DERMACLAIM
Test & Trust

Project Reference

DC.132.01.001

Sponsor and Test Products

WANKA TANKA LTD

BRACELET TANKA T-FLEX EXTRACT

Title of Study

IN VITRO ASSESSMENT OF THE ANTIOXIDANT CAPABILITIES IN HUMAN KERATINOCYTES

TABLE OF CONTENTS

1. EXECUTIVE SUMMARY

OBJECTIVE: To evaluate the antioxidant capabilities of "Bracelet Tanka T-Flex Extract" (hereafter, T-Flex extract) after *in vitro* treatment in human HaCaT keratinocytes during 6 hours.

PROCEDURE: Human HaCaT keratinocytes were cultured in the presence of the tested sample during 6 hours at different concentrations (1 % and 10 %) for 6 hours. After the incubation period, total RNA was purified, quantified, and complementary DNA (cDNA) was synthesized. Relative gene expression of *SOD1 (Superoxide dismutase 1), CAT (Catalase), and NFE2L2 (Nuclear factor erythroid 2-related factor 2)* was determined through RT-qPCR using $2^{-\Delta\Delta Ct}$ Method, with *ACTB* used as housekeeping gene.

RESULTS: Results showed the treatment with T-Flex extract at 10 % during 6 hours significantly activated the expression of *SOD1*, *CAT*, and *NFE2L2* by 23.6 %, 41.0 %, and 106.3 %, compared to the untreated control. When the sample was applied at 1 %, a significant activation of *CAT* (+35.6 %) was also obtained, whereas no significant neither relevant results were obtained for *SOD1* nor *NFE2L2*.

