Biological Activity of Polynesian *Calophyllum inophyllum* Oil Extract on Human Skin Cells*

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Key words
- *Calophyllum inophyllum*
- Calophyllaceae
- French Polynesia
- human skin cells
- wound healing
- transcriptomics

Abstract

Oil from the nuts of *Calophyllum inophyllum*, locally called "Tamanu oil" in French Polynesia, was traditionally used for wound healing and to cure various skin problems and ailments. The skin-active effect of "Tamanu oil emulsion" was investigated on human skin cells (keratinocytes and dermal fibroblasts) and showed cell proliferation, glycosaminoglycan and collagen production, and wound healing activity. Transcriptomic analysis of the treated cells revealed gene expression modulation including genes involved in the metabolic process implied in O-glycan biosynthesis, cell adhesion, and cell proliferation. The presence of neoflavonoids as bioactive constituents in Tamanu oil emulsion may contribute to these biological activities. Altogether, consistent data related to targeted histological and cellular functions brought new highlights on the mechanisms involved in these biological processes induced by Tamanu oil effects in skin cells.

Abbreviations

GAG:	glycosaminoglycans
GO:	gene ontology
HaCaT:	cultured human keratinocyte (cells)
HDF:	human dermal fibroblast
TOE:	Tamanu oil emulsion
XTT:	(2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2h-tetrazolium-5-carboxanilide)

Supporting information available online at http://www.thieme-connect.de/products

Introduction

Traditional uses of medicinal plants for centuries have inspired their modern uses in pharmacology or cosmetology. *Calophyllum inophyllum* L. (Calophyllaceae) is an evergreen pantropical tree distributed in Africa, Asia, and Pacific countries [1]. This plant, locally called "Tamanu" in French Polynesia, having a great heritage symbol as a sacred tree in Polynesia, was used for centuries in Polynesian pharmacopoeia and cosmetopoeia [2–6]. Its different organs (leaves, bark, fruits, seeds) were used in traditional medicine to cure many diseases, but its nuts and containing oil were mostly used for skin care. Beside its well-known traditional uses, oil or nuts were reported to treat different kinds of skin affections (all kinds of burns, most dermatoses, eczema, certain skin allergies, acne, psoriasis, herpes, chilblains, skin cracks, diabetic sores, hemorrhoids, dry skin, hair loss, etc.) or conjunctivitis, and to heal all kinds of burns and flat wounds [7–9]. In addition to independent long-term traditional uses, the effectiveness of "Tamanu oil" was also proven by *in vivo* studies performed on hospitalized patients showing that its topical application on resistant wounds led to significant diminution of scars and impressive wound healing including postsurgical treatments [10,11]. Literature data revealed interesting biological activity related to skin affections and Tamanu oil content beneficial effects such as antibacterial [12], antifungal [13], anti-inflammatory [14], and wound healing [15]. Such biological properties may explain the use of Tamanu oil as an active cosmetic ingredient

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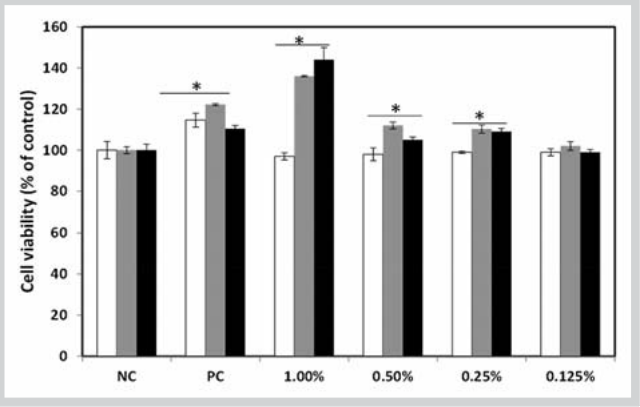

Fig. 1 Cell viability of human keratinocytes (HaCaT cells) treated with TOE during 4 h (white bars), 18 h (grey bars), and 24 h (black bars) at 37 °C. The assay was performed 24 h following the incubation period. The negative control (NC) corresponds to cells treated with free emulsions. The positive control (PC) corresponds to cells treated with 25 µg/ml of vitamin C. Data were calculated as % of the value obtained with non-treated cells (*$p < 0.05$).

