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Modulatory effects of cinnamomi cortex and its components epicatechin and linalool on skin circadian rhythms

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Circadian rhythms, intrinsic 24-h cycles regulating physiological processes, are crucial for skin homeostasis. Disruptions in these rhythms are linked to various skin disorders and impaired barrier function. Circadian rhythms can be modulated by botanical compounds, which hold therapeutic potential. However, the effect of cinnamomi cortex (CC), an anti-inflammatory, antioxidant, and antimicrobial agent, on the circadian rhythm of keratinocytes remains unclear. This study aimed to examine the effects of CC extract and its 18 individual components on the circadian rhythm of HaCaT, an immortalized human keratinocyte line. CC extract and its bioactive components epicatechin (EC) and linalool (LO) significantly enhanced the circadian amplitude without altering the period. Gene expression analysis revealed that CC extract, EC, and LO altered the mRNA and protein levels of clock genes in a time-dependent manner. During molecular docking simulations, both EC and LO exhibited strong binding affinities for RORA, a key nuclear receptor involved in circadian regulation. Enhanced BMAL1 promoter activity following EC and LO treatments corroborated these findings. Furthermore, EC and LO demonstrated significant antioxidant activities, as evidenced by reduced reactive oxygen species levels and increased expression of antioxidant enzymes. EC and LO also upregulated skin barrier-related and ceramide synthesis genes and modulated the expression of cellular longevity-promoting genes. In conclusion, CC extract, particularly the components EC and LO, modulated circadian rhythms, reduced oxidative stress, and enhanced skin barrier function in keratinocytes. These findings highlight the potential of CC extract and its components as novel dermatological treatments to improve skin health and combat aging.

Keywords Circadian rhythm, Keratinocyte, Epicatechin, Linalool, Cinnamomi cortex

Abbreviations

BMAL1	Brain and muscle ARNT-like 1
CAT	Catalase
CC	Cinnamomi cortex
CDK	Cyclin-dependent kinase
CDKN1A	Cyclin-dependent kinase inhibitor 1A
CDKN1B	Cyclin-dependent kinase inhibitor 1B
CRY1	Cryptochrome 1
DSP	Desmoplakin
EC	Epicatechin
FLG	Filaggrin
LO	Linalool
NR1D1	Nuclear receptor subfamily 1 group D member 1
PER1	Period 1
PER2	Period 2
RORA	Retinoic acid-related orphan receptor alpha
ROS	Reactive oxygen species
SOD1	Superoxide dismutase 1

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SOD2	Superoxide dismutase 2
SPTLC	Serine palmitoyltransferase long chain base subunit
TERT	Telomerase reverse transcriptase
TGM1	Transglutaminase 1

Circadian rhythms are intrinsic, nearly 24-h physiological cycles regulated by the circadian clock, a molecular mechanism present in cells[1]. These physiological rhythms are primarily influenced by external cues, such as light, which play a critical role in sleep–wake cycles, hormonal release, metabolic pathways, and overall homeostasis[1,2]. In humans, the skin—the largest organ—is directly exposed to environmental changes, and its constituent cells, including keratinocytes, are highly responsive to such external stimuli.

Skin cells, including keratinocytes, possess an autonomous circadian clock that is essential for maintaining skin homeostasis and function[3,4]. Processes such as epidermal moisture loss, corneal cell proliferation, skin blood flow, and skin temperature regulation each undergo circadian changes[5,6]. Antioxidant production and repair enzyme activity are generally higher during the day; this helps to prevent the damaging effects of ultraviolet radiation and environmental pollutants[7,8]. Conversely, as light exposure decreases at night, the circadian rhythm of the skin switches to recovery and regeneration, facilitating processes such as cell regeneration, collagen synthesis, and increased blood flow[3,9,10].

Disruptions in the circadian rhythms of these cells have been linked to various skin disorders and impaired barrier function[11]. Dysregulated circadian rhythms can also lead to other skin issues, including premature aging, impaired wound healing, and increased sensitivity[11–14]. Thus, regulating and ensuring normal circadian clock activity will aid in disease management, and the regulation of skin circadian rhythms is a major focus in dermatology because of its crucial role in maintaining skin homeostasis and overall health. Understanding how these rhythms are normally controlled underscores the importance of researching the modulators that influence them[15–18]. However, the mechanisms by which circadian rhythms are modulated in keratinocytes remain largely unexplored.

Certain botanical compounds modulate circadian rhythms in various organs, with promising implications for the development of novel therapeutic interventions[17,19]. Although several compounds have been studied for the treatment of abnormal circadian rhythms, developing a new drug involves considerable time and financial investments to identify an effective drug target, conduct clinical trials, obtain manufacturing approval, and achieve sales, while being accompanied by a high probability of failure. Therefore, a recent trend in drug development is the repurposing of existing drugs or compounds by identifying new functions or applications for them[17].

According to traditional Korean and Chinese medicinal systems, Yin and Yang—concepts describing the interconnected and interdependent property of apparent opposing or contrary forces in the nature—govern the body's natural rhythms, including the daily cycles of activity and rest[20]. This mirrors the function of circadian rhythms. For instance, during the day (Yang), the body's energy is outward and active, whereas at night (Yin), it turns inward for rest and rejuvenation[20]. Although Yin–Yang is not a circadian rhythm itself, it symbolically represents the cyclical, rhythmic changes that circadian rhythms biologically manifest.

In both these traditions, herbal medicines, including cinnamomi cortex (CC), are used to treat diseases and restore the Yin–Yang balance, thereby promoting overall health[20–22]. CC, commonly known as cinnamon bark, is widely recognized for its pharmacological properties, including anti-inflammatory, antioxidant, and antimicrobial effects[23]. CC contains bioactive compounds that have drawn scientific interest for their potential health benefits. However, despite its extensive use, the impact of CC extract or its bioactive components on the circadian rhythm of keratinocytes remain unexplored.

In this study, we aimed to experimentally demonstrate the effects of CC extract and its individual components on the regulation of circadian rhythms in keratinocytes. By elucidating the modulatory effects of CC and its bioactive compounds on these cellular clocks, we sought to uncover novel approaches to enhance skin health and resilience. The findings can pave the way for innovative therapeutic strategies that leverage the chronobiological properties of natural extracts to promote dermatological well-being.