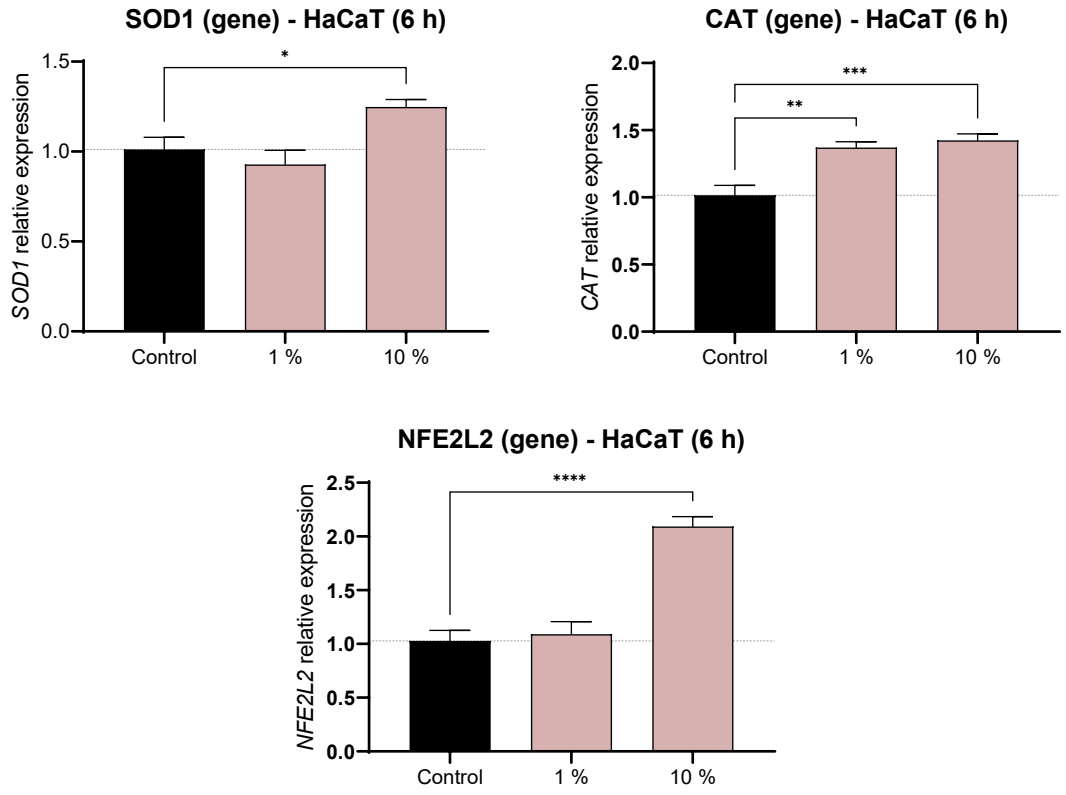

Figure 1. Effects in the gene expression of SOD1, CAT, and NFE2L2*. Bar graph represents relative gene expression of SOD1 (Superoxide dismutase 1), CAT (Catalase), and NFE2L2 (Nuclear factor erythroid 2-related factor 2), after 6 hours of treatment in human HaCaT keratinocytes with Bracelet*

Tanka T-Flex Extract (1 % and 10 %). Asterisks (*) over bars indicate statistical significance vs Control (ordinary one-way ANOVA test followed by Dunnett's multiple comparisons test).

CONCLUSION: The *in vitro* treatment with "**Bracelet Tanka T-Flex Extract**" in human **HaCaT keratinocytes** displays **antioxidant effects, substantiated in a significant upregulation of *SOD1 (Superoxide dismutase 1), CAT (Catalase), and NFE2L2 (Nuclear factor erythroid 2-related factor 2)***, compared to the untreated control.

2. IDENTIFICATION OF STUDY

PROJECT REFERENCE: DC.132.01.001

SPONSOR: Wanka Tanka Ltd

TEST PRODUCTS: Bracelet Tanka T-Flex Extract

TITLE: *In vitro* assessment of the antioxidant capabilities in human keratinocytes.

STUDY DIRECTOR: Alejandro Pérez Fernández, PhD

3. PROVIDER AND TESTING FACILITIES

COMPANY: Dermaclaim Lab S.L.

VAT NUMBER: B16909699

ADDRESS: Parc Cientific Universitat de Valencia (PCUV). Calle Agustín Escardino Benlloch, 9. 46980, Paterna (Valencia), España

EMAIL: dermaclaim@dermaclaim.com **PHONE**: +34 644 41 61 12

TESTING FACILITIES: SCSIE (UV) - Edificio Jerónimo Muñoz, 5ª planta. Carrer del Dr. Moliner, 50. 46100 Burjassot, Valencia (España)

EMAIL: laboratorio@dermaclaim.com **PHONE**: +34 697 44 56 40

4. SPONSOR

COMPANY: Wanka Tanka LTD

ID TAX: 516061769

ADDRESS: Rehov Hasadna 8. 1173836 Beit Shean, Israel

PROJECT RESPONSIBLE: Nimrod Vardi, CEO

EMAIL: amnonvardi@gmail.com

5. TEST PRODUCTS

PRODUCT NAME: BRACELET TANKA T-FLEX

REFERENCE & BATCH: Not provided

INCI: Not provided

DERMACLAIM REFERENCE: DC.0212

GALENIC: Not applicable

NUMBER AND TYPE OF SAMPLES: 1

CONTENT: 1 Bracelet

STORAGE CONDITIONS: Room temperature (21 ± 2 ºC)

Upon arrival at Dermaclaim Lab S.L. facilities, the test material was assigned a unique laboratory code number and registered into a daily log identifying sponsor, product name, batch number, number of units and quantity received, date of reception, status of the reception, storage conditions and reference of the project in which the samples are being analyzed.

Samples are kept within 24 months after submission of final report, unless otherwise specified by the sponsor.

