# *Cereus grandiflorus* flower extract modulates genes involved in circadian rhythm and cell migration to favorize night cell processes

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**Abstract (Maximum of 250 words)**

**Background**: The objective was to assess if *Cereus grandiflorus* extract might improve keratinocytes migration and modulate the circadian rhythm.

**Methods**: An artificial human keratinocyte wound healing test was performed. The cells were treated with the *C. grandiflorus* extract to observe its action on cells migration at times 14, 18 and 24 hours.

A second test carried out on human keratinocytes studied the modulation of the *C. grandiflorus* extract on 14 circadian genes expression on basal situation during 48h via a qPCR array to follow modulation due to circadian rhythm.

**Results**: In the artificial keratinocyte wound healing test, *C. grandiflorus* extract (0.3mg/mL) significantly stimulated cells migration (on average 128% of the control (no treatment) over the 3 analysis times). The second vitro studies demonstrated that both, melatonin and *C. grandiflorus* extract modulate 6 circadian genes, including PER1 and the melatonin receptors, in a similar pattern.

**Conclusion**: The *C. grandiflorus* extract stimulates keratinocyte migration and modulates specific genes involved in circadian rhythm, these two actions are major processes that participate to skin night regeneration.

**Keywords:** skin chronobiology; regeneration; night processes; circadian rhythm

**Introduction**.

*Cereus grandiflorus* belonging to the Cactaceae familly (cactus), is native from the desert regions of the West Indies, tropical America, and Mexico. Its flowers, sweet scented, bloom with white petals at night for six hours only. This medicinal flower is traditionally used to treat heart diseases. It is rich in sugars such as glucose, fructose, amino acids, acids (citric, fumaric, malonic…), alkaloids (including cactine) and flavonoids.

The developed extract of flower for cosmetic is a complex ingredient whom composition has been described, containing flavonols, organic acids, phenolic acids, sugars, nucleosides.

It has been demonstrated that many metabolic pathways are under the control of clock genes [1], present in each cell of the body that function in a circadian and autonomous manner. It is well demonstrated that the release of melatonin during night-time plays a role in the circadian rhythm and involves interaction with clock genes.[2]

At skin level, these clock genes control the rate and intensity of expression of genes involved in the quality of the skin barrier, in hydration [3], in defense and / or regeneration of the skin. Interestingly, during nighttime the circadian rhythm favorize the regeneration processes. [4]

The objective was to assess if *C. grandiflorus* extract might improve keratinocytes migration and modulate the circadian rhythm, as compared to melatonin.

**Materials and Methods**.

1- Keratinocyte migration on wound healing model

   a. Materials

Cellular types used are Normal Human Epidermal Keratinocytes (NHEK), Bioalternative reference K593, after 3$^{rd}$ passage, under the following culture condition: 37°C, 5% CO2 in a culture medium Keratinocyte-SFM complemented with Epidermal Growth Factor (EGF) 0.25 ng/ml, Pituitary extract 25 µg/ml and Gentamycin 25 µg/ml.

The assay medium is Keratinocyte-SFM complemented with Gentamycin 25 µg/ml.

Tested compound is a 100% *Cereus grandiflorus* flower extract powder tested at 2 concentrations 0.1mg/mL and 0.3mg/mL diluted in test medium; the concentration was selected after cytotoxicity test on NHEK.

Positive reference of the test is EGF (Gibco) at 10ng/mL.

b. Method

The keratinocytes were seeded in culture medium in a 96-well plate dedicated to migration analysis (ref. Platypus OrisTM Collagen I Coated Plate).

In this plate, the wells were “coated” with a collagen solution and a mask was placed in the center of each well, preventing cell adhesion in this area, thus forming an artificial wound (migration zone).

A few hours after seeding the cells and after cell adhesion, the medium was replaced with test medium and for the conditions with preincubation, the cells were treated with *the C. grandiflorus* extract. After 24 hours of preincubation, the caches were removed then the cells were labeled with calcein-AM, incubated for 30 minutes and images were taken (T0). The medium was then replaced by test medium containing or not containing (control) the compounds, the combinations under test or the reference (EGF). The cells were incubated, and image analyzes were carried out in kinetics at 14, 18 and 24 hours. Before imaging at time 18 hours, cells were again labeled with calcein-AM and incubated for 30 minutes.

All the experimental conditions were carried out in n=3, except the control condition carried out in n=6.

The cell migration zone was monitored after 0, 14, 18 and 24 hours of incubation with a high-resolution imaging system, automated microscope INCell Analyzer™2200 (GE Healthcare) (x4 objective) and the wound surface was analyzed with the Image J software.