Results

CC modulates the circadian clock and rhythm of skin keratinocytes

To assess the cytotoxicity of cinnamomi cortex (CC) extract on keratinocytes, various concentrations of CC extract were applied and incubated for 24 or 72 h, followed by a cell viability assay. CC at concentrations of 100 μg/mL or less showed no cytotoxicity after either 24 or 72 h of incubation. However, a concentration of 1000 μg/mL exhibited cytotoxic effects (Fig. 1A). To further investigate the modulatory function of CC on circadian rhythms, synchronized HaCaT—an immortalized human keratinocyte line—stably expressing *BMAL1* promoter-driven luciferase were synchronized using dexamethasone for 2 h. The cells were then treated with CC extract, and bioluminescence activity was measured. The bioluminescence count increased in a concentration-dependent manner after treatment with CC extract (Fig. 1B). Although the period of the circadian rhythm remained unaffected by treatment with CC extract (Fig. 1C), the amplitude increased significantly at a concentration of 100 μg/mL (Fig. 1D). Notably, cells treated with 50 μg/mL CC extract also exhibited enhanced circadian amplitude; however, this increase was not statistically significant.

Based on the finding that CC extract modulates the circadian activity of *BMAL1* promoter-driven luciferase, the expression levels of core circadian clock genes were examined. Treatment with 100 μg/mL of CC extract decreased the cryptochrome 1 (*CRY1*) mRNA levels (Fig. 1F) and increased *BMAL1*, D-box binding PAR bZIP transcription factor (*DBP*), nuclear receptor subfamily 1 group D member 1 (*NR1D1*), and period 2 (*PER2*) mRNA levels in a time-dependent manner (Fig. 1E,G,H,I). Furthermore, the levels of CLOCK, PER1, and

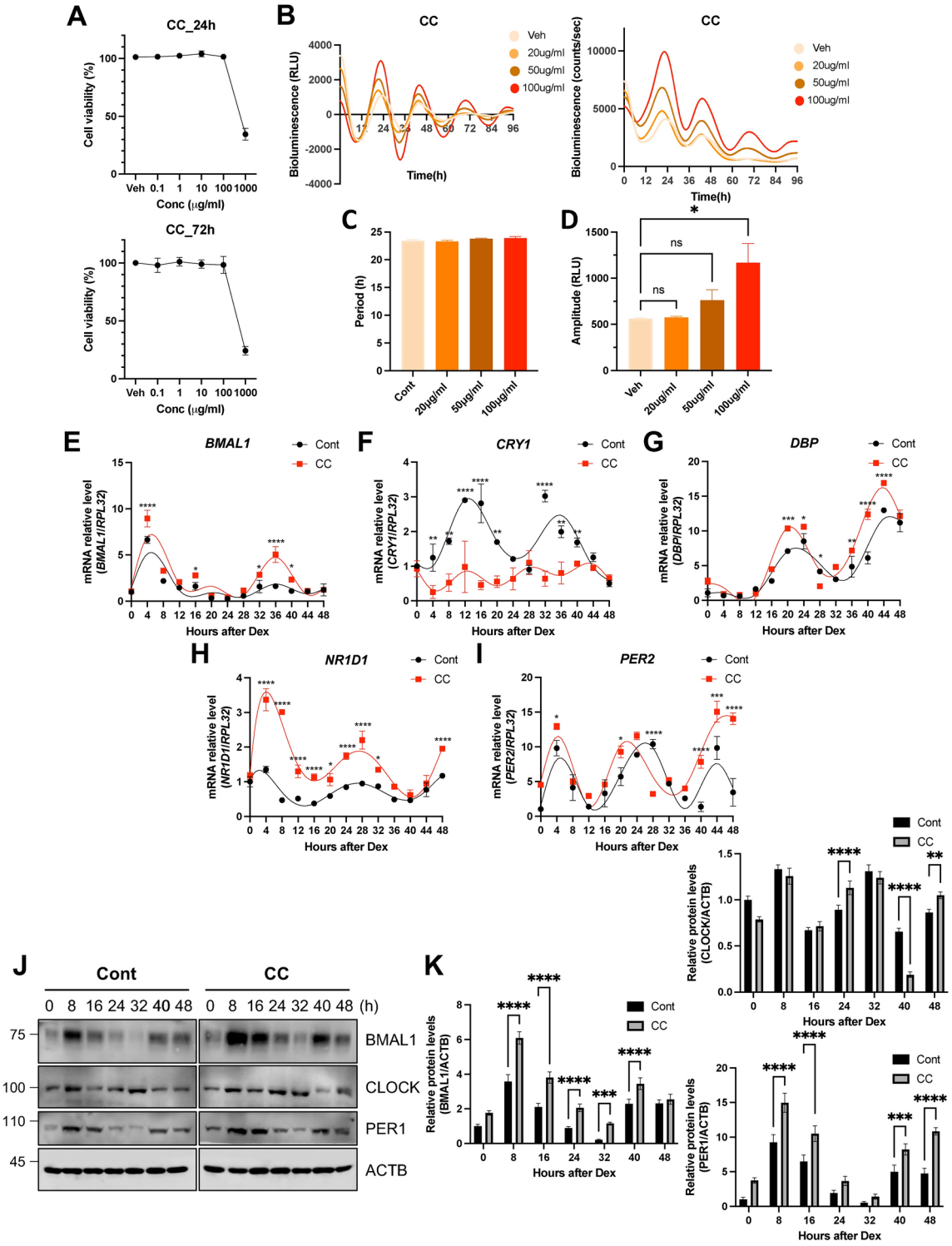


Fig. 1. CC modulates the circadian rhythm of skin keratinocytes. (**A**) HaCaT cells were treated with cinnamomi cortex (CC) extract for 24 or 72 h and subjected to Cell Counting Assay-8 (CCK-8) analysis. The cell viability of control was set to 100. (**B**) Dexamethasone-mediated synchronized HaCaT cells that express *BMAL1* promoter-driven luciferase were treated with the indicated concentration of CC, and bioluminescence activity was measured for 96 h in real-time (right). The measured bioluminescence activity was detrended using the Kronos HT software (ATTO). (**C**) Period and (**D**) amplitude of detrended bioluminescence data were analyzed using the BioDare2 platform. (**E**–**I**) Dexamethasone-mediated synchronized HaCaT cells were cultured with or without 100 μg/mL of CC extract, and cells were harvested at indicated time points. The mRNA levels of circadian clock genes (**E**) *BMAL1*, (**F**) *CRY1*, (**G**) *DBP*, (**H**) *NR1D1*, and (**I**) *PER2* were analyzed using quantitative real-time PCR. The mRNA level of control at 0 h were set to 1.0. (**J**) The same cell extracts as shown in (**E**–**I**) were used to determine protein expression levels using indicated antibodies. (**K**) The relative protein levels shown in (**J**) were quantified from the blots and plotted (n = 3).

BMAL1 proteins increased but not at all time points (Fig. 1J,K, and Supplementary Fig. S1). These results suggest that CC extract modulated both the cellular and molecular aspects of the circadian rhythm.

