C heyneana*

is known to include germacrone, dehydrocurdione, isocurcumenol, curcumenol, curcumanolides A and B, zerumbone, oxycurcumenol, and zedoarondiol (Cho et al., 2009). Based on its CUR content and antioxidant activity, in the current research, we performed *in vitro* and *in vivo* bioassays to investigate the anti-aging properties of this plant, in order to provide a scientific basis for its development as an anti-aging medicine or as a phytocosmeceutical.

2. Methods and Materials

2.1. Chemicals and solvents

The following reagents were analytical quality: curcumin (Sigma-Aldrich, Singapore), mushroom tyrosinase, a collagenase from ChC, DPPH (Sigma-Aldrich, Japan), and ((7-methoxycoumarin-4-yl) acetyl-L-prolyl-L-leucylglycyl-L-leucyl-[Nβ -(2,4- dinitrophenyl)-L-2,3-diaminopropionyl]-L-alanyl-L-arginine amide) (Peptide Institute, Osaka, Japan). Water was purified in a Millipore Milli-Q system (Bedford, MA). Solvents and reagents were purchased from TCI (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and Nacalai Tesque, Merck (Indonesia), unless otherwise specified. All chemicals and solvents were of analytical grade.

2.2. Plant material and extract preparation

C. heyneana rhizomes were obtained from a cultivated area in Pucangan hill, Jombang, East Java, Indonesia, in March 2015 (coordinates 112° 17’ 7’’ E and 7° 23’ 10’’ S). A voucher specimen was identified and deposited in the Herbarium of Airlangga University (KLN CH032015). The rhizomes were washed, cut into pieces, and dried using an oven at 40°C for three days. Dry rhizomes were ground into powder and stored in a sealed container until use. Ethanolic extract was obtained by extracting the plant material (100 g of rhizomes) using

70% ethanol (plant:solvent, 1:10, w/v), in a microwave (30% generator power), for one min. Dry rhizome powder (100 g) was also extracted, separately, with the same conditions, using n-hexane, ethyl acetate, and methanol, respectively. The extracts were dried by evaporating the solvent under reduced pressure to obtain the crude ethanol extract (CE), n-hexane extract (HEX), ethyl acetate extract (EA), and methanol extract (MEOH).

2.3. Simultaneus determination of C, DMC and BDMC content

Curcuminoid standard was dissolved in methanol at 1.0 mg/mL to obtain a stock solution. Five concentrations were derived from the stock solution in order to determine the calibration curve of Curcuminoid, and were applied on the TLC plate to obtain the final amounts of 65-130 ng/spot. The mobile phase condition was optimized to chloroform:methanol (40:1) through our study.

Each sample was prepared in methanol at 2.5 mg/mL, and was spotted 1.0 µL with a 7.0 mm bandwidth on an aluminum TLC plate 60F254 (20 × 10 cm; E. Merck, Germany) using a Camag Linomat 5 syringe, in triplicate, under the following conditions: application rate, 150 nL/s; space between each band, 7.0 mm; slit dimension, 5.00 mm × 0.45 mm; and scanning speed, 20 mm/s. The TLC plate was eluted for 8 cm using chloroform:methanol (40:1) as mobile phase, in the automated developing chamber version 2 (Camag, Muttenz, Switzerland). The plates were inspected using a CAMAG visualizer under 365 nm, and were photographed and uploaded to the computer software (WinCats). Densitometric scanning was carried out and operated by Camag Videoscan software. The C, DMC and BDMC content in the sample was determined by the peak area using linear regression, and was expressed in mg/g of the extract.

2.4. Antioxidant assay

The antioxidant activity was determined using a DPPH assay (Matsunami et al., 2006). The extract solution was prepared in DMSO at 10 mg/mL, and serially diluted into different concentrations. One microliter of each different concentrations of extract solution, in triplicate, was mixed with 100 µL freshly prepared DPPH methanolic solution (250mM), on a 96-well microplate; and the final concentrations of the extracts were 100, 50, 25, 12.5, and 6.25 ug/mL. After incubation in the dark for 30 min, at room temperature, DPPH level of each well was evaluated by detection of the absorbance at 515 nm, using a Multiscan Go Thermo Scientific microplate reader. DMSO was used as a negative control and trolox as a positive control. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

Inhibition ratio (%) = $[(A_{control} - A_{test}) / A_{test}] \times 100$

where $A_{control}$ is the absorbance of the control (DMSO) and A_{test} is the absorbance of the extracts. The IC50 of the inhibition ratio was determined graphically.