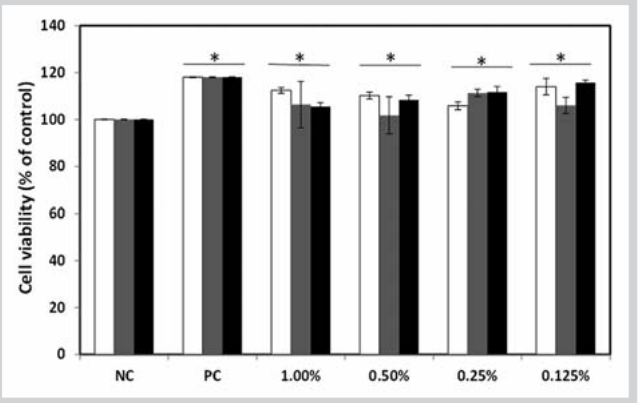

Fig. 2 Cell viability of normal human fibroblasts treated with emulsions of Tamanu oil (TOE) during 4 h (white bars), 18 h (grey bars), and 24 h (black bars) at 37 °C. The assay was performed 24 h following the incubation period. The negative control (NC) corresponds to cells treated with free emulsions. The positive control (PC) corresponds to cells treated with 25 µg/ml. Data were calculated as % of the value obtained with non-treated cells (*$p < 0.05$).

recorded as "*Calophyllum inophyllum* seed oil" by the INCI (International Nomenclature of Cosmetic Ingredients) [16,17]. Therefore, this oil is known to be included in regenerating and protective cosmetic formulas such as skin restorative, sunburn protection, soothing, and wrinkle or stretch mark prevention [18]. Most of the bioactive properties of Tamanu oil are attributed to the presence of the resinous content in the oil, which constitutes a unique characteristic of this healing oil [3,7]. Tamanu oil is still used nowadays in modern context, therefore biological activity of this cosmetic ingredient should be assessed to bring more understanding on its mode of action. For that purpose, we present herein an investigation on the healing effects of Tamanu oil on skin cell cultures (keratinocytes and fibroblasts). We performed different experiments to assess the effects of TOE on cell proliferation, GAG, collagen production, and wound healing activity. Transcriptomic analysis on the treated cells was also performed to evaluate the genetic modulation induced by Tamanu oil treatment.

Results and Discussion

In this study, Tamanu oil was emulsified as emulsions to improve intracellular delivery. We evaluated the effect of those formulations on the viability of human keratinocytes and normal human skin fibroblasts. These cells were treated with different concentrations (0.125% to 1%) of TOE during 4, 18, and 24 h. As shown in **Fig. 1**, there was no effect of free emulsions on the cell viability of human keratinocytes or fibroblasts, whatever the concentrations and time of incubation used. This indicates that lipid emulsions *per se* were not cytotoxic. TOE used at 0.125% did not affect the cell viability of keratinocytes (**Fig. 1**). However, cell proliferation was observed at higher TOE mass fractions for only the longest incubation times (18 or 24 h). Particularly, an increase in the range of 10 to 20% relative to the control cells was measured when the cells were treated with 0.25% and 0.5% TOE, respectively. The best increase of proliferation was obtained with 1% TOE (40% more than the control cells). At this TOE concentration, the benefit is even more important than the effect of plain vitamin C used as a standard positive control. It induced only a 20% increase for both HaCaT and HDF, as reported previously [19]. The effect of TOE on dermal fibroblasts was quite different, as can be seen in **Fig. 2**. When cells were incubated for 4 h, no improvement was induced for all concentrations used. The results of cell viability on fibroblasts showed a slight proliferation around 5 to 20% at all dilutions of TOE no matter the incubation time.

Since collagen production is frequently associated with cell proliferation, we checked if cell incubation with TOE could positively impact collagen production by either HaCaT cells or HDF. The experiments were carried out with 1% TOE incubated for 4 and 24 h. Data of **Fig. 3A** indicated that TOE promoted an increase of collagen production between 10 to 40% with a similar level for both cell types. The stimulation was dependent on the duration of incubation. It was ~ 1.10- and 1.23-folds higher after 4 h for HaCaT and HDF, respectively. The level increased up to ~ 1.4-fold after 24 h incubation for both cell types. For keratinocytes, this could be a consequence of the increase of keratinocytes proliferation (**Fig. 1**). This is unlikely in the case of fibroblasts, since their proliferation was not improved (**Fig. 2**). Therefore, the increase of collagen production is likely a genuine effect of the treatment.

The effect of TOE on cells induced a huge amount of GAG production. After 24 h, it was about 350% compared to the control. However, the trend for GAG production (**Fig. 3**) was cell type-dependent. HaCaT cells produced more GAG when the incubation was carried out for 4 h (1.75-fold) and it further improved at 24 h (3.70-fold). Note that there was no effect of free emulsions on those productions.

