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Curcuma longa extract inhibits the activity of mushroom tyrosinase and the growth of murine skin cancer B16F10 cells

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ABSTRACT

Introduction: Tyrosinase is considered an important target of melanin biosynthesis inhibitors. *Curcuma longa* L. has been used in the Javanese traditional whitening cosmetics. This work aimed to explore the effect of *C. longa* extracts on mushroom tyrosinase activity and the cytotoxicity of the extract towards murine skin cancer B16F10 cells.
Methods: *C. longa* rhizomes were cold-extracted using ethanol 70% and yielded 15.3% w/w of extract (ECL). The presence of curcuminoids in ECL was determined by reversed-phase high-performance liquid chromatography (RP-HPLC). ECL was assessed for its inhibitory effects on mushroom tyrosinase activity using L-DOPA as substrate and kojic acid as the positive control drug. The cytotoxicity of ECL and curcumin was studied in B16F10 cells.
Results: Triplet peaks of RP-HPLC chromatogram revealed that curcuminoids were available in ECL. The level of bisdemethoxycurcumin was 6.3306% (tR = 12.646 minutes), demethoxycurcumin was 3.1414% (tR = 13.675 minutes), and curcumin was 8.3754% (tR = 14.802 minutes). ECL had a weak inhibitory activity towards mushroom tyrosinase with IC_{50} = 564.8 µg/mL, while the IC_{50} = of kojic acid was 55.70 µg/mL. Both ECL and kojic acid had moderate toxicity to B16F10 cells (IC_{50} survival growth rates were 98.06 µg/mL and 65.54 µg/mL, respectively). Curcumin was highly toxic to B16F10 cells (IC_{50} = 14.42 µg/mL).
Conclusion: Taken together, ECL might be able to prevent melanogenesis via the inhibition of tyrosinase activity, and interestingly, it could inhibit the growth of murine skin cancer B16F10 cells. However, further studies are needed to verify its antimelanogenesis and anticancer properties.

Implication for health policy/practice/research/medical education:
This study supports the scientification and development of *Curcuma longa* L. rhizome as an active component of skin whitening cosmetics.

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Introduction

The pigmentation on the skin is influenced by various factors, including tyrosinase, a copper-containing enzyme, which catalyzes the hydroxylation of L-tyrosine (the enzyme's substrate) to L-DOPA, and eventually, the oxidation of L-DOPA to dopaquinone. Polymerization of these quinones results in the formation of melanin (1,2). L-tyrosine and L-DOPA function as substrates and intermediates of melanin production (3). Alteration in melanin biosynthesis can lead to various skin diseases in humans (4). It has been known that plants with antioxidant properties might be utilized for the treatment of skin disorders.

In Indonesia, the rhizomes of turmeric or *Curcuma longa* L. (synonym *C. domestica* Valeton; local name *kunyit*) have traditionally been used as *lulur*, to cleanse, soften, and whiten the skins of Javanese princesses. The major active compounds contained in *C. longa* rhizomes are curcumin, demethoxycurcumin, and bisdemethoxycurcumin (5). *In vitro* studies on curcumin reported its activity in reducing the melanin content and tyrosinase activity and blocking the expression of

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melanogenesis-related proteins in human melanocytes. Curcumin also activates phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase-3β (PI3K/Akt/GSK-3β), extracellular-signal-regulated kinase (ERK), and p38 mitogen-activated protein kinase (p38 MAPK) pathways (6). Curcumin decreases melanin levels and blocks the tyrosinase activity in alpha-melanocyte stimulating hormone-stimulated B16F10 cells (7). Nonetheless, curcumin is notable for its antioxidant property indicating high reactivity in scavenging peroxyl radicals (8).

In fact, clinical trials in several countries have reported the safety of (mostly) curcumin prepared as a pharmaceutical dosage form in healthy subjects (8).

An open-label, prospective clinical study of a standardized *C. longa* extract has been performed on 12 healthy adult Indian ethnicity participants for 90 days and revealed that the liver function and other hematological parameters were not significantly altered, which proved the safety of the extract (9). Another clinical study of a low dose of curcumin on healthy middle-aged males and females in Ohio, USA, resulted in a significant decrease in plasma triglyceride, plasma beta-amyloid, plasma alanine aminotransferase, salivary amylase, and an increase in plasma catalase, plasma myeloperoxidase, and plasma nitric oxide (10). A randomized-control double-blind prospective trial on 59 healthy non-smoking adults in Texas, USA, treated with 200 mg oral curcumin for 8 weeks resulted in a clinically significant rectification in endothelial function thus reducing the risk of cardiovascular diseases (11). One hour after treatment with 400 mg oral curcumin, healthy elderly participants (n = 60, aged 60-85 years) significantly improved performance on sustained attention and working memory tasks (12).