2.5. Tyrosinase inhibitor

Tyrosinase inhibitory activity was determined using the dopachrome method with L-DOPA as the substrate (Widyowati et al., 2016). The sample solution (10 µL) was mixed with 40 µL of 2.5 mM L-tyrosine solution in 0.05 M phosphate buffer (pH 6.80), and 50 µL of mushroom tyrosinase (100 U/mL) in the same buffer, in a 96-well microplate, and the final concentrations of the extracts were 100, 50, 25, 12.5, and 6.25 µg/mL, in triplicate. After incubation at 25°C for 5 min, the amount of dopachrome was determined by measuring the optical density (OD) at 475 nm using a Multiscan Go Thermo Scientific microplate reader. Arbutin was used as a positive control. The inhibition of tyrosinase was determined using the following formula:

Inhibition ratio (%) = $[1 - (A_{sample} – A_{blank}) / (A_{control} - A_{blank})] \times 100$

Where $A_{control}$ is without the test sample and A_{blank} is without both the test sample and tyrosinase. The IC50 of the inhibition ratio was determined graphically.

2.6. Collagenase inhibitor

Collagenase inhibitory activity was measured using the modified method described by Teramachi et al. (Teramachi et al., 2005). The sample mixture (10 μL), 10 μg/mL of the enzyme (collagenase derived from *Clostridium histolyticum*) and 50 mM Tricine buffer (pH 7.5), was added to a 96-well microtiter plate, the final concentrations of the extracts were 100, 50, 25, 12.5, and 6.25 μg/mL in triplicate. After 10 min incubation at 37°C, the substrate solution ((7-methoxycoumarin-4-yl) acetyl-L-prolyl-L-leucylglycyl-L-leucyl-[Nβ -(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-alanyl-L-arginine amide) ~~(Peptide Institute, Osaka, Japan)~~ was added, to obtain a final concentration of 10 μM, and was allowed to react. The fluorescence values were measured after 0 and 30 min incubation at 37°C, at an excitation of 320 nm and an emission of 405 nm, using a fluorescence plate reader (EnSpire, Perkin Elmer, Inc., Japan). Caffeic acid was used as a positive control, and the IC50 of the activity was determined graphically

2.7. Animals and histomorphologic analysis of UV exposure on rat skin

The research was designed based on the ethical standards for animal use, and was approved by the Airlangga University Ethical Committee of Animal Experimentation (protocol number 1146/10). UV model of animal experiment was done using the modified method described by Saric and Sivamani (Saric and Sivamani, 2016). Forty male rats (ICR), aged 3 months and weighing 150-200 g, were used in this study. They were obtained from the laboratory animal center.

The back of each rat was shaved every three days; the area shaved measured 3 x 3 cm. Rats were randomly grouped into four groups: normal, vehicle, crude extract, and retinoic acid as positive controll (Kong et al., 2015).

The observation time was six weeks. In the normal group (n = 10), animals did not receive UV ray exposure. In the vehicle group (n = 10), all animals received UV ray exposure for 21 days, followed by application of the vehicle (Natrium CMC 0.05%) topically for a 21 day period. In the crude ethanolic extract group (n = 10), all animals received UV ray exposure for 21 days, followed by application of the crude ethanolic extract (10% in the vehicle) topically for a 21 day period. In the retinoic acid group (n = 10), all animals received UV ray exposure for 21 days, followed by application of retinoic acid (0.05% in the vehicle) topically for a 21 day period.

2.7.1. Ultraviolet radiation (UVR)

UV light exposure was performed using a wooden box, which at the top of the box was installed a TL20W / 12RS UV lamp. Animals are placed inside the wooden box, where the distance of the UV lamp is 20 cm from the back of the animals. All animals from all groups, except animal of control group, were exposed for 60 seconds every day (exposure was increased 60 seconds every two days) for 21 days. The exposure time was optimized based on the strength of the lamp, according to the dose of erythematous on the skin of the animal.