Specific components of CC regulate the circadian rhythm

To identify the most functional CC component involved in circadian rhythm modulation, viability of cells treated with various concentrations of the 18 individual components constituting CC was assessed (Table 1, Supplementary Fig. S2 and Fig. 2A,E). Most components were not cytotoxic at concentrations below 100 μM. Thereafter, real-time bioluminescence activity was measured in synchronized BMAL1::Luc HaCaT cells treated with each component. Two compounds, epicatechin (EC) and linalool (LO), altered the circadian rhythm, whereas the other 16 compounds did not (Fig. 2B,F and Supplementary Fig. S3). Treatment with EC increased the bioluminescence activity, and the increase was the most prominent at 100 μM concentration (Fig. 2B). Although EC did not change the periods of the circadian rhythms substantially, treatment with 100 μM of EC significantly increased the circadian amplitude (Fig. 2C,D). Treatment with LO also increased the bioluminescence activity in a dose-dependent manner (Fig. 2F); the periods were unchanged, whereas the amplitude increased significantly (Fig. 2G,H). Thus, EC and LO were identified as the key molecules that regulate circadian rhythms among the 18 constituent components of CC extract.

EC and LO directly activate the circadian clock system

Because EC and LO enhanced the amplitude of *BMAL1*-driven cellular circadian rhythms, we sought to determine whether EC or LO directly interacts with proteins of the core clock system. Using the three-dimensional structures of the proteins and ligand, the binding sites and affinities were simulated using the CB-DOCK2 server[24,25]. The binding affinities of EC and LO for different clock proteins were measured (data not shown), and both EC and LO exhibited the highest binding affinity for the RORA protein, also known as nuclear receptor ROR alpha, with a Vina score of −9.1 and −5.6, respectively. The binding score of EC indicated a strong interaction and a high likelihood of actual binding[26–28] (Fig. 3A), whereas that of LO indicated a weaker binding (Fig. 3B). Simulations using AlphaFold 3, similar to those using CB-DOCK2, also predicted a binding between RORA and EC, as well as between RORA and LO (data not shown). This ensured the consistency of our findings and reinforced the potential of these compounds to interact with RORA. RORA aids in the functioning of *BMAL1* promoter by binding to the ROR element[29]. Based on the predicted interaction of EC and LO with the RORA protein, *BMAL1* promoter activity in response to EC or LO treatment was measured. Treatment with 50 or 100 μM EC or LO significantly enhanced *BMAL1* promoter-driven luciferase activity (Fig. 3C), and the activity was dose-dependent (Fig. 3D). Thus, the CC extract components EC and LO directly bind to the RORA protein to increase its transactivation.

EC and LO modulate circadian functions

Because the circadian rhythm is generated and regulated by core clock genes, the expression levels of several core clock genes were examined in synchronized cells after treatment with EC or LO. Treatment with CC extract, EC, and LO increased *BMAL1* mRNA levels (Fig. 4A,F). Treatment with EC did not alter the *CRY1* mRNA levels; however, LO downregulated *CRY1* mRNA, resembling the effect exerted by CC extract (Fig. 4B,G). Similar to the effect of CC extract on the mRNA levels of *DBP*, treatment with EC increased *DBP* mRNA levels; however, LO treatment did not (Fig. 4C,H). *NR1D1* mRNA levels increased slightly after both EC and LO treatments but not at all time points; the effect of CC extract on the *NR1D1* mRNA levels, however, was more prominent (Fig.

	Single compounds	Cas. No
1	(-)-Borneol	464–45-9
2	(-)-Caryophyllene oxide	1139–30-6
3	(R)-(+)-Limonene	5989–27-5
4	1,8-Cineole	470–82-6
5	4-Hydroxy-3-Methoxycinnamaldehyde	458–36-6
6	Cinnamaldehyde	104–55-2
7	Cinnamic acid	140–10-3
8	Cinnamyl acetate	103–54-8
9	Cinnamyl alcohol	104–54-1
10	Coumarin	91–64-5
11	(-)-Epicatechin	490–46-0
12	Eugenol	97–53-0
13	Linalool	78–70-6
14	Nerolidol	7212–44-4
15	Terephthalic acid	100–21-0
16	Terpinolene	586–62-9
17	α-Terpineol	98–55-5
18	β-Caryophyllene	87–44-5

Table 1. List of single compounds in CC extract.