Previous studies have reported the advantage of curcumin in maintaining human health and its mechanism in blocking the melanogenesis process. However, there is limited study on the activity of the plant extract in melanogenesis. This work aimed to explore the inhibitory activity of *C. longa* extract towards mushroom tyrosinase as well as its effect on the survival rate of murine skin cancer B16F10 cells.

Materials and Methods

Plant materials and identification

Turmeric rhizomes were obtained from the Experimental Plantation of the Research Institute for Spices and Medicinal Plants (BALITTRO) Manoko, Cikahuripan, Lembang, West Java, Indonesia. The plants were taxonomy identified by a certified botanist at the Laboratory of Identification and Determination, School of Life Sciences and Technology, Bandung Institute of Technology, and based on the characteristics described by Newman et al (13) and two older references; the sample was confirmed as *Curcuma longa* L. (Letter No. 5727/I1.CO2.2/PL/2019). The rhizomes were washed under tap water to remove soil and dirt and oven-dried at a maximum temperature of 40ºC for 2 days. The dried rhizomes were ground to powder and sieved using a sieve mesh-16.

Preparation of the extract

The rhizome powder (1.0 kg) was extracted with ethanol 70% (10 L) for 5 days at 25-26˚C. The extract was filtered and the solvent was removed by a vacuum rotary evaporator at 45-50˚C. The yield of the viscous ethanol extract of *C. longa* (ECL) was 15.3% w/w.

HPLC analysis to determine curcumin in ECL

Determination of curcumin in ECL was carried out by employing a reversed-phase high-performance liquid chromatography (RP-HPLC) (Waters Alliance 2695) following a procedure described elsewhere (14) with a few modifications. Samples (20 µL) were injected into the C18 column (Merck LiChroCART 250 mm × 4.6 mm) and were eluted in a mixture of water-acetonitrile in a gradient-elution mobile phase with an initial of 60% acetonitrile increased to 80% acetonitrile in 12 minutes, maintained for 5 minutes, increased to 90% acetonitrile in 17 minutes with a flow rate of 0.5 mL/min. Detection (Waters 2489 UV-visible detector) was set at 425 nm. The chromatogram of ECL was compared to that of standard curcumin.

Chemicals, cells, and cell culture

Tyrosinase inhibitor screening kit (colorimetric) (Sigma-Aldrich product number MAK257), kojic acid K3125 (Sigma-Aldrich CAS number 501-30-4), murine melanoma cell lines B16F10 (ATCC® CRL-6475™) (a collection of the Cell and Molecular Biology Laboratory, Faculty of Pharmacy, Universitas Padjadjaran) were prepared. B16F10 is a cell line exhibiting a morphology of spindle-shaped and epithelial-like cells isolated from the skin tissue of a mouse with melanoma (https://www.atcc.org/products/crl-6475).

The cells were grown at 37°C with 5% CO2 in the Dulbecco's Modified Eagle Medium (DMEM) produced by ATCC, added 10% fetal bovine serum (FBS) (GIBCO) and 1% of penicillin-streptomycin (10 000 U penicillin and 10 mg streptomycin/mL) (Sigma-Aldrich). After the cells reached a confluency of 80%, they were subcultured in 0.05% of TrypLE enzymes (Gibco).

Cytotoxicity assay

The cytotoxicity of ECL against the B16F10 cells (8000 cells/well) was assessed using the water-soluble WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium/Cell Counting Kit-8. ECL solutions were prepared by dissolving the extract in 1% DMSO in a culture medium. The solution was serial-diluted to final concentrations of 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, and 500 µg/mL, and incubated at 37ºC for 48 hours. The absorbance of

the formazan product was measured using a microplate reader (Infinite M200 Pro, NanoQuant Tecan). The IC_{50} was calculated using GraphPad Prism 8.4.2. Furthermore, the cytotoxicity of kojic acid was also assessed using the same procedure. Kojic acid solutions were prepared to final concentrations of 7.813 µg/mL, 15.625 µg/mL, 31.25 µg/mL, 62.5 µg/mL, and 125 µg/mL. The morphology of the cells was observed by using ZEISS Axio Vert A1 Bio inverted microscope in 100x magnification.