2.7.2. Biopsy procedures and histologic analysis

Rat skin histology samples were obtained by fixing rat skin biopsies into 10% formalin solution, and were then embedded into paraffin blocks for sectioning and staining using a combination of hematoxylin–eosin. All of the sample histology slides were observed by one pathologist using an optical microscope (Olympus BX50, USA), and were captured by Software Cell D (Olympus).

2.8. Statistical analysis

The results are expressed as means ± SEM (standart error of the mean. Statistical differences between groups were estimated using one-way analysis of variance (ANOVA) with Duncan's test, and were considered statistically significant at $p < 0.05$.

3. Results

The extraction yield of CE obtained with the extraction process using 70% ethanol was 9.69 g. The extraction processes using n-hexane, ethyl acetate, and methanol, respectively, produced 6.4956 g HEX, 7.5991 g EA, and 7.7003 g MEOH (Table 1).

Table 1. The extraction yield of extract and the C, DMC and BDMC content in extracts of *C. heyneana* rhizome

Samples	Extraction Yield (g)	C (mg/g extract)	DMC (mg/g extract)	BDMC (mg/g extract)
CE	9.6900	3.11 ± 0.110a	15.97 ± 0.059a	3.25 ± 0.004a
HEX	6.4956	0.11 ± 0.091d	0.07 ± 0.011d	NA
EA	7.5991	0.73 ± 0.056b	3.62 ± 0.017b	2.40 ± 0.088b
MEOH	7.7003	0.27 ± 0.120c	1.77 ± 0.103c	0.93 ± 0.106c

Values are expressed as mean ± SEM (n = 3); means in the same column followed by different letters are significantly different at $p < 0.05$ using Duncan's tests. CE, crude ethanolic extract; HEX, n-hexane extract; EA, ethyl acetate extract; MEOH, methanol extract; C, Curcumin; DMC, demethoxy curcumin ; BDMC, bis-demethoxy curcumin.

3.1. Determination of C, DMC and BDMC content

CUR, consisting of C, DMC, and BDMC, is the principal active compound that contributes to the activity of plants in the genus *Curcuma* (Anand et al., 2008; Dutta, 2015; Gonçalves et al., 2014; Gul and Basheer, 2016; Jadoon et al., 2015; Sarvalkar et al., 2011). In figure 1A shows that DMC has a larger spot than C and BDMC in all extracts.