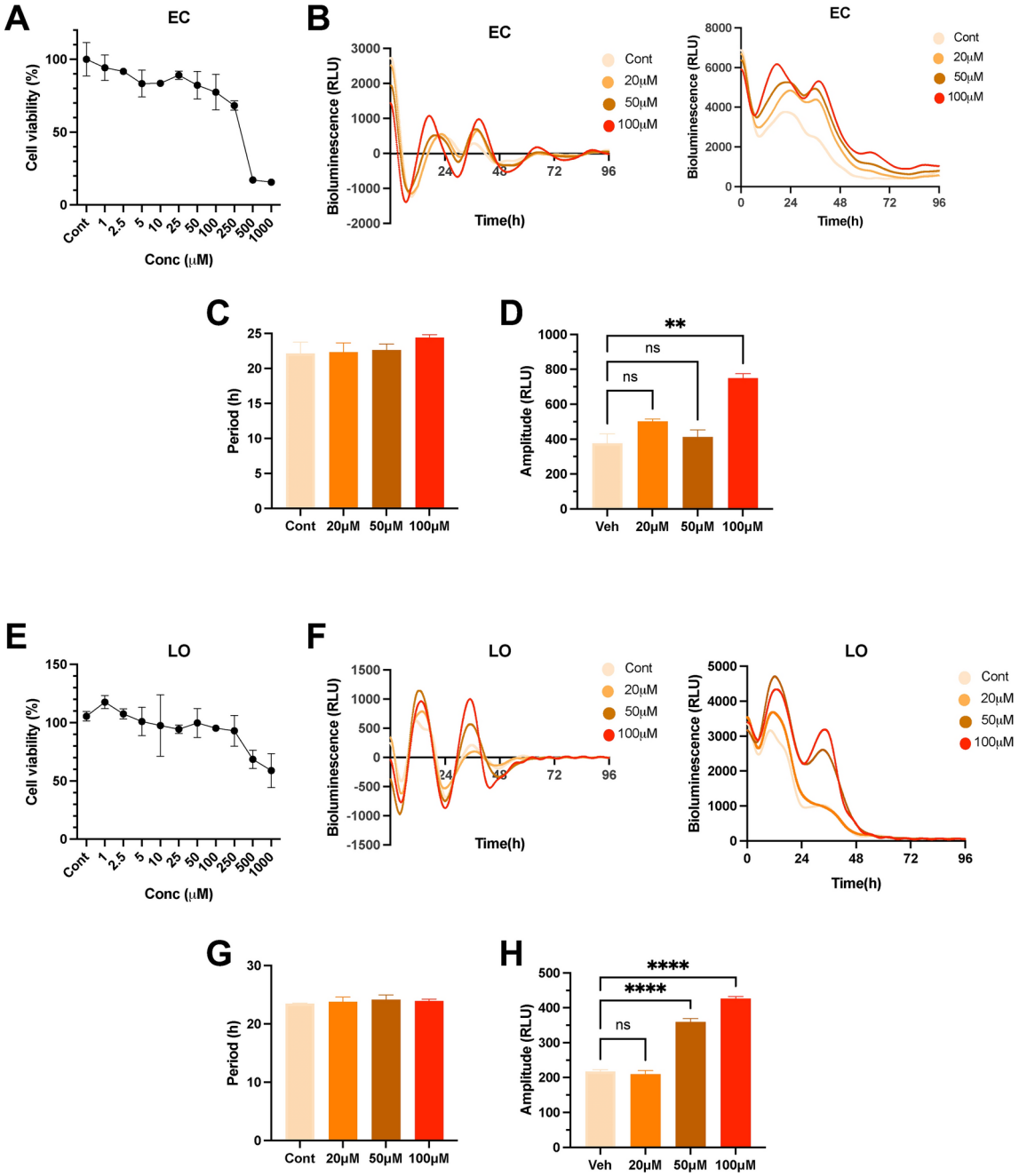
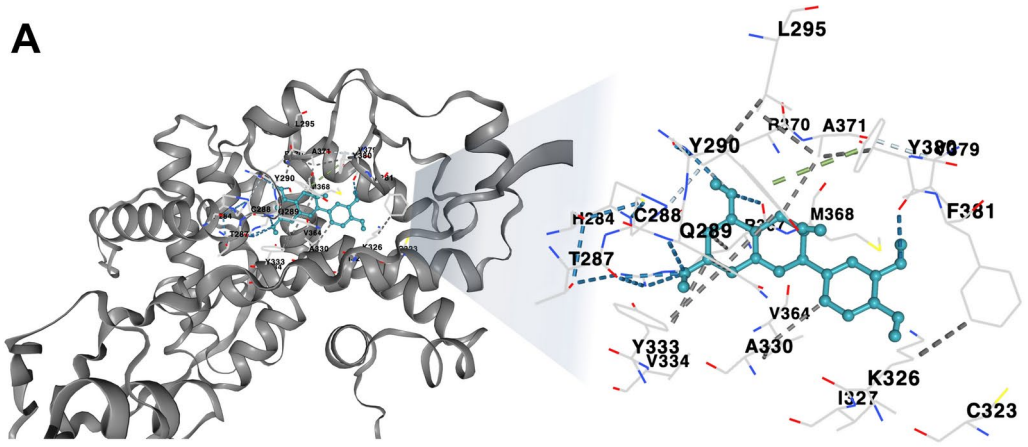


Fig. 2. Epicatechin (EC) and linalool (LO) regulate circadian rhythm. (**A**,**E**) HaCaT cells were treated with EC or LO for 24 h and subjected to CCK-8 analysis. The value of control was set to 100. (**B**,**F**) Dexamethasone-mediated synchronized BMAL1::Luc cells were treated with indicated concentrations of EC or LO, and bioluminescence activity was measured for 96 h in a real-time manner. The bioluminescence data was detrended, and the (**C**,**G**) period and (**D**,**H**) amplitude were analyzed.

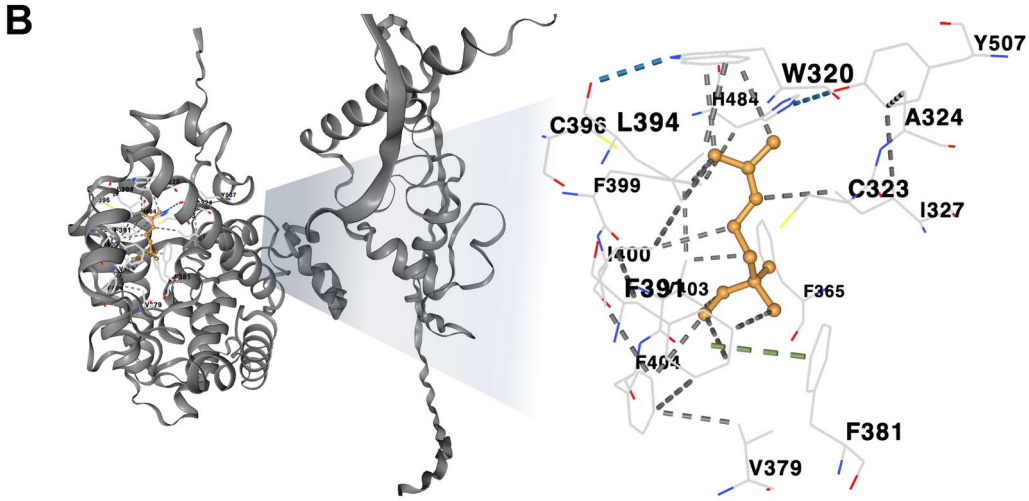
4D,I). Treatment with CC extract also increased the mRNA levels of *PER2*, whereas the fold change was minor when treated with either EC or LO and was not observed at all time points (Fig. 4E,J). Thus, EC and LO altered the mRNA expression pattern of the clock genes but in a manner slightly distinct from that of CC extract.

EC and LO modulate antioxidant defense systems in skin cells

The circadian-modulating function of EC and LO in keratinocytes might affect diverse cellular physiology because the circadian rhythm affects approximately 60% of all genes[30]. As the circadian rhythm is associated with oxidative stress control[7], we examined the influence of EC and LO on the antioxidant system in keratinocytes. Treatment with hydrogen peroxide increased the fluorescence signal of CellROX™ oxidative stress reagents, which are used to measure reactive oxygen species (ROS) levels in live cells (Fig. 5A,B). Co-treatment of hydrogen peroxide with EC or LO significantly decreased ROS levels. As EC and LO decreased the oxidative stress-induced ROS



RORA-EC	Vina score	Cavity volume (Å)	Center (x, y, z)	Docking size (x, y, z)
	-9.1	1290	-7, 2, 4	21, 21, 21



RORA-LO	Vina score	Cavity volume (Å)	Center (x, y, z)	Docking size (x, y, z)
	-5.6	1290	-7, 2, 4	18, 18, 18

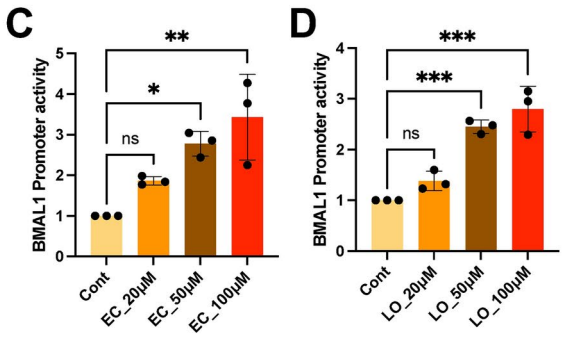


Fig. 3. EC and LO directly activate the circadian clock system. (**A**,**B**) Molecular docked structures of EC and LO with the core circadian clock protein RORA, conducted using the CB-DOCK 2 server. (**C**,**D**) HaCaT cells that stably express BMAL1::Luc were treated with indicated concentrations of EC or LO, and luciferase activity was measured after 24 h of incubation. The luciferase activity of control was set to 1 (n = 3).