One of the main applications of Tamanu oil is its use as a topical application for wound healing. It has been reported that it improved patient's scars when applied topically. We determined the wound healing stimulating capacity of TOE on a scratched fibroblast monolayer. Wound healing assays were performed using the best conditions (1% TOE, 24 h) that stimulated both cell proliferation as well as collagen and GAG production. Data from **Fig. 4** indicates that TOE accelerated the wound closure of the scratched fibroblast monolayer. The gap was closed after 14 h,

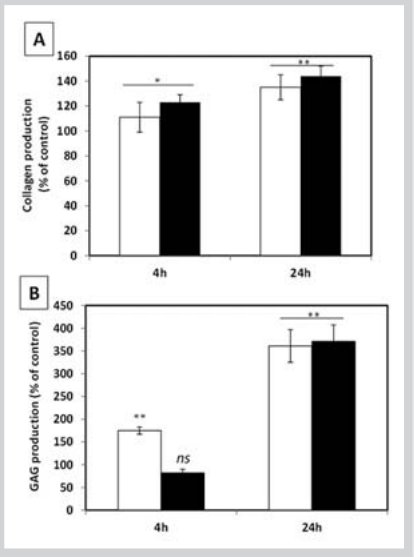

Fig. 3 Quantification of collagen (**A**) and GAG (**B**) production of HaCaT cells (□) and fibroblasts (▪) treated with 1% TOE during 4 h or 24 h. Quantification assays were performed 18 h post-treatment (*$p < 0.05$; **$p < 0.01$).

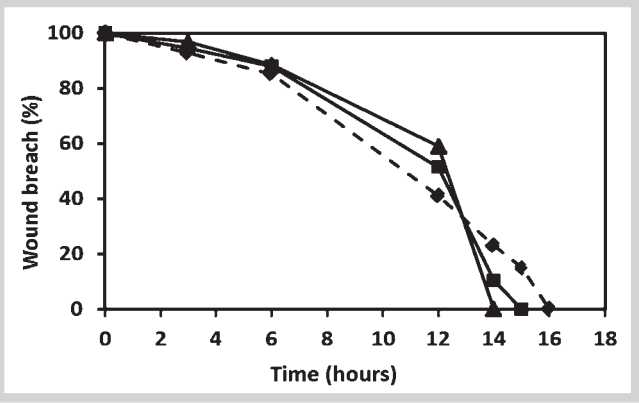

Fig. 4 CytoSelect™ 24-well wound healing assay on normal human fibroblasts after 24 h incubation of 1% TOE. The wound closure was followed by time-lapse videomicroscopy. The wound breach left was expressed as the percent of initial breach surface. (▲, straight line): Cells treated with 1% TOE; (▪, straight line): cells treated with 25 µg/ml of vitamin C; (✦, dashed line): cells treated with free emulsions.

even faster than in vitamin C-treated cells, which required 15 h for complete wound closure. In cells treated with free TOE, the wound closed only after 16 h. This positive effect of TOE on wound closure is in line with the production of collagen and GAG. These two extracellular matrix proteins are known as important players of wound healing. They are involved in the cell-matrix interaction and known to be implied in cell proliferation and cell migration via the induction of specific signaling pathways [20].

Recently, it has been reported that Tamanu oil enhanced the wound closure of a breach made on a monolayer of HaCaT cells [15]. In this study, Tamanu oil from Polynesia diluted with olive oil exhibited the highest wound closure compared to other Tamanu oils from Indonesia, the Fiji Islands, and New Caledonia. The improvement of patient's scar by topical application of Tamanu oil can likely be attributed by the capacity of Tamanu oil to foster the wound closure of a breach made on fibroblasts and keratinocytes monolayers.

As Tamanu oil treatment generated biological effects, we analyzed the gene expression of HaCaT and HDF cells to decipher the cellular mechanisms of action and the genes induced or inhibited by such treatment. The differential gene expressions between cells treated or not with TOE cells showed that 223 genes were significantly differently expressed after 24 h. Among them, 218 have a GO annotation (**Table 1 S**, Supporting Information). Of the 201 sequences, whom the cellular component was assigned, 59.7% are membrane products (**Fig. 1 S**, Supporting Information). A significant assignation for the extracellular relationships was observed with 37.3% of the sequences to cell periphery, 27.36% to the extracellular region, and 13.93% to the cell junction. Most of them are cell surface glycoproteins or binding proteins, such as collagen, protocadherins, claudins, and mucins, which participate in the extracellular matrix and cell adhesion. For the organelle component (76.12%), 9.95% were cytoskeleton components and 37.8% nuclear components. For the biological process of these gene products (192 sequences were assigned), 56.25% were involved in the response to a stimulus (response to abiotic, chemical, endogenous stimuli, etc.) such as cell migration and hypoxia, 74.28% were involved in a metabolic process such as 2-galactosyltransferases and 1-fucosyltransferase (FUT9) implied in O-glycan biosynthesis, 11.98% were involved in cell adhesion, and 13.20% were involved in cell proliferation.