The area of the artificial wound (central area without cells) was measured at time T0, after 14, 18 and 24 hours of incubation. In order to monitor and quantify wound coverage, wound measurements after 14, 18 and 24 hours of incubation are related to the initial surface area measured at T0 (see Figure A). The effect of compounds on migration was compared to the untreated control.

The raw data were transferred and processed using Microsoft Excel software. Intergroup comparisons were made using the unpaired two-tailed Student's t test. Statistical analyzes can be interpreted if $n \geq 5$; however, for $n < 5$, the calculated data is only provided as an indication.

We evaluated the percentage of coverage of the migration zone:

Coverage (%) = 100 – (Surface of the wound /Initial surface x 100)

2- Circadian genes activity

a. Materials

Cellular types used are NHEK, Bioalternative reference K341, after 3rd passage, under the following culture condition: 37°C, 5% CO2 in a culture medium Keratinocyte-SFM complemented with Epidermal Growth Factor (EGF) and Pituitary extract.

The assay medium is Keratinocyte-SFM.

Tested compounds are an hydroglycerin extract concentrated at 1.4% of *Cereus grandiflorus* flower extract and tested at 2 concentrations 1mg/mL and 3.3mg/mL diluted in assay medium; the concentration was selected after cytotoxicity test on NHEK. And melatonin (Sigma) diluted in Ethanol, tested concentration are 66.7 µg/ml and 200 µg/ml. The concentration was selected after cytotoxicity test on NHEK.

b. Method

Keratinocytes were seeded in 24-well plates and cultured in culture medium for 24 hours and in assay medium for a further 24 hours. The medium was then replaced by assay medium containing or not (control) the test compounds or the solvent control (ethanol – 0.4%) and the cells were then incubated for 1, 3, 6, 24, 36 or 48 hours. All experimental conditions were performed in n=3.

At the end of incubation, the cells were washed in phosphate buffered saline (PBS) solution and immediately dry-frozen at -80°C.

The expression of markers was analyzed using RT-qPCR method on total RNA extracted from the cell monolayers of each experimental condition (before RNA extraction, the replicates of the same experimental condition were pooled).The analysis of transcripts was performed in n=2 using a PCR array (‘‘GT210512 mQPA-16 customized’’) dedicated to research and adapted to ‘screening’ format (Marker qPCR array or ‘mQPA’ designed by Bioalternatives) and targeting 16 genes selected for their role in circadian rhythm.

Total RNA was extracted from each sample using TriPure Isolation Reagent® according to the supplier's instructions. The quality of RNA was evaluated using capillary electrophoresis (Bioanalyzer 2100, Agilent technologies). The quantity of RNA was evaluated using a spectrophotometer (Synergy H1, Biotech Instruments). The complementary DNA (cDNA) was synthetized by reverse transcription of total RNA in presence of oligo(dT) and «Transcript or Reverse Transcriptase» (Roche). The cDNA quantities were then adjusted before PCR step.

The PCR (Polymerase Chain Reaction) were performed using the « Light Cycler® » system (Roche Molecular System Inc.) according to the supplier's instructions. The reaction mix (10 all final) was prepared as follows: -2.5 all of cDNA, -primers (forward and reverse), -reagent mix (Ozyme) containing taq DNA polymerase, SYBR Green I and MgCl2.

The incorporation of fluorescence in amplified DNA was continuously measured during the PCR cycles. This resulted in a "fluorescence intensity" versus "PCR cycle" plot allowing the evaluation of a relative expression (RE) value for each marker. The value selected for RE calculations is the "output point" (Ct) of the fluorescence curve. For a considered marker, the highest is the cycle number; the lowest is the mRNA quantity. The RE value was calculated with the formula: (1/2number of cycles) x 106.

The PCR array used in the present study included 1 reference gene (RPS28). This housekeeping gene was used for data normalization since its expression is constitutive and theoretically stable. Consequently, the level of expression of the target markers was compared to the expression level of this marker for all test conditions.

Classification of effects ("treated" conditions versus "control")