6. DATES OF STUDY

STUDY START DATE: 17/08/2022

DATE OF SAMPLES' RECEPTION: 07/10/2022

PROCEDURE'S START DATE: 14/11/2022

PROCEDURE'S END DATE: 22/11/2022

FINAL REPORT DATE: 01/12/2022

REPORT REVIEWED DATE: 01/12/2022

REPORT DELIVERY DATE: 01/12/2022

7. IN VITRO PLATFORM

C ELL LINE: HaCaT immortalized human keratinocytes

ORIGIN: Caucasian, adult human skin.

SUPPLIER: AddexBio.

LOT NUMBER: 0003798.

CELL PASSAGE: 26.

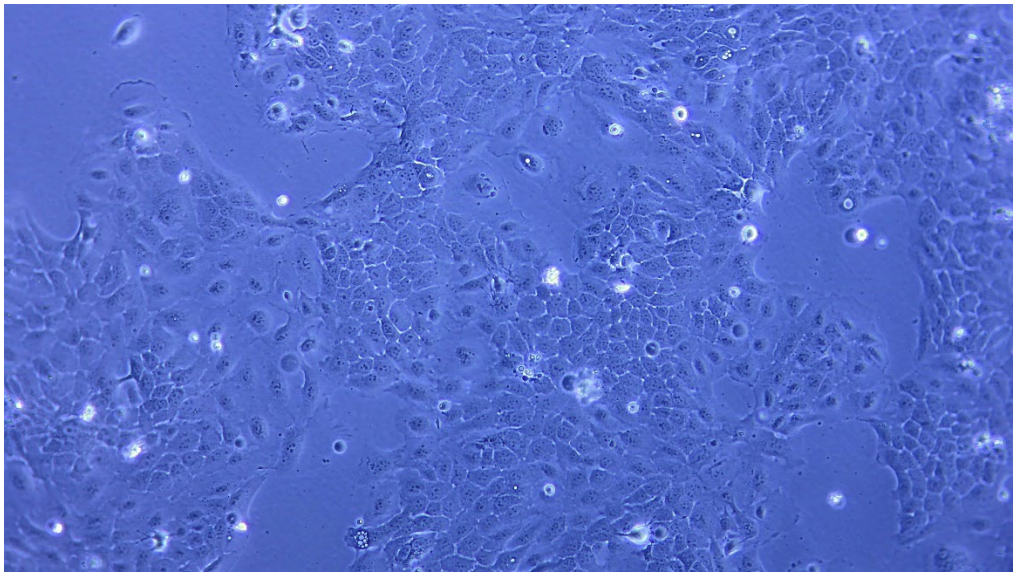

***Figure 2. Representative image of HaCaT cells in culture**. Bright field optical microscopy picture of cultured HaCaT cells at 80 % confluence. This is the typical stage of a HaCaT culture before trypsinization and subculture or cell seeding for subsequent experiments.*

8. ETHICAL RELEVANCE

Cultured primary human keratinocytes are frequently employed for studies of immunological and inflammatory responses; however, interpretation of experimental data may be complicated by donor-to-donor variability, the relatively short culture lifetime, and variations between passages. To standardize the *in vitro* studies on keratinocytes, we used *HaCaT* cells, a long-lived, spontaneously transformed aneuploid immortal keratinocyte cell line from adult human skin, widely used in scientific research [*Boukamp et al.., 1988*]. *HaCaT* cells are utilized for their high capacity to differentiate and proliferate *in vitro* [*Schoop et al., 1999*]. Their use in research allows for the characterization of human keratinocytes, using a model that is reproducible and solves issues such as short culture lifespan and donot-to-donor variations associated to primary cultures. Additionally, *HaCaT* cells have shown to be a suitable model to follow the release of inflammatory and repair mediators [*Colombo et al., 2017*].

This study has been conducted according to the general conditions of Dermaclaim Lab S.L., established for research studies involving human cell lines.

Before the beginning of the study, Dermaclaim internally assessed and approved its suitability for the product type and the methodology to be used. The study aimed at a better knowledge of the efficacy of the test products.