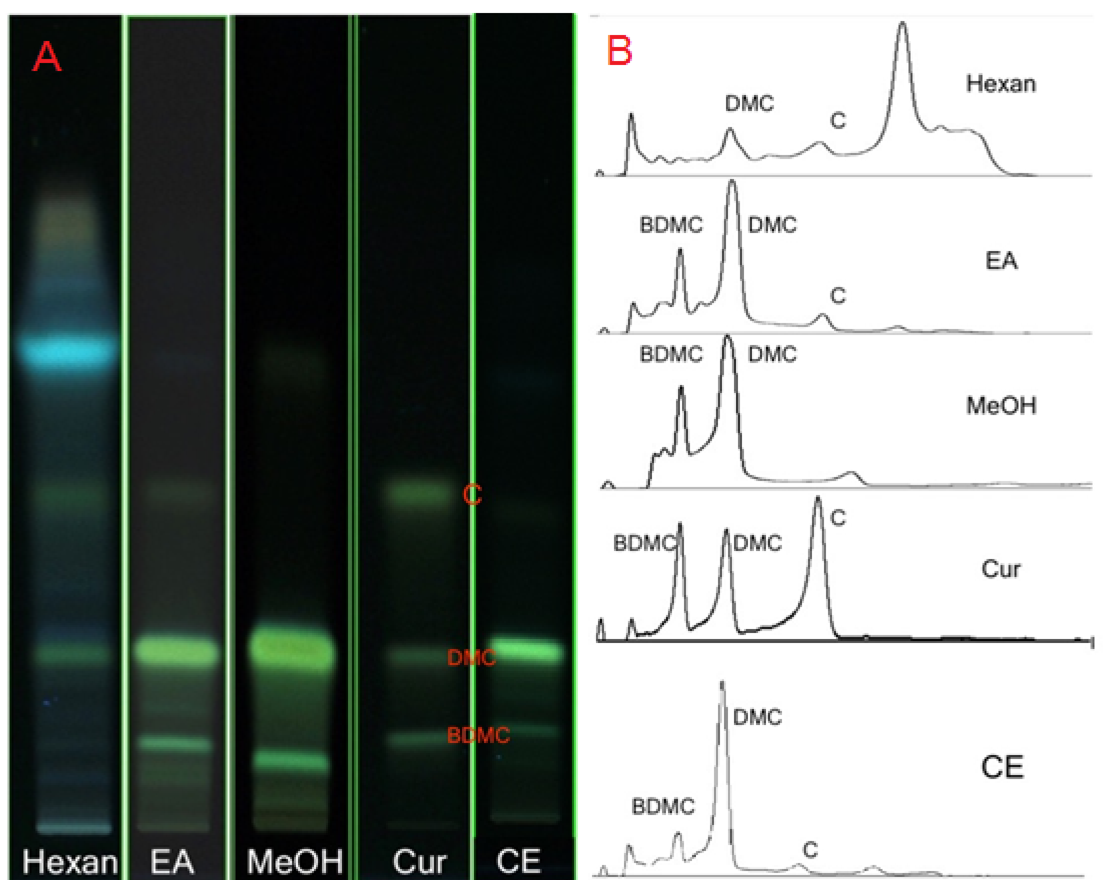


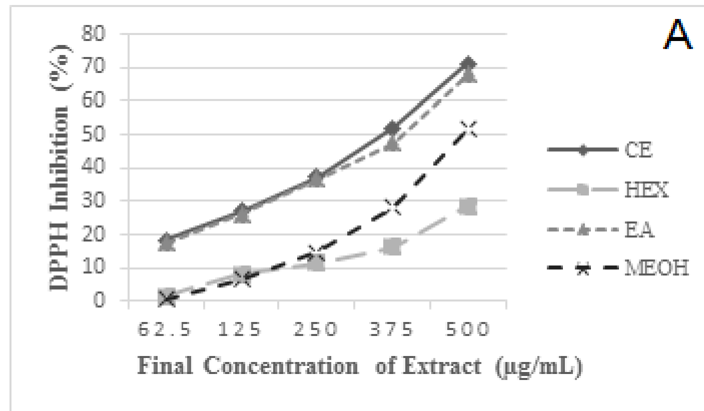
Figure 1. TLC profiles of HEX, EA, MEOH, CE and CUR (color online only).

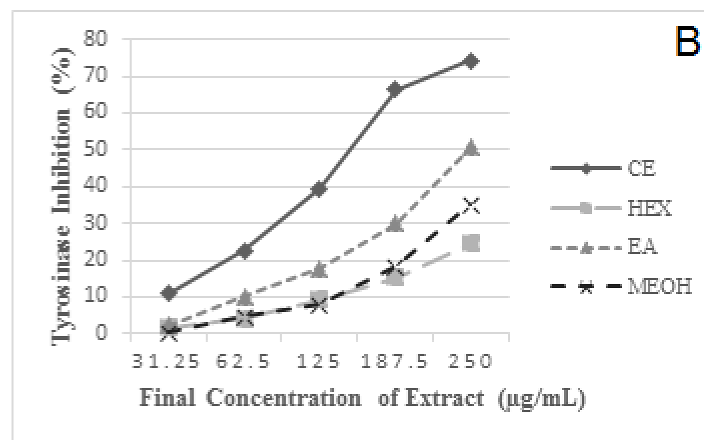
TLC plate was developed in chloroform:methanol (40:1) and was visualized as the spots with fluoresence color under 365 nm.

C, DMC and BDMC content was quantified using the TLC densitometric method, (Figure 1B). The results showed that the DMC has a larger peak area and content than C and BDMC in all extracts (Table 1).

3.2. Antioxidant, tyrosinase inhibitor, and collagenase inhibitor activity

In all assays, all of the extracts showed dose-dependent activity in the final concentration range from 62.5–500 µg/mL for the DPPH assay and 31.25–250 µg/mL for the tyrosinase inhibitor and collagenase inhibitor assays (Figure 2).