The biological processes of reepithelialization following a wound are well known [20]. They imply epidermal cell migration and proliferation, restoration of barrier function by the consolidation of the extracellular matrix, and remodeling with collagen fiber rearrangement and cell junction development [21,22]. It is important to note that the differently expressed and annotated genes are mostly implied in these different processes.

Overall, those observations are completely in agreement with the increase of cell proliferation, collagen and GAG production as well as wound healing. But, this transcriptomic analysis clearly showed the expression modulation of genes related to targeted tissue and cellular functions. It brought new highlights on the mechanisms involved in these biological processes beyond the already shown biological activities of the TOE extract bioactive content.

Amongst vegetable oils, Tamanu oil is characterized by the presence of a resinous part (not found in any other vegetable oil), which is soluble in ethanol and may reach a concentration from 10 to 20% of oil. The occurrence of this resinous part (corresponding to an ethanol-soluble fraction) was considered a unique characteristic of Tamanu oil from which many of its biological activities were attributed [3,7,16,17,23,24]. Actually, the resinous part of Tamanu oil is known to contain bioactive secondary metabolites mostly constituted by neoflavonoids, including pyranocoumarin derivatives [25,26]. Such bioactive components were identified in this French Polynesian Tamanu oil extract (**Fig. 2 S**, Supporting Information), namely, calophyllolide (**1**), tamanolide (**2**), calanolide Gut 70 (**3**), inophyllum D (**4**), inophyllum P (**5**), inophyllum C (**6**), and inophyllum E (**7**). Minor constituents such as calanolide A, calanolide B, calanolide D, tamanolide D, and tamanolide P were previously identified in Tamanu oil resin (**Fig. 5**). These compounds have been previously found in *C. inophyllum* oil from French Polynesia [23,24,27,28].

Altogether, coherent data brought new insights and partly proved the wound healing activity traditionally attributed to Tamanu oil.

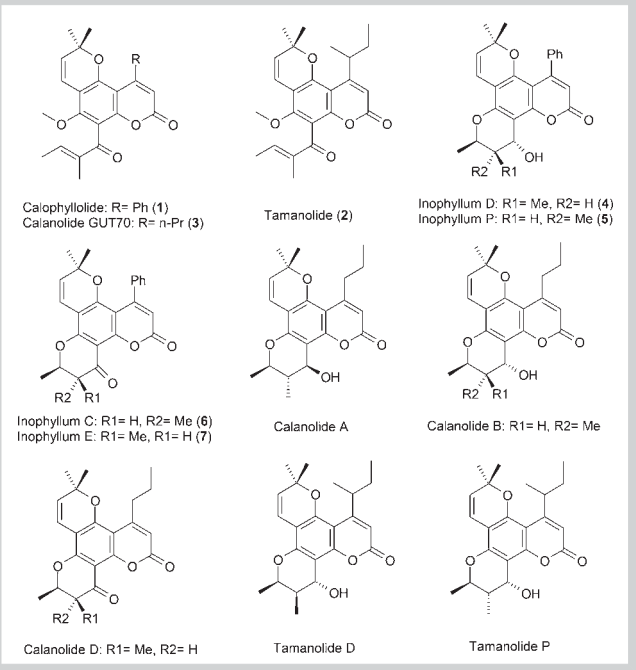

Fig. 5 Structures of neoflavonoid constituents of the resinous part (ethanol soluble fraction) of Tamanu oil.

Materials and Methods

Plant material

C. inophyllum (Tamanu) nuts were collected during the fruit flushing season on 20th October 2013. The plants were identified by Dr. J.F. Butaud (botanist). Voucher specimens (N°T-N4) are kept at the herbarium of phytochemistry laboratory, University of French Polynesia.

Oil extraction

First, the shells of *C. inophyllum* (Tamanu) nuts were removed, and then the kernels were air- and sun-dried during 2 months. During the drying process, the kernels lost 20% of their weight and become brownish with an increase of their oil content. The transformation was completed within 2 months.