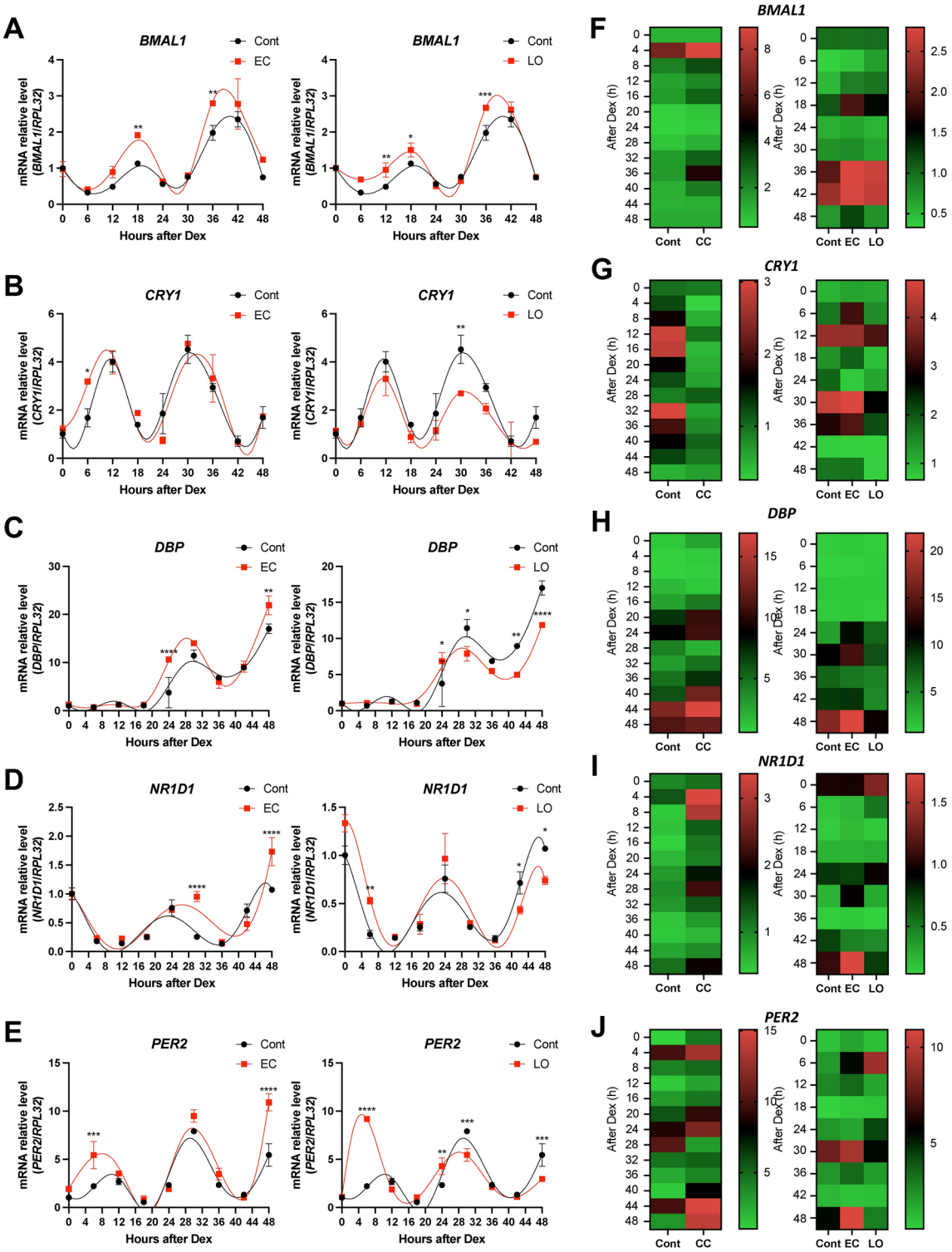


Fig. 4. EC and LO modulate the expression of clock genes. (**A**–**E**) Synchronized HaCaT cells were treated with 100 μM of EC or LO, and the cells were harvested at indicated time points. mRNA levels of the clock genes were measured using quantitative real-time PCR by using gene-specific primers. The mRNA level of control at 0 h was set to 1. (**F**–**J**) To better visualize the temporal changes in clock gene expression across treatments, heatmaps were generated for *BMAL1*, *CRY1*, *DBP*, *NR1D1*, and *PER2*, depicting the relative mRNA expression levels at different time points.