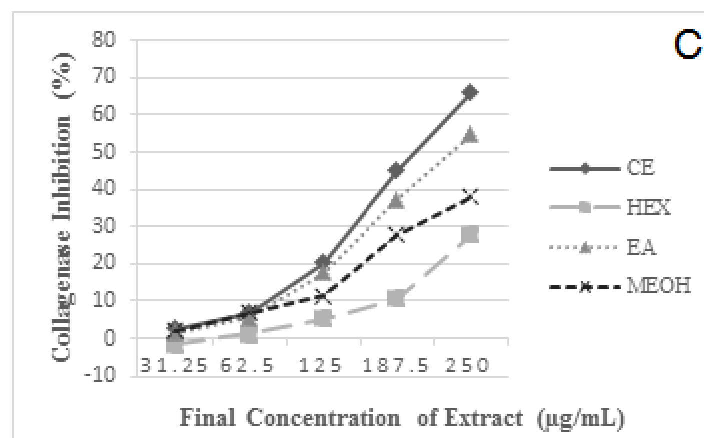


Figure 2. Dose-dependent antioxidant (A), tyrosinase inhibitor (B), and collagenase inhibitor (C) activity evaluated with *in vitro* assays. The tests were performed in triplicate. CE, crude ethanolic extract; HEX, n-hexane extract; EA, ethyl acetate extract; MEOH, methanol extract.

The IC50 calculation for each assay used the curves for each standard and the samples of *C. heyneana*. The curves were calculated using five concentration in the ranges described in Figure 2 and showed the linear relationships and strong correlation coefficients ($r > 0.95$). CUR, the principal active constituent of the *Curcuma* genus, showed antioxidant, tyrosinase inhibitor, and collagenase inhibitor activity greater than all *C. heyneana* extracts. All of the extracts had an IC50 greater than 300 µg/mL, and n-hexane and methanol extract had an IC50 greater than 500 µg/mL. The IC50 of tyrosinase inhibitor activity for all the extracts exhibited a wide range, from 155.50 to 403.99 µg/mL, while the collagenase inhibitor activity IC50 ranged from 204.80 to 454.75 µg/mL (Table 2).

Table 2. IC50 values of antioxidant, tyrosinase inhibitor, and collagenase inhibitor activity, for all extracts

Samples	IC50 (µg/mL)		
	Antioxidant	Tyrosinase Inhibitor	Collagenase Inhibitor
CUR	60.08 ± 1.17a	59.65 ± 1.19b	140.33 ± 2.01b
CE	338.18 ± 8.17b	155.50 ± 3.99c	204.80 ± 2.12c
HEX	more than 500	403.99 ± 0.41f	454.75 ± 5.52f
EA	363.26 ± 7.21b	265.44 ± 1.70d	238.58 ± 1.07d
MEOH	more than 500	359.04 ± 12.15e	323.44 ± 5.61e
T	28.97 ± 0.41a	-	-
AR	-	38.02 ± 0.25a	-
CA	-	-	30.16 ± 0.55a

Values are expressed as mean ± SEM (n = 3); means in the same column followed by different letters are significantly different at $p < 0.05$ using Duncan's tests. CUR, curcuminoid; CE, crude ethanolic extract; HEX, n-hexane extract; EA, ethyl acetate extract; MEOH, methanol extract; T, trolox; AR, arbutin; CA, caffeic acid.

Linear range (µg/mL) for antioxidant assay: T = 6.25–50; C = 12.50–100; samples = 62.50–500.

Linear range (µg/mL) for tyrosinase inhibitor assay: AR = 6.25–50; C = 12.5–100; samples = 62.5–500.

Linear range (µg/mL) for collagenase inhibitor assay: CA = 6.25–50; C = 31.25–250; samples = 62.5–500 .

3.3. Histomorphologic effects of UV exposure on rat skin

Crude ethanolic extracts was selected for further rat *in vivo* study, because the activity of antioxidant, tyrosinase inhibitor and collagenase inhibitor of CE was higher than the hexane, ethyl acetate and methanol fractions. Histology analysis showed changes caused by UV exposure and changes after treatment with the samples. In Figure 3, after UV exposure epidermal thickness (ET) of vehicle group increased three-fold from 4.07 ± 0.27 µm (normal skin) to 12.21 ± 0.41 µm. Topical application of the extract for 21 days decreased the ET to 8.09 ± 0.10 µm, while topical application of retinoic acid as a reference drug decreased the ET to 4.14 ± 0.27 µm, which was not significantly different to the normal group. UV ray exposure caused pyknotic nuclei of epidermal cells, considered to be sunburn cells (SBCs). SBCs incidence was calculated per linear millimeter of the epidermis for each animal. There was only 4.40 ± 0.28 SBC/mm2 in the epidermis of normal rats. UV exposure increased the SBCs in vehicle group to 60.80 ± 2.56 SBC/mm2. Topical application of the extract decreased six-fold in the number of SBC. Normal dermis showed orderly arranged collagen,

abundant elastic fibers, and extrafibrillar matrix. In vehicle group, UV exposure damaged the arrangement of collagen so that the distance between collagen increased by approximately five times, as compared to normal rat skin. Topical application of the extract decreased the distance between collagen by 22% compared to normal group, while application of retinoic acid regenerated the distance between collagen to a normal group.