Mushroom tyrosinase inhibition assay

Tyrosinase inhibition assays were carried out by following the protocol in the kit that employs mushroom tyrosinase. Mushroom tyrosinase was chosen because it possesses high similarity and homology to human tyrosinase (15). L-DOPA was used as the enzyme substrate. The reaction mixture (1000 µL) contained 685 µL of phosphate buffer (50 mM, pH 6.5), 50 µL of mushroom tyrosinase (333 U/mL in phosphate buffer), 20 µL of ECL dissolved in DMSO, and 100 µL of 5 mM L-DOPA. After the addition of L-DOPA, the reaction was immediately measured at 510 nm for the formation of the product (dopachrome). Kojic acid (concentration range of 12.5-200 µg/mL) was used as a positive control. The concentration range of ECL used for the mushroom tyrosinase inhibition assay was 125-2000 µg/mL. Each measurement was made in triplicate. The IC_{50} value, a concentration giving 50% inhibition of tyrosinase activity, was determined by interpolating the logarithmic concentration against the % inhibition of tyrosinase activity (16):

$$\textit{Inhibition of tyrosinase activity } (\%) = [(A - B)/A] \times 100\ \%$$

where A is the absorbance of the control with the enzyme at 510 nm; B is the absorbance of the test sample with the enzyme at 510 nm.

Results

Determination of curcumin in ECL

RP-HPLC chromatogram revealed that curcumin was positively contained in ECL as a curcuminoid triplet with demethoxycurcumin and bisdemethoxycurcumin. The level of bisdemethoxycurcumin was 6.3306%, eluted at 12.646 minutes; demethoxycurcumin was 3.1414%, eluted at 13.675 minutes; and curcumin was 8.3754%, eluted at 14.802 minutes (Figure 1).

The effect of ECL on mushroom tyrosinase activity was depicted as a plot between the logarithmic concentration of ECL or kojic acid against % tyrosinase activity. ECL had a weak inhibitory activity towards mushroom tyrosinase (IC_{50} = 564.8 µg/mL) (Figure 2A) compared to that of kojic acid (IC_{50} = 55.70 µg/mL) (Figure 2B). By increasing the concentration of ECL or kojic acid, the % inhibition of mushroom tyrosinase activity was also elevated in a sigmoidal pattern curve.

The effect of ECL on murine skin cancer B16F10 cells

The ECL exhibited a weak inhibitory on mushroom tyrosinase activity. Hence, its ability to influence the survival growth rate of murine skin cancer B16F10 cells was assayed. The cells were treated with ECL at concentrations ranging from 31.25 µg/mL to 500 µg/mL for 48 hours at 37°C and examined using WST-8/CCK-8. The results revealed that ECL started inhibiting the survival growth rate of the murine skin cancer cells at 62.5 µg/mL with a survival growth rate of 60.13%, while concentrations of 125, 250, and 500 µg/mL resulted in a survival growth rate of 31.56%, 1.69%, and 0.65%, respectively. The cytotoxicity was depicted as a plot between the logarithmic concentration of ECL against the % survival growth rate of the cells. ECL was confirmed as moderate cytotoxicity to murine skin cancer B16F10 cells with IC_{50} of 98.06 µg/mL (Figure 3A), whereas curcumin was categorized as a compound with high cytotoxicity with IC_{50} of 14.42 µg/mL (Figure 3B). Kojic acid was similar to ECL of moderate cytotoxicity with IC_{50} of 65.54 µg/mL (Figure 3C).

The microscopic examination of the cells treated with ECL, curcumin, or kojic acid is depicted in Figure 4. Normal murine skin cancer B16F10 cells (Figure 4A) revealed high-density cells, while treating the cells with

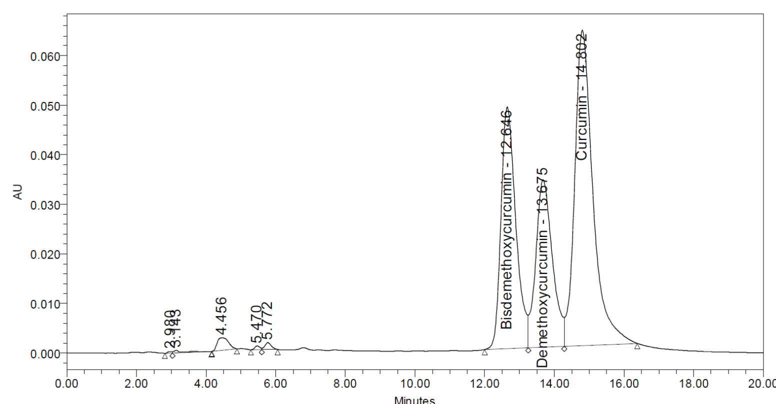

Figure 1. Reversed-phase high-performance liquid chromatography chromatogram of *Curcuma longa* extract using a gradient-elution system of water and acetonitrile revealing triplet peaks of bisdemethoxycurcumin at retention time of 12.646 min; demethoxycurcumin at retention time of 13.675 min; and curcumin at retention time of 14.802 min.