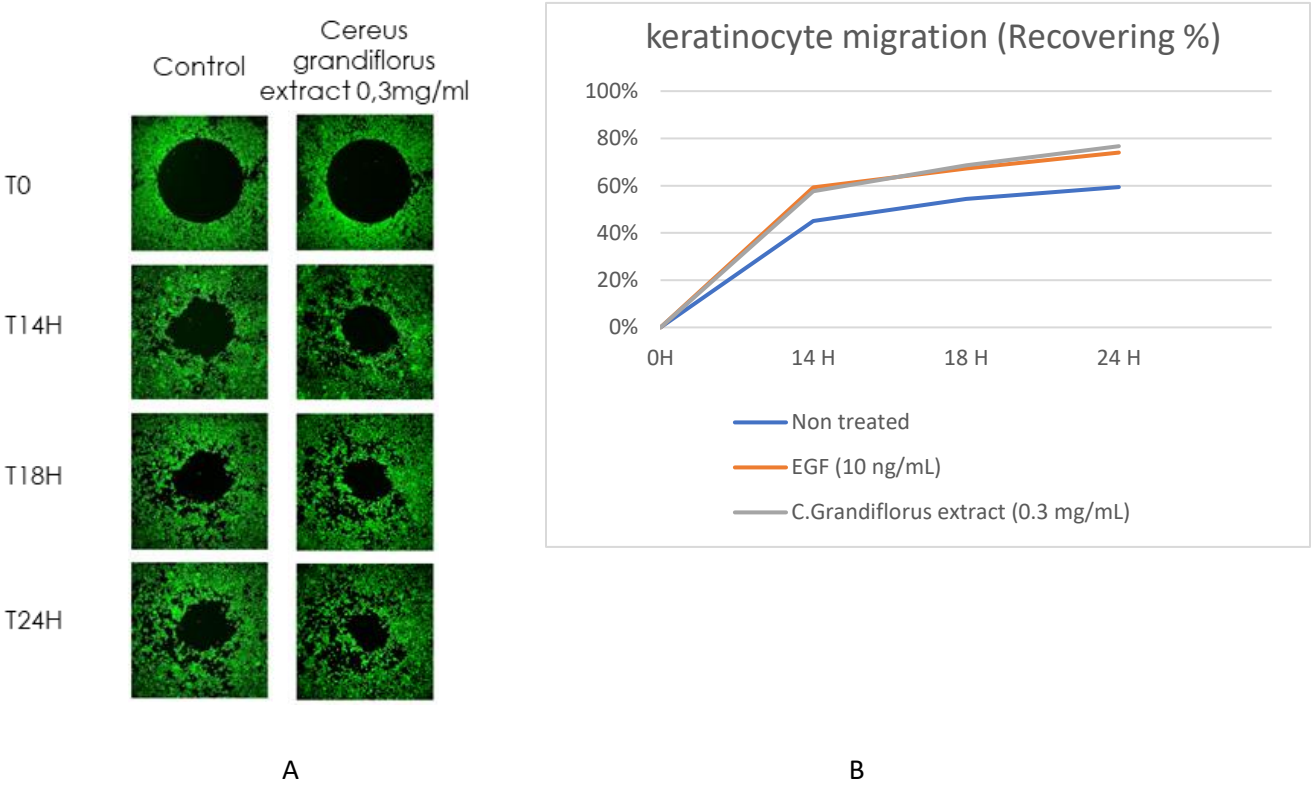
table:

| Relative expression(% of control) | Classification of effects |
|---|---|
| > 200% and < 300% | Stimulation |
| > 150% and < 200% | Slight stimulation |
| > 50% and < 70% | Moderate inhibition |
| > 30% and < 50% | Inhibition |

**Results**.

1- Keratinocyte migration on wound healing model

In the artificial keratinocyte wound healing test, *C. grandiflorus* extract (0.3mg/mL) significantly stimulated cells migration with an average 128% of the control (no treatment) over the 3 analysis times (figure 1).




A B

Figure 1 – A/ Representative images of the effect of the *C. grandiflorus* extract (0.3mg/mL) with 24H preincubation on the migration of keratinocytes after 14, 18 and 24 hours of incubation versus untreated. B/ graph representating % of recovering of the 3 conditions (untreated, EGF 10ng/mL, *C. grandiflorus* extract 0.3mg/mL at 14h, 18h, 24h.

2- Circadian genes activity

The qPCR array on circadian genes demonstrated that both, melatonin and *C. grandiflorus* extract modulate 6 circadian genes, including PER1 and the melatonin receptors, following similar patterns:

**PER1**

This gene is one of the well-known clock gene which encode for components of the circadian activity, it is highly involved in skin metabolism rhythm [5]. At 3H, we observed a small-significant increase of expression of PER1 with melatonin and *C. grandiflorus* extract compared to the control Ethanol that did not increase. At 6H, we observed an increase of expression of PER1 with melatonin and *C. grandiflorus* extract when the control Ethanol led to no change. At 48H, we observed a moderate decrease of PER1 expression, when the control Ethanol led to no change. (Figure 2)

| PER 1 | 1H | 3H | 6H | 24H | 36H | 48H |
|---|---|---|---|---|---|---|
| Melatonine at 200 µg/ml (in Ethanol) | 129 | 152 | 195 | 81 | 71 | 64 |
| C. Grandiflorus extract at 3.3mg/mL | 96 | 161 | 296 | 105 | 81 | 67 |
| Ethanol | 90 | 114 | 127 | 89 | 97 | 105 |