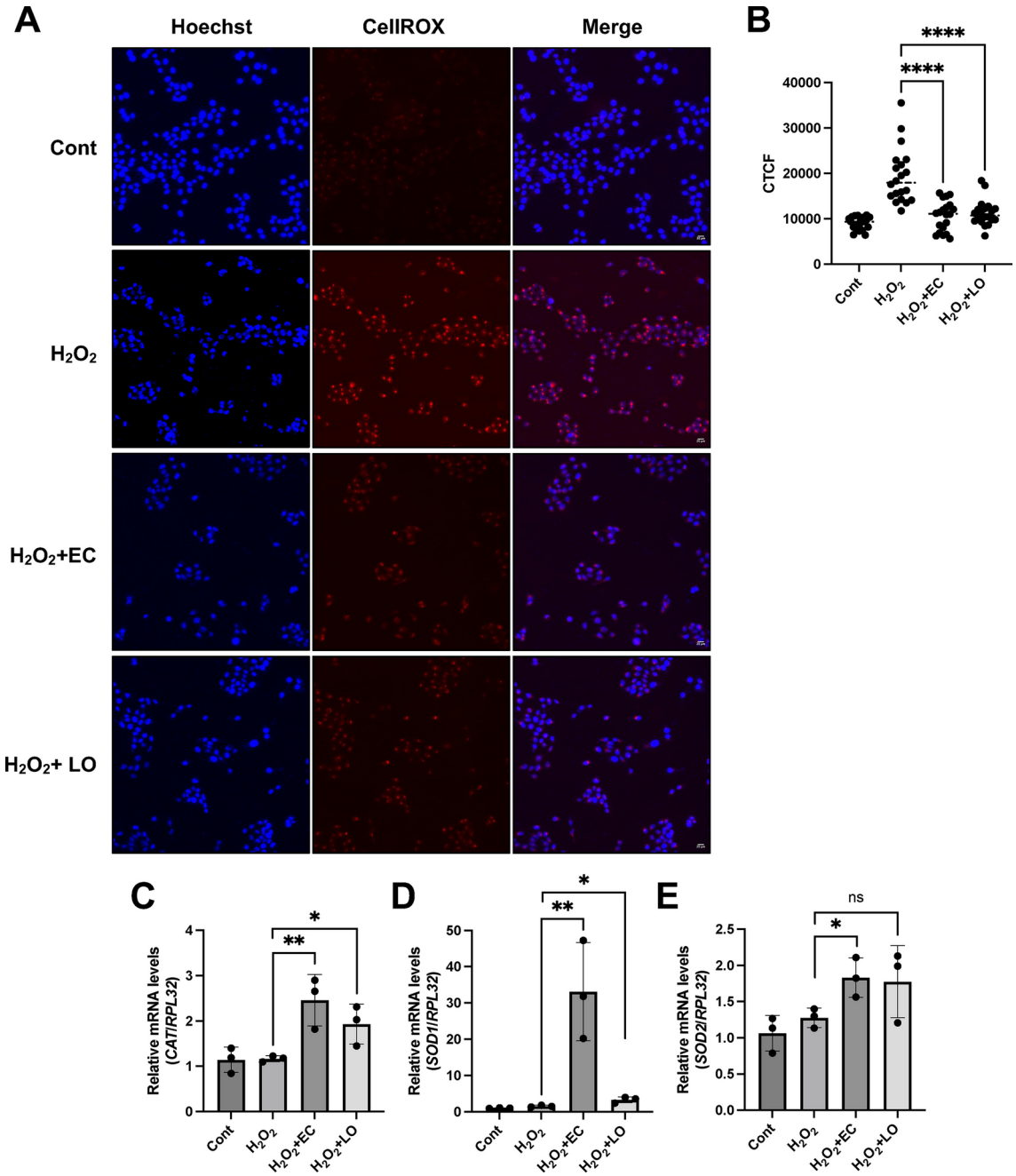


Fig. 5. EC and LO modulate antioxidant activity. (**A**) HaCaT cells were treated with H_2O_2 and EC or LO. After 24 h of incubation CellROX and NucBlue reagents were added. The nuclear signal is indicated in blue. Scale bar, 200 µm. (**B**) The fluorescence intensity of CellROX per cell was quantified. (**C**–**E**) HaCaT cells were treated with H_2O_2 with EC or LO for 24 h, and mRNA levels were quantified using quantitative real-time PCR by using the indicated gene-specific primers (n = 3).

levels, the expression levels of antioxidant enzymes such as catalase (CAT), superoxide dismutase 1 (SOD1), and superoxide dismutase 2 (SOD2) were examined in the presence of EC or LO. *CAT* expression increased when cells were treated with hydrogen peroxide and EC or LO (Fig. 5C). Treatment with EC considerably increased the expression level of *SOD1* under oxidative stress (Fig. 5D). LO also upregulated *SOD1* but to a lesser extent than that by EC. EC and LO tended to slightly increase *SOD2* expression (Fig. 5E). These results indicate that the CC extract components EC and LO have the potential to regulate circadian rhythms and modulate antioxidant defense systems in keratinocytes.

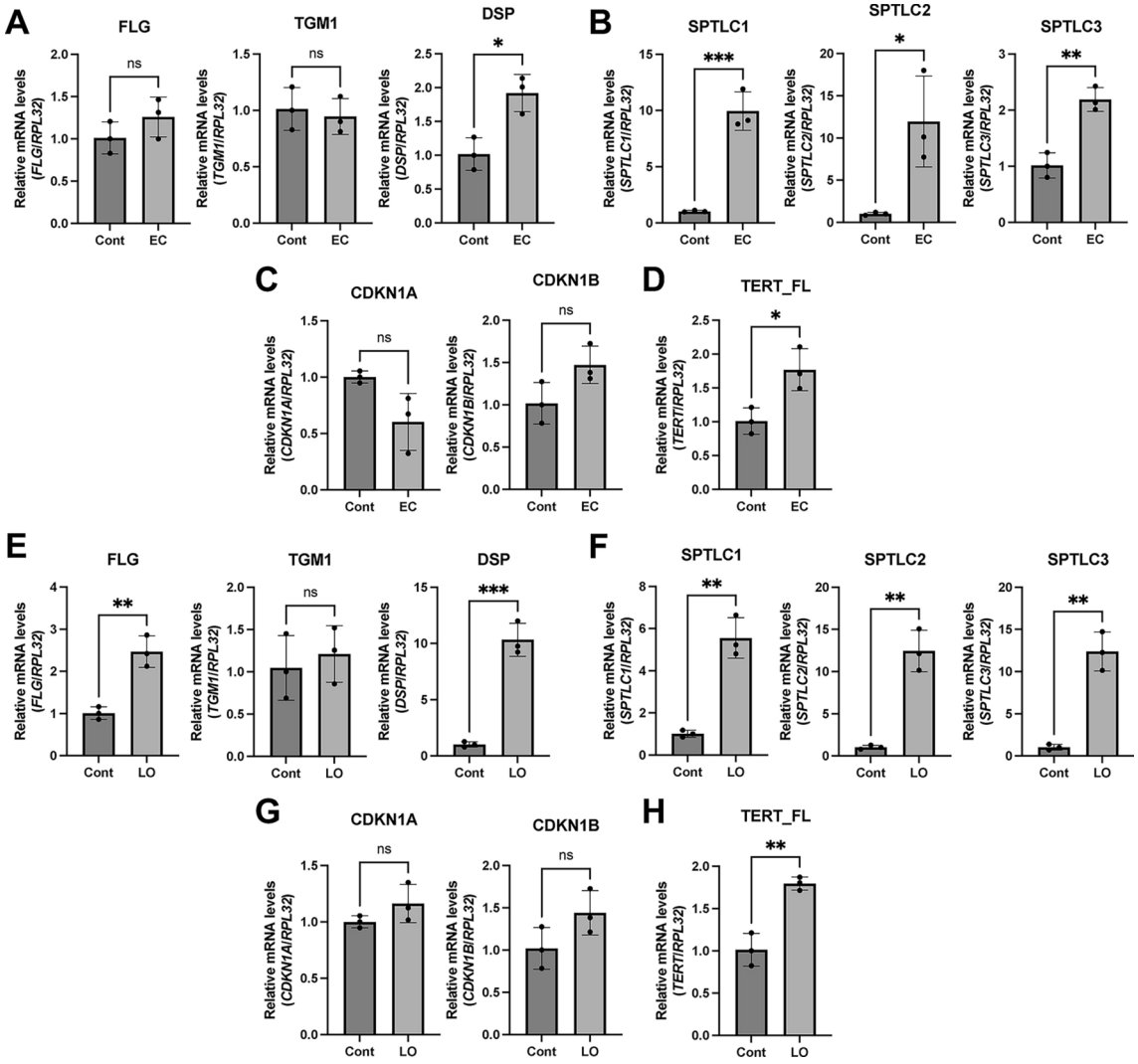


Fig. 6. EC and LO enhance skin barrier function. HaCaT cells were treated with EC or LO for 24 h and the expression levels of (**A**,**E**) barrier-related genes, (**B**,**F**) ceramide synthesis genes, and (**C**,**D**,**G**,**H**) anti-aging-related genes were analyzed using quantitative real-time PCR (n = 3).