The *in vitro* experimental conditions adopted (human cell line, quantity of product applied, duration of treatment, application of external agent, etc.) try to mimic the environmental conditions of our skin. Nevertheless, results obtained from *in vitro* experiments cannot often be directly extrapolated to the same *in vivo* biological responses.

9. INTRODUCTION

Skin, the largest organ of the body, functions as the necessary interface between the internal and the external environment. Thus, it continuously protects the body from noxious stimuli, e.g., microorganisms, ultraviolet (UV) irradiation, allergens, and irritants. Its unique role and function is a direct result of its structure and makeup, particularly of the most superficial part, the epidermis [*Lin et al., 2017*].

The main cellular component of the epidermis includes keratinocytes. In the basal layer of the epidermis, keratinocytes preserve their ability to proliferate upward to form the spinous layer and the granular layer. Beyond the granular layer, the keratinocytes terminally differentiate into corneocytes in the horny layer. In the outmost part of epidermis, corneocytes, together with the intercellular lamellar compartment (lipids), contribute to the structure and function of the stratum corneum (SC).

The skin serves as a protective barrier against the environment and is constantly exposed to insults which can lead to the generation of reactive oxygen species (ROS). While low levels of ROS act as intracellular signaling messengers [*Finkel et al., 2011*], high ROS levels lead to oxidative stress, which is deleterious, as it damages cellular macromolecules [*Casares et al., 2020*]. Oxidative stress-induced cell damage can lead to chronic inflammation and is involved in the pathogenesis of skin diseases, skin disorder and skin aging.

All life processes are governed by redox signaling; thus, the maintenance of a physiological level of oxidants is mandatory for proper cellular functioning. This can be obtained by switching on/off some regulation pathways or programmed cell death. Oxidants are responsible for a well-known process, senescence, as they are involved in telomere shortening. Different authors demonstrated that cells grown in the presence of strong oxidative environments have a shorter life span compared with cells grown in low oxygen tension [*Chen et al., 1994*]. With aging, a decreased performance of cell endogenous antioxidant system occurs; thus, elderly people are more susceptible to oxidative stress [*Spector, 2000*].

To counteract the harmful accumulation of ROS, healthy skin presents a battery of defense mechanisms including antioxidant and detoxification systems. Many of these systems are under the control of nuclear factor erythroid 2-like 2 (NRF2), which regulates the expression of numerous detoxifying enzymes and antioxidant proteins and is highly conserved throughout evolution [*Sykiotis and Bohmann, 2010*]. Nrf2 knockout (ko) mice show a reduction in the basal and inducible expression of cytoprotective genes, and are

more susceptible to the toxicity of ROS-inducing agents and electrophiles. Viceversa, Nrf2 activation efficiently protected cells from ROS damage *in vitro* and *in vivo*. Most importantly, a variety of Nrf2-activating substances showed a potent chemopreventive and anti-inflammatory function in animal models of cancer [*Kundu and Surh, 2010*]. Promising results with Nrf2 activators have also been obtained in animal models of neurodegenerative diseases [*de Vries et al, 2008*].