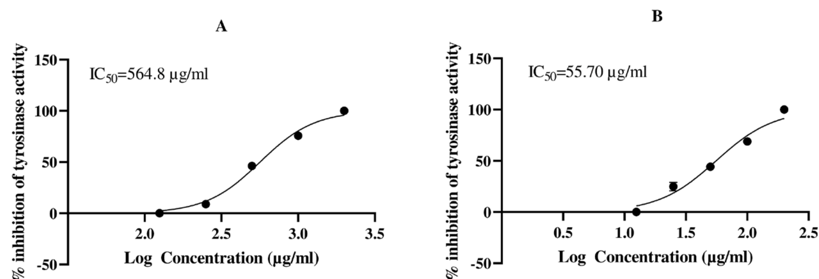

Figure 2. Effect of (A) *Curcuma longa* extract (IC_{50} = 564.8 µg/mL) and (B) kojic acid (IC_{50} = 55.70 µg/mL) on mushroom tyrosinase activity.

ECL (Figure 4B) and/or curcumin (Figure 4C) and/or kojic acid (Figure 4D) showed shrunken cells and low-density.

Discussion

In our work, curcumin was analyzed by an RP-HPLC using a gradient-elution system of water–acetonitrile as a mobile phase. The RP-HPLC chromatogram revealed that curcumin was positively contained in ECL as a curcuminoid triplet with demethoxycurcumin and bisdemethoxycurcumin. The level of bisdemethoxycurcumin was 6.3306%, demethoxycurcumin was 3.1414%, and curcumin was 8.3754% (Figure 1). The level of curcumin was the highest of the three curcuminoids. The presence of triplets in the HPLC chromatogram was also reported by previous studies (14,18).

Our work revealed that ECL inhibits the activity of mushroom tyrosinase (IC_{50} = 564.8 µg/mL) (Figure 2A). The methanol extract, instead of ethanol extract, of *C. longa* rhizome demonstrated an inhibition towards tyrosinase activity (19). However, a previous study on curcumin also reported similar results to ours. In their work, it was confirmed that curcumin exhibited moderate inhibition against the monophenolase activity of tyrosinase with an IC_{50} of 326.5 µM (20). The activity of curcumin is attributed to its two phenyl structures, each with -OH and -OCH3 groups attached to the orto position (21), which the other two curcuminoids lack.

The cytotoxicity of drugs or compounds is categorized as high if IC_{50} is less than 20 µg/mL, moderate if IC_{50} ranges between 21-200 µg/mL, weak if IC_{50} ranges 201-500 µg/mL, and no cytotoxicity if IC_{50} exceeds 500 µg/mL (22). ECL was confirmed as moderate cytotoxicity to murine skin cancer B16F10 cells and curcumin exhibited high cytotoxicity. These findings suggest that ECL and curcumin might possess anticancer properties on murine skin cancer B16F10 cells.

Kojic acid inhibited the activity of mushroom tyrosinase (IC_{50} = 55.70 µg/mL) (Figure 2B). Phenolic compounds, e.g., kojic acid and curcumin, may be utilized as antimelanogenesis due to their similarity in structure to tyrosine, the substrate of tyrosinase (19). A previous study reported that kojic acid was observed bound at the opening of the active site of tyrosinase, implicating a competitive inhibition to the substrate (tyrosine). Two residues, Arg209 and Val218, located in the second outer layer of the active site, function a role in the binding of tyrosine (23). Our preliminary *in silico* study visualized that curcumin and other phenolic constituents of *C. longa* bound to tyrosinase and tyrosinase-related protein-1 with binding modes similar to those of kojic acid (24).