EC, LO, and CC extract enhance skin barrier function

Because keratinocytes are pivotal in maintaining the overall integrity and protective function of the skin in a circadian rhythm-dependent manner[31,32], the effects of the bioactive compounds in CC extract on the expression of skin barrier genes were investigated.

FLG, which encodes filaggrin and is crucial for keratin network formation and skin hydration, was significantly upregulated by LO treatment; however, EC had no effect on *FLG* (Fig. 6A,E). *TGM1*, which encodes transglutaminase 1, an enzyme involved in cross-linking structural skin proteins, showed no significant changes in expression after EC or LO treatment. However, *DSP*, encoding desmoplakin, a critical desmosomal protein critical for epidermal integrity, was significantly upregulated by both EC and LO. Ceramide synthesis, a circadian-regulated process essential for skin barrier function, was evaluated by measuring the expression levels of *SPTLC1*, *SPTLC2*, and *SPTLC3*, which are key genes involved in ceramide production. Treatment with EC, LO, as well as CC extract significantly upregulated these genes, indicating a shared effect on ceramide synthesis (Fig. 6B,F; Supplementary Fig. S4A).

To assess the potential effects of EC, LO, and CC extract on cellular aging, the expression levels of senescence-associated genes *CDKN1A* and *CDKN1B* were measured. Neither EC nor LO significantly altered the expression of these genes (Fig. 6C,G). However, the transcript levels of *TERT*, a core component of the reverse transcriptional activity of telomerase, increased following treatment with EC or LO (Fig. 6D,H). Notably, CC extract inhibited *CDKN1A* and *CDKN1B* expression, thereby preventing cell cycle arrest, and also increased *TERT* mRNA levels (Supplementary Fig. S4B). These results suggest that EC, LO, and CC extract stimulate skin barrier function by enhancing structural integrity, increasing ceramide levels, and inhibiting cellular aging.

Discussion

We demonstrated that CC extract and its components EC and LO exert significant effects on both cellular and molecular circadian mechanisms in keratinocytes.

Bioluminescence assays revealed that CC extract enhanced the amplitude of circadian rhythms in a concentration-dependent manner, with the maximum effect observed at 100 μg/mL. This finding highlights the potent modulatory effect of CC extract on circadian rhythms, consistent with previous findings that link circadian amplitude modulation to improved cellular function and resilience to environmental stressors[13,18,33,34].

Gene expression analysis elucidated how CC modulates the circadian clock in keratinocytes, leading to changes in circadian rhythms. Treatment with CC extract decreased *CRY1* mRNA levels and increased *DBP, NR1D1,* and *PER2* levels. These changes suggest that CC extract can reprogram the expression of core clock genes, potentially enhancing the robustness of the circadian clock.

The identification of EC and LO as the circadian rhythm-modulating active components of CC highlights their potential as therapeutic agents. Both compounds significantly increased the bioluminescence activity and circadian amplitude in a dose-dependent manner, mirroring the effects of CC extract. *CRY1* suppression by CC and LO may reduce feedback inhibition on CLOCK and BMAL1, indirectly enhancing *BMAL1* promoter activity. Furthermore, CC and LO may directly activate *BMAL1* transcription via RORA, as supported by molecular docking results. These mechanisms collectively contribute to enhanced circadian amplitude and robustness. Notably, docking simulations suggested strong binding affinities of EC and LO for the RORA protein, a nuclear receptor integral to circadian regulation. This interaction may explain the observed enhancement of *BMAL1* promoter activity following EC and LO treatment. These findings suggest that EC and LO directly activate RORA to modulate circadian clock genes; however, further studies are needed to identify specific binding regions and confirm the direct interaction.

While CC extract broadly upregulated clock genes including *DBP*, *NR1D1*, and *PER2*, EC and LO demonstrated distinct effects on the expression of these genes. EC selectively increased *DBP* expression, whereas LO specifically downregulated *CRY1*. These findings suggest that the mechanistic pathways of EC and LO may differ from that of CC extract, with EC primarily enhancing transcriptional activators and LO potentially modulating repressive components of the circadian machinery. This differentiation underscores the unique contributions of these compounds to circadian regulation.

Previous studies have demonstrated that the circadian clock system, through key regulators such as BMAL1 and CLOCK, plays a critical role in managing oxidative stress and maintaining skin barrier function. For example, BMAL1:CLOCK complexes regulate antioxidant enzymes such as CAT and superoxide dismutases (SOD1 and SOD2), aligning their expression with circadian rhythms to mitigate oxidative stress. BMAL1 inactivation disrupts CAT expression, leading to increased oxidative stress[35], whereas BMAL1 activates SOD1 and SOD2 through E-box motifs in their promoters[36–39].

Similarly, the circadian clock influences lipid metabolism and ceramide synthesis, which are essential for maintaining the skin barrier. Genome-wide analyses have revealed that BMAL1 regulates ceramide synthesis genes such as *SPTLC1*, *SPTLC2*, and *SPTLC3*, by aligning their expression with circadian rhythms[38,40,41]. These findings provide a strong basis for linking circadian modulation to changes in skin barrier function.

In the context of our study, CC extract and its components EC and LO, modulate the circadian clock system in keratinocytes. Although we did not perform additional experiments to explicitly demonstrate how these circadian changes affect oxidative stress and skin barrier function, the findings align with the established role of circadian rhythms in regulating these physiological processes. This consistency with existing evidence suggests that the observed effects of CC extract, EC, and LO on skin physiology are mediated, at least partially, through circadian clock modulation. To further explore these connections, we plan to conduct chromatin immunoprecipitation assays to confirm BMAL1/CLOCK binding to antioxidant and lipid metabolism gene promoters, gene knockdown experiments to assess the direct effects of circadian clock disruption, and temporal gene expression analyses to investigate circadian rhythms in key pathways under CC extract treatment.