The dried kernels (80.7 g) were placed in cheese cloth in a mortar and thoroughly crushed with a pestle to allow oil expression, which then yielded a green brownish crude Tamanu oil (34.6 g) after filtration.

HPLC-PDA analysis

Chromatographic analysis were performed by an HP-1260 HPLC system with an autosampler and a UV-DAD detector using silica column (silica Uptisphere type from Interchrom, porosity 120 Å, granulometry 5 µm, size 150 × 4.6 mm, I.D.) to obtain the HPLC profile of Tamanu oil neoflavonoid constituents. TOE (10.5 mg) was dissolved in ethanol (2 mL), and the soluble part was then freed of solvent, which yielded the Tamanu oil ethanolic extract (1.87 mg). TOE ethanol-soluble extract (15 µl) was dissolved in cyclohexane-ethylacetate (50 : 50, v/v) to obtain a concentration of 1 mg/ml and injected at 280 nm. The eluent (0.8 mL·min^{-1}) was a gradient of cyclohexane-ethylacetate from 8 to 25% of ethylacetate (v/v) during 40 min, followed by a stabilization period of 5 min of cyclohexane-ethylacetate (75 : 25, v/v). The compounds were identified using previously isolated standards.

Preparation of Tamanu oil emulsions

Tamanu oil was emulsified with lipids and sterically stabilized by a copolymer, prepared as described before [29]. The lipid used was a commercial grade of monolinolein (Dimodan® U/J purchased from DANISCO A/S Braband) composed of monoglycerides (mostly C18-chains). It comprises 96% monoglycerides, from which 62% are linoleate. Equal amounts of Dimodan and Tamanu oil were mixed on a hot plate. Then, triblock copolymer Pluronic®F127, PEO99-PPO67-PEO99 (BASF, Germany) dissolved in deionized water (0.375 wt %) was added to the volume of monolinolein. To emulsify the obtained bulk phase, solutions were sonicated with a VibraCell 75186 apparatus (Sonics & Materials, Inc.) for 8 min (with 1 pulse after every 1 second).

Cells and cell culture

The HaCaT cells were *in vitro* spontaneously transformed human keratinocytes purchased from Cell Lines Services. Normal human dermal fibroblasts (ECACC) were derived from the dermis of normal human neonatal foreskin or adult skin (HDF). They were cultured in specific DMEM growth medium with 4.5 g/L glucose (Invitrogen) that was supplemented with 10% fetal bovine serum and 1% L-Glutamine. Cells were used between passages 5 and 20. All cells were cultured in a 37 °C, 5% CO_2 humidified incubator and they were routinely checked for mycoplasma infection.

Cell treatments with Tamanu-containing emulsions

Two days prior to the treatment, HaCaT cells and HDF (2×10^5 cells) were seeded into 24-well plates. Cells were rinsed in PBS and an indicated dilution of TOE was added into fresh culture medium during 24 h at 37 °C. As a negative control, cells were treated with emulsions devoid of Tamanu oil emulsion.

Cell proliferation assay

Cell viability and cell proliferation were evaluated using a cell proliferation kit II (Roche). It is an effective method to measure cell growth. XTT is a colorless or slightly yellow compound that becomes bright orange upon reduction by cellular effectors such as mitochondrial oxidoreductases. Cells were plated at a density of 3×10^4 per well for HDF and 5×10^4 per well for HaCat. After 1 day, they were incubated at 37 °C in the presence of indicated dilutions of TOE during 4, 24, or 48 h. The medium was then removed and cells were further incubated in normal medium for 24 h before performing the XTT assay according to manufacturer's instructions. Briefly, XTT solution at a final concentration 0.3 mg/ml was added to each well and the incubation was carried out for 4 h at 37 °C. During this incubation, orange formazan solution was formed, resulting from the conversion of the yellow tetrazolium salt XTT by viable cells. The intensity of coloration was quantified by measuring the absorbance with a specific absorbance filter at 450 nm using a Victor spectrophotomer (PerkinElmer).