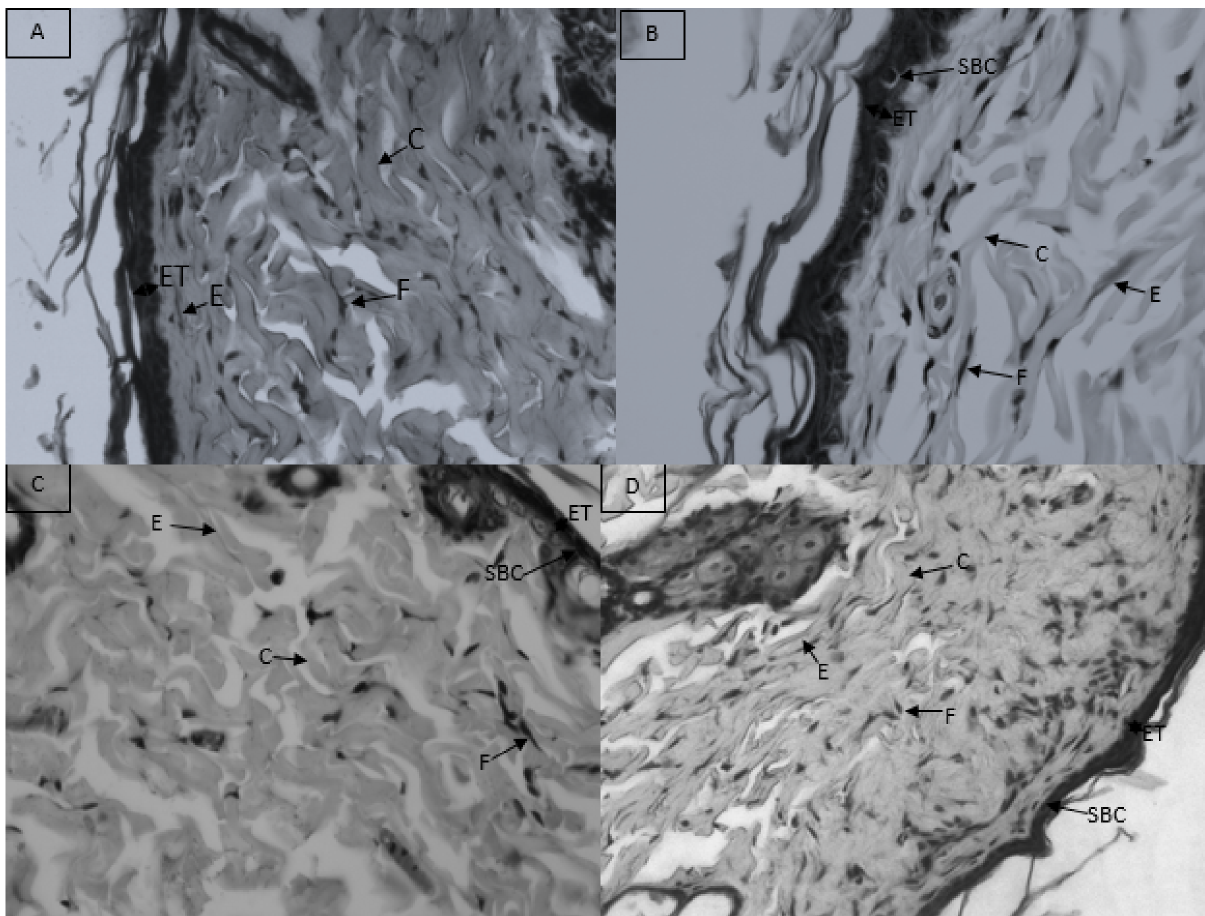


Figure 3. Histologic analysis of rat skin specimen.