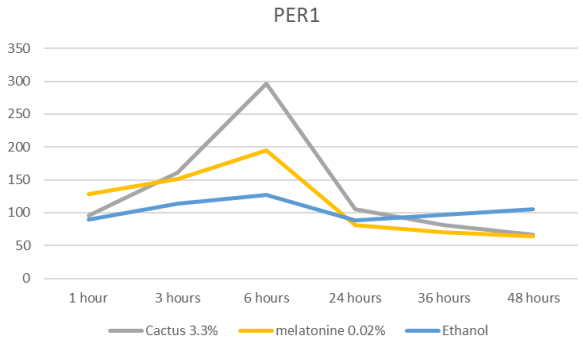



Figure 2 - Effect of C. *Grandiflorus* extract, Melatonin and control (ethanol) on the expression of PER 1 gene in normal human epidermal keratinocytes –Rt-qPCR assay.

**BHLHE40**

This gene encodes protein that can promote PER1 and repress CLOCK/ARNTL's transactivation of PER1. This gene is believed to be involved in the control of circadian rhythm [6]. At 3H, we observed a decrease of BHLHE4 expression with melatonin and *C.grandiflorus* extract, when the control Ethanol led to a low decrease. At 6H, we observed an increase of expression of BHLHE4 with melatonin and *C. grandiflorus* extract when the control Ethanol led to no change. (Figure 3)

| BHLHE4 | 1H | 3H | 6H | 24H | 36H | 48H |
|---|---|---|---|---|---|---|
| Melatonine at 200 µg/ml (in Ethanol) | 46 | 37 | 161 | 126 | 102 | 98 |
| C. Grandiflorus extract at 3.3mg/mL | 48 | 34 | 258 | 120 | 75 | 86 |
| Ethanol | 41 | 66 | 108 | 79 | 106 | 93 |

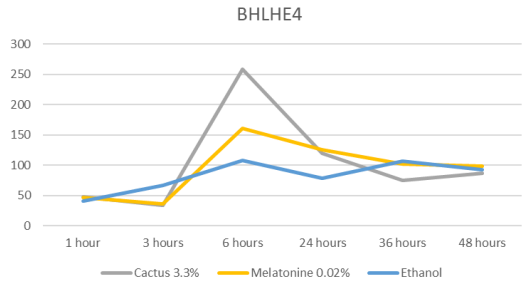



Figure 3 - Effect of C. *Grandiflorus* extract, Melatonin and control (ethanol) on the expression of BHLHE40 gene in normal human epidermal keratinocytes –Rt-qPCR assay.

**MTNR1B**

This gene encodes one of two high affinity forms of a receptor for melatonin. It is thought to may be involved in the effects of melatonin.[7]

*C. Grandiflorus* extract and melatonin follow a similar pattern, at the opposite of Ethanol, specifically at T24H. (Figure 4)

| MTNR1B | 1H | 3H | 6H | 24H | 36H | 48H |
|---|---|---|---|---|---|---|
| Melatonine at 200 µg/ml (in Ethanol) | 80 | 132 | 72 | 73 | 158 | 134 |
| C. Grandiflorus extract at 1mg/mL | 86 | 123 | 89 | 83 | 137 | 97 |
| Ethanol | 83 | 145 | 104 | 177 | 128 | 128 |

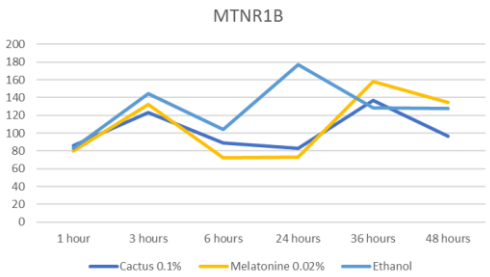



Figure 4 - Effect of C. *Grandiflorus* extract, Melatonin and control (ethanol) on the expression of BHLHE40 gene in normal human epidermal keratinocytes –Rt-qPCR assay

**Discussion.** The efficacy of *C. grandiflorus* flower extract on skin is poorly documented, those new data on keratinocytes allow us to complete previous generated data and go further in the understanding of this new active. Interestingly this active showed a capacity to promote epidermal regeneration processes by increasing migration of keratinocyte on the wound model. Moreover, the results of *C. grandiflorus* flower extract on circadian gene expression in parallel of Melatonin show interesting action to promotes potential skin night processes, by modulating gene expression with similar pattern as melatonin.

Further investigations would be necessary to understand its method of action to explain its efficacy.

**Conclusion**. The presented studies help to complete our knowledge in the ingredient *C. grandiflorus* flower extract, they demonstrated stimulation of keratinocyte migration and modulation specific genes involved in circadian rhythm, these two actions are major processes that participate to skin night regeneration.

**Conflict of Interest Statement**. NONE. However, all authors are employed by L'Oréal R&I or YSL Beauty.

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