Measurement of soluble collagens concentration

Sircol™ soluble collagen assay (Biocolor Ltd.) was used to quantify total soluble collagens, according to the manufacturer's instructions. Briefly, cells were incubated with TOE during either 4 or 24 h cells and then starved for serum overnight. Supernatants were collected and centrifuged at 1500 rpm for 5 min to pellet the extracellular matrix. Supernatants containing soluble colla-

gens were mixed with Sircol dye containing Sirius red in picric acid (ratio 1 : 5, v/v) for 30 min and then centrifuged at 10 000 rpm for 10 min to drop the formed collagen-dye complex. After removing the suspension, the pellets were dissolved in Sircol Alkali reagent (containing 0.5 M sodium hydroxide). A collagen standard curve was prepared using bovine skin collagen provided in the Sircol Assay kit. The total collagen concentration was determined by measuring the relative absorbance at 540 nm using a multiple detection plate reader (Victor 3 V, PerkinElmer).

Measurement of sulfated glycosaminoglycans concentration

Blyscan™ sulfated glycosaminoglycan assay (Biocolor Ltd.) was used to quantify total sulfated GAG according to the manufacturer's instructions. Briefly, after the cells incubation with 1% TOE during either 4 or 24 h, the cells were starved for serum overnight. Next, they were rinsed with PBS and submitted to papain digestion (0.5% papain in 0.1 M sodium acetate, 0.01 M EDTA, 0.5 mM cysteine HCl; pH 6.4) for 3 h at 65 °C. The supernatants were collected and centrifuged at 12 000 rpm for 10 min. The supernatants containing sulfated GAG were mixed with Blyscan dye containing 1,9-dimethyl-methylene blue (ratio 1 : 5, v/v) for 30 min and centrifuged at 12 000 rpm for 10 min to pellet the GAG-dye complex. The supernatants were removed and the pellets were dissolved in dissociation reagent (containing sodium salt of an anionic surfactant). A standard curve was prepared using chondroitin 4-sulfate provided in the Blyscan™ assay kit. The total sulfated GAG concentration was determined by measuring the relative absorbance at 656 nm using a multiple detection plate reader (Victor 3 V, PerkinElmer).

Wound healing migration assay

We used a CytoSelect™ 24-well wound healing assay (Cell Biolabs Inc.) for the wound healing assay. This allows creating a standardized gap 0.9 mm wide. Two days before the experiments, 1×10^5 cells were seeded on the CytoSelect™ 24-well wound healing assay. When the cell confluency was reached, cells were treated during 24 h at 37 °C with different concentrations of TOE or 25 µg/ml free vitamin C, which is known to stimulate cell proliferation and synthesis of extracellular matrix proteins. Each condition was performed at least in triplicate. After washing, a new medium was added and the wound closure under various experimental conditions was followed by video microscopy using a Zeiss Axiovert 200 M (Carl Zeiss Inc.) fully motorized microscope during 24 h (scan speed: 1 image every hour). Note that cells were incubated in an atmosphere- and temperature-controlled chamber at 37 °C and 5% CO_2. Quantitative analysis of the cell free area was performed using the Axiovision Rel. 4.7 (Carl Zeiss Inc.). The level of wound healing for a time x was evaluated by calculating the percentage of the cell free area at T_x divided by the cell free area at the initial state.

Transcriptomics

HaCaT and HDF were treated during 24 h with 1% TOE. Total RNA was isolated from cultured cells using QIAGEN RNeasy kit according to the manufacturer's instructions. The integrity and quality of total RNA was assessed using a Bioanalyzer (Agilent Technology). Only samples showing intact RNA (RNA Integrity number > 9) were used for RNA-seq analysis. RNA Sequencing was conducted by Macrogen (Korea) using Illumina SBS technology, 2 × 100 bp (454) on HiSeq 2000, with a total lecture of 5.9 Gb sequences for the 8 samples. Only reads with a Q > 30 were conserved. This resulted in over 50 million reads per sample.

Raw data were mapped to the human genome [30] using Bowtie2 2.2.6 [31]. Expression levels were evaluated using RSEM v1.2.25 [32] and differential expression between control samples and treated samples was performed using the edgeRun package [33]. Gene and transcript annotation used in the analysis correspond to Gencode v23, and molecular functions and assignations to the Gene Ontology Consortium [34] were performed with the Blast2 GO program [35].

Statistical analysis

All experiments were performed at least three times in triplicate, and the value corresponds to the mean value of triplicates. The arithmetic value of triplicates was attributed to the analyzed value, and an expanded uncertainty with a coverage Student's factor for a 95% confidence interval was calculated.

Supporting information

A list of the 223 differentially expressed genes with GO annotation, a diagram of the genes with a significant differential expression according to the cellular component (GO), and the HPLC-PDA chromatogram profile of the bioactive components (neoflavonoids) of the TOE ethanolic extract are available as Supporting Information.

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Conflict of Interest

The authors declare no conflicts of interest.

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