Histologic rat skin specimens was performed using H & E staining. (A) Normal group, no UV exposure + no treatment; (B) vehicle group, UV exposure + vehicle treatment; (C) crude extract group, UV exposure + crude extract treatment; (D) retinoic acid group, UV exposure + retinoic acid treatment. Cellular morphology was viewed at 40x magnification. ET, epidermal thickness; SBC, sunburn cell; C, collagen fiber; F, fibroblast; E, elastic fiber.

Normal skin had 41.53 ± 0.60 fibroblasts/10,000 μm^2. In vehicle group, after UV exposure, the number of fibroblast decreased to 9.74 ± 0.26 fibroblasts/10,000 μm^2. After topical application of the crude extract, the number of fibroblast increased to 16.60 ± 0.33 fibroblasts/10,000 μm^2; but the application of retinoid acid increased the number of fibroblast larger. In addition, in the normal dermal layer, the elastic fibers were neatly arranged, but in the vehicle group, after UV exposure, the number of elastic fibers reduced, and the structure became irregular. Topical application of crude extract increased the number of elastic fibers so that the arrangement was tightly and regularly (Table 3).

Table 3. Histologic analysis of UV exposed rat skin specimens

Groups	Epidermal Thickness (μm)	Number of SBC/mm^2 in Epidermis	Number of Fibroblasts/10,000 μm^2	Distance Between Collagen Fibers (μm)	Elastic Fibers
Normal	4.07 ± 0.27b	4.40 ± 0.28b	41.53 ± 0.60b	3.09 ± 0.10b	++++
Vehicle	12.21 ± 0.41a	60.80 ± 2.56a	9.74 ± 0.26a	14.70 ± 0.40a	+
CE	8.09 ± 0.10a,b	11.80 ± 0.95ab	16.60 ± 0.33a,b	11.60 ± 0.42ab	++
RA	4.14 ± 0.27b	28.00 ± 1.76ab	40.86 ± 0.30b	3.02 ± 0.16ab	++++

Values are expressed as mean ± SEM (n = 10); means in the same column followed by different letters are significantly different at $p < 0.05$ using Duncan's tests. (a) Significantly different from the normal group at $p < 0.05$. (b) Significantly different from the vehicle group at $p < 0.05$. Normal group, no UV exposure + no treatment; vehicle group, UV exposure + vehicle treatment; CE group, UV exposure + crude extract treatment; RA group, UV exposure + retinoic acid treatment.

The results showed that the topical application of the crude extract of *C. heyneana* produced significant improvement in the UV-induced damage to the skin structure.

4. Discussion

The chromatographic profile of the extract indicates the pattern of biological activity or the chemical content present in the extract (Bhise and Salunkhe, 2009; Sanja et al., 2009). All compounds, not only active compounds, has a contribution to pharmacological activity of the extract so that chromatographic profile analysis is a comprehensive approach to the quality consistency and stability of the extract. In addition, other approaches are also made by selecting one or more compounds used for herbal product quality assurance. This selected compound is usually known as an active principle of a medicinal plant (Xie et al., 2006). In the *Curcuma* genus, CUR have been known as the principal active constituents that play a role in the various biological activities; these consist of C, DMC, and BDMC (Amalraj et al., 2017; Sasikumar, 2005). The total curcuminoid content found in the *C. heyneana* samples was lower than other *Curcuma* species reported in the literature (Dutta, 2015; Nahak and Rajani, 2011). The CUR content in turmeric comprises ≈77% C, ≈17% DMC, and ≈3% BDMC (Goel and Aggarwal, 2010). All extracts of *C. heyneana* exhibited higher DMC than C (Figure 1). These differences result in lower antioxidant activity of the *C. heyneana* extract, compared to C. CE had higher total CUR content than HEX, EA and MEOH. CE also exhibited higher antioxidant activity, tyrosinase inhibitor activity and collagenase inhibitor activity than HEX, EA and MEOH. This suggests that differences in total CUR content in the extracts affect their biological activity.