Conclusion

The inhibitory activity of the ethanol ECL on the activity of mushroom tyrosinase and the cytotoxicity of the extract towards murine skin cancer B16F10 cells were evaluated. ECL weakly inhibited the activity of mushroom tyrosinase and was of moderate cytotoxicity to murine skin cancer B16F10 cells as proven by its ability to reduce the survival growth rate of the cells. Curcumin, a major constituent contained in *C. longa* rhizome, has shown high cytotoxicity. Taken together, ECL, which positively contains curcumin,

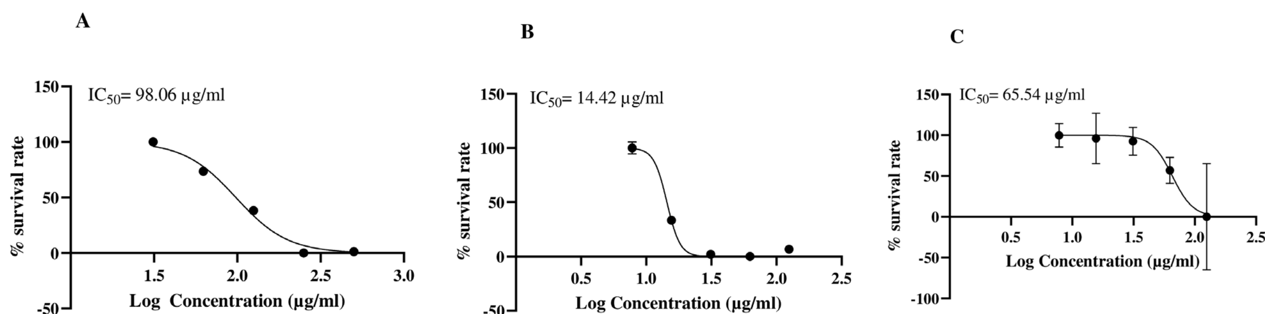

Figure 3. The effects of (A) *Curcuma longa* extract, (B) curcumin, and (C) kojic acid on the survival growth rate of B16F10 cells.

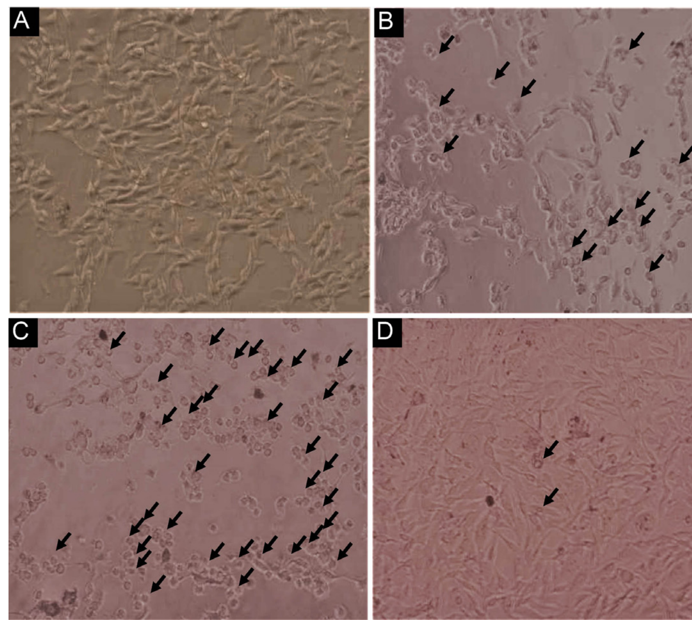

Figure 4. The microscopic examination of the cells treated with *Curcuma longa* extract-treated, curcumin, or kojic acid. A) normal high-density B16F10 cells, B) *C. longa* extract-treated B16F10 cells, C) curcumin-treated B16F10 cells, and D) kojic acid-treated B16F10 cells. Bold arrows indicate shrunken-abnormal or dead cells.

bisdemethoxycurcumin, and demethoxycurcumin might be able to prevent melanogenesis via the inhibition of tyrosinase activity; interestingly, it could inhibit the growth of murine skin cancer B16F10 cells. Considering this, ECL is prospective to be developed as an active component in cosmetics. However, further studies are needed to verify its antimelanogenesis and anticancer properties.

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Authors' contributions
JL was responsible for the study design and methodology. SAS and JL contributed to the data interpretation. DF contributed to the investigation, data collection, data validity, and statistical analysis. JL, SAS, and NMS contributed equally to the supervision of the project. JL and DF contributed to manuscript preparation and revision. All authors have read and approved the final manuscript.

Conflict of interests
None.

Ethical considerations
This work has been approved by the Ethical Committee of Padjadjaran University (document No. 586/UN6.KEP/EC/2020).

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