Finally, the analysis of senescence-associated genes revealed that EC and LO upregulated the mRNA transcripts of *TERT* without significantly affecting *CDKN1A* or *CDKN1B* expression. This suggests that EC and LO promote cellular longevity through telomerase activation, thus providing a potential mechanism for their anti-aging effects.

In future, we plan to utilize 3D artificial skin tissue models to further explore the functional applications of CC extract and its components EC and LO. Using 3D models may offer a more physiologically relevant environment than traditional monolayer cell cultures, closely mimicking the complex architecture and cellular interactions of human skin. By employing 3D skin models, we aim to deepen our understanding of how CC extract, EC, and LO modulate skin-related processes, including circadian rhythm regulation, barrier function, and cellular repair. This approach will also allow us to explore their potential therapeutic effects on various skin conditions, thereby contributing to the development of more targeted and effective dermatological treatments.

In conclusion, our study demonstrated that CC extract and its components EC and LO have significant potential to modulate circadian clock, resulting in enhanced circadian rhythms, reduced oxidative stress, and enhanced skin barrier function in keratinocytes. This study provides compelling evidence for the potential therapeutic applications of CC extract in skin health and anti-aging treatments for use in the development of novel dermatological treatments. Further research is warranted to elucidate the clinical applications and long-term benefits of these compounds in skin care.

Materials and methods

Cell culture

Human epidermal keratinocytes (HaCaT cells) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich). The cells were maintained in a humidified incubator with 95% ambient air and 5% CO_2 at 37 °C.

Preparation of extracts and individual compounds

The plant extract (cinnamomi cortex) used in this study was obtained from the Korea Plant Extract Bank at the Korea Research Institute of Bioscience and Biotechnology (CA02-011; Daejeon, Korea). The extract was prepared by using 95% ethyl alcohol as the extraction solvent, according to the manufacturer's instruction.

Several individual components of CC have been identified (Table 1)[42–46]. The following individual components of cinnamomi cortex were used in this research: (−)-borneol (CAS-464-45-9, Sigma-Aldrich); (−)-caryophyllene oxide (CAS-1139-30-6, Sigma-Aldrich); (R)-(+)-limonene (CAS-5989-27-5, Sigma-Aldrich); 1,8-cineole (CAS-470-82-6, Sigma-Aldrich); 4-hydroxy-3-methoxycinnamaldehyde (CAS-458-36-6, Sigma-Aldrich); cinnamaldehyde (CAS-104-55-2, Sigma-Aldrich); cinnamic acid (CAS-140-10-3, Sigma-Aldrich); cinnamyl acetate (CAS-103-54-8, Sigma-Aldrich); cinnamyl alcohol (CAS-104-54-1, Sigma-Aldrich); coumarin (CAS-91-64-5, Sigma-Aldrich); (−)-epicatechin (CAS-490-46-0; Sigma-Aldrich), eugenol (CAS-97-53-0, Sigma-Aldrich); linalool (CAS-78-70-6, Sigma-Aldrich); nerolidol (CAS-7212-44-4, Sigma-Aldrich); terephthalic Acid (CAS-100-21-0, Sigma-Aldrich); terpinolene (CAS-586-62-9, Sigma-Aldrich); α-terpineol (CAS-98-55-5, Sigma-Aldrich); and β-caryophyllene (CAS-87-44-5, Sigma-Aldrich).

Cell viability analysis

Cells were seeded into 96-well plates at a density of 5×10^4 cells/well and incubated overnight. Cell viability was determined after 24 and 72 h in the presence or absence of the test extracts or compounds using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Plates were read using an Infinite M Nano microplate reader (Tecan, Zurich, Switzerland) according to the manufacturer's instructions.

Real-time bioluminescence

Human HaCaT cells, engineered to express mouse *BMAL1* promoter-driven luciferase (obtained from Dr. Kramer, Charité—Universitätsmedizin Berlin, Berlin, Germany)[4] were treated with 100 nM dexamethasone for 2 h. The medium was then replaced with fresh culture medium containing CC extract or 20, 50, or 100 μM of either of the components, along with 100 μM luciferin. Luciferase activity was measured in real-time over four days using a luminometer (Kronos HT, ATTO, Tokyo, Japan). Next, bioluminescence activity was determined using detrending data processing, and the respective graphs were generated. The period and amplitude of the cellular circadian rhythms were analyzed using the online platform BioDare2 (https://biodare2.ed.ac.uk)[47,48].

Molecular docking simulations

We used the CB-DOCK2 server to perform molecular docking simulations. The binding pocket was determined using a cavity detection algorithm, and the docking was executed with AutoDock Vina for affinity scoring. Grid sizes were auto-generated to fit the identified binding pockets, and RORA was docked against EC and LO as ligands.

RNA quantification

Total RNA was extracted from the cells using TRIzol™ reagent (Invitrogen™). RNA was reverse-transcribed using GoScript™ Reverse Transcriptase (Promega) with oligo(dT) primers, following the manufacturer's instructions. mRNA levels were quantified using quantitative real-time PCR with a QuantStudio 3 real-time PCR instrument (Applied Biosystems) and SYBR Green PCR Master Mix (Thermo Fisher Scientific) as previously reported[49]. Most primers used in this study were designed to span exon-exon junctions to avoid amplification of genomic DNA. Primers that did not span exon-exon junctions were subjected to additional validation measures. RNA samples were treated with DNaseI before reverse transcription to eliminate genomic DNA contamination. The sizes of real-time PCR products were verified to match the expected product sizes using gel electrophoresis, and melting curve analysis consistently showed a single sharp peak for all reactions, confirming the specificity of amplification.

Determination of intracellular ROS levels

Intracellular ROS levels were measured using the fluorogenic CellROX™ Orange reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The CellROX reagent was added to the cultured cells at a concentration of 5 μM for 30 min at 37 °C. Nuclear staining was conducted using NucBlue Live ReadyProbes Reagent (Thermo Fisher Scientific) for 5 min. Fluorescent images were acquired using an EVOS FL Auto Imaging System (Thermo Fisher Scientific) with a magnification of 200×.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism software. Comparisons between two groups were performed using a two-tailed unpaired Student's *t*-test. For comparisons involving more than two groups, one-way analysis of variance (ANOVA) with Tukey's post-hoc test was used. To evaluate the interaction between treatment groups and time points for circadian clock gene expression, two-way ANOVA was performed. Post-hoc multiple comparisons were conducted using Šídák's test. Statistical significance was set at $p < 0.05$. Statistical parameters, including the definitions and exact values of n (number of biological replicates), distributions, and

deviations, are reported in the figures and their corresponding legends. All quantitative data are presented as mean ± standard error of the mean.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions

JYK, EMJ, and KHL conceived the study and wrote the manuscript. JYK, JL and SHK performed the experiments. KHL obtained funding. All authors have read and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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