Photoaging is caused by chronic exposure to UV light. This leads to incomplete repair of damage, which ultimately results in abnormal structures and functions; this is termed premature aging (Chiu et al., 2017; Fore, 2006). UV exposure on rat skin for 21 days,

according to erythema dose, caused changes in the structure and affected the function of the skin. Epidermal thickness increased three-fold compared to normal conditions. This is due to the regeneration of cells in basal keratinocytes. Mature keratinocytes develop into stratum spinosum, stratum granulosum, and stratum corneum. This is reflected by an increase in epidermal thickness, acting as a protective function of the epidermis against UV exposure (Fore, 2006). The thickness of rat skin epidermis treated with topical CE and retinoic acid was lower than that treated with the vehicle only. Retinoic acid has been used for skin aging therapy. The use of topical retinoic acid shows histologic improvements such as increased collagen synthesis through blocking collagenase activity which is the basis of skin aging therapy (Kong et al., 2015). The ability of retinoic acid to suppress the increase in ET was greater than that of the CE. Both retinoic acid and the CE induced intrinsic skin protection, in order to restore normal skin conditions.

The histological appearance of UV exposed rat skin revealed a large number of SBCs. UV exposure is believed to cause direct injury to nuclear DNA in keratinocytes, thus stimulating the formation of SBCs. SBCs are easily recognized by their pyknotic nuclei and eosinophilic cytoplasm. Ultrastructurally, SBCs show the characteristics of apoptotic keratinocytes, with the appearance of nuclear chromatin condensation and dark cytoplasm tonofilament due to densely packed bundles. The decrease in the number of SBCs suggests a mechanism of defense or repair of DNA damage so there is no stimulation of apoptotic keratinocyte (Okada et al., 2003).

UV exposure causes dermal changes in both cellular and cell matrix components. The dermal layer contains connective tissue composed of various types of collagens, elastin, fibronectin, proteoglycan, and other extracellular matrix molecules. UV exposure causes an imbalance between the synthesis and degradation of the extracellular components. This imbalance caused the amount of fibroblasts decreases and thus affects its functional ability. The

synthesis of new collagen decreases, collagen fibers become messy, also collagen fragmentation that causes disorganization of the whole skin. Structure and composition Elastic fibers undergo irreversible changes so elastin becomes amorphous and functional activity is poor. The structure and composition of elastic fibers changes, causing fibers with amorphous elastin, which have poor functional activity. Collagen fibers become disorganized, randomly oriented, and fragmented (Ghersetich et al., 1994; Hwang et al., 2012; Pasquali-Ronchetti and Baccaeani-Contri, 1997; Zouboulis and Boschnakow, 2001).

Chronic UV skin exposure causes photoaging which is characterized by thickening, roughness, coarse wrinkles, mottled pigmentation, and histologic changes, including impairment of collagen fibers, extreme accumulation of abnormal elastic fibers, and an enhancement of glycosaminoglycans (Serafini et al., 2014). Synthetic or natural substances that can inhibit, delay, or repair damage caused by UV exposure are referred to as photo-chemoprevention.

The results of this study indicated that the antioxidant activity of crude extract *C. heyneana* was smaller than curcuminoid. This was related to the content of demethoxy curcumin greater than curcumin in *C. heyneana*. This research also showed that curcuminoid levels in crude *C. heyneana* extract plays a significant role in antioxidant activity. Crude extract C. heyneana also had activity of tyrosinase inhibitor and collagenase inhibitor. This corresponded to improvements in the appearance of UV induced-rat skin histomorphometry with parameters such as epidermal thickness, number of sunburn cells, collagen tissue, fibroblasts and elastin.

5. Conclusions

C. heyneana known ~~as~~ Temu giring has been used traditionally as skin care. The present study showed that crude extract of *C.heyneana* has the potential to be used as an anti-aging compound, and may be developed into phytocosmeceutical products.

Glossary

AR : Arbutin

BDMC : Bis-demethoxy Curcumin

C : Cucumin

CA : Caffeic Acid

CE : Crude Ethanol Extract

CUR : Curcuminoid

DMC : Demethoxy Curcumin

EA : Ethyl Acetate Extract

ET : Epidermis Thickness

HEX : n-Hexane Extract

MEOH : Methano Extract

RA : Retinoic Acid

SBC : Sunburn Cell

SEM : Standart Error of Mean

T : Trolox

TLC : Thin Layer Chromatography

UV : Ultra Violet

Acknowledgments

This work was supported by an International Research Collaboration and Scientific Publication grant from The Ministries Of Research, Technology, and Higher Education, Republic of Indonesia (Grant no. 597/UN3.14/LT/2017). Thanks to PT. Rembaka who has given the drug reference used in the in vivo test.

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