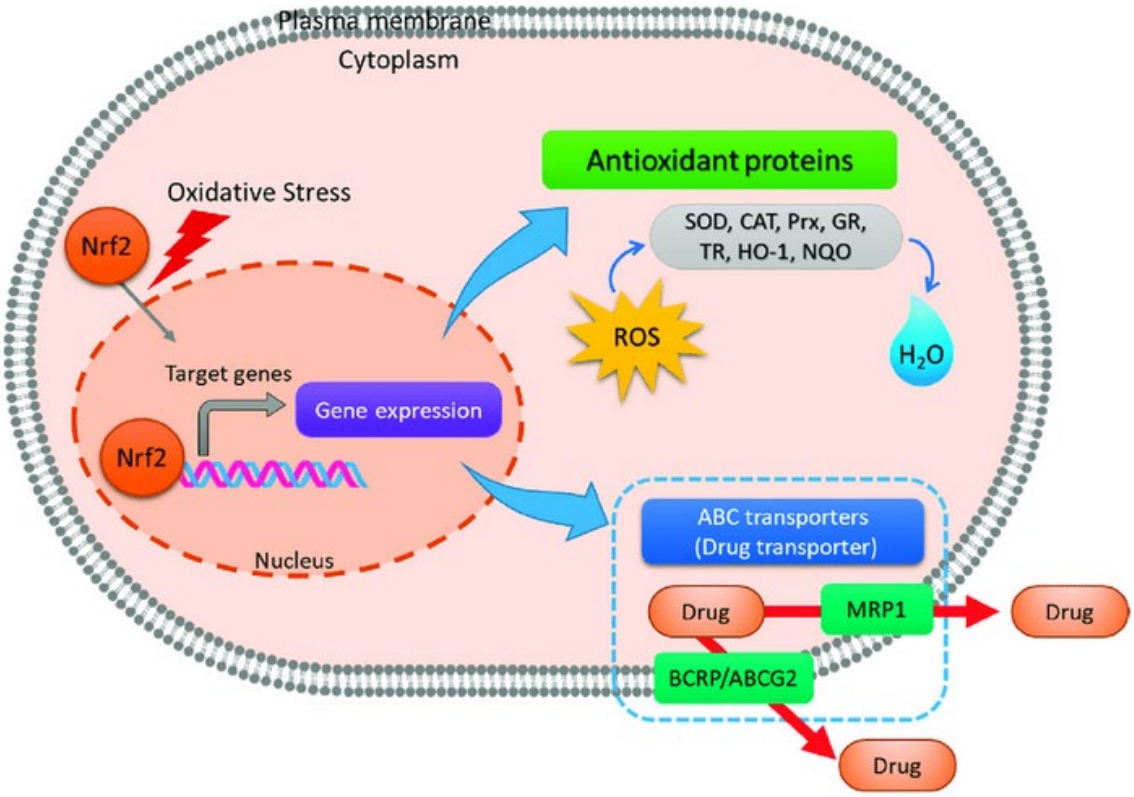

***Figure 3**. Nuclear factor erythroid 2-related factor 2 (Nrf2) regulates redox-homeostasis and chemoresistance in cells. Nrf2 induces antioxidant proteins such as superoxide dismutase (SOD), catalase (CAT), peroxiredoxin (Prx), glutathione reductase (GR), thioredoxin reductase (TR), heme oxygenase-1 (HO-1), and NAD(P)H quinone oxidoreductase 1 (NQO) [Kim et al., 2019].*

On the other hand, superoxide (O2−) is an anion free radical primarily produced in mitochondria due to leakage during electron transport. As much as one to two percent of oxygen consumed by mammalian cells is estimated to be converted to superoxide [*Stowe and Camara, 2009*]. Supraphysiological concentration of superoxide and hydrogen peroxide, and their more reactive products such as hydroperoxyl radical (HOO•), hydroxyl radical (HO•), and reactive aldehydes, can oxidize DNA, RNA, lipids, and proteins, producing not only aberrant regulatory responses to alter cellular processes,

but also reversible and irreversible damage to cells and tissues [*Alfadda and Sallam, 2012*].

Because of its severely deleterious effects, superoxide is rapidly dismutated through enzyme-catalyzed reactions into hydrogen peroxide, that in turn is converted to water and molecular oxygen. Superoxide dismutases (SODs) are enzymes responsible for scavenging superoxide into hydrogen peroxide and molecular oxygen via the following chemical reaction: 2O2•− + 2H+ → H2O2 + O2. All cellular organisms express SODs with heavy metals in their catalytic centers. Mammalian cells possess three SOD isoforms: SOD1, a Cu2+/Zn2+ enzyme; SOD2, a Mn2+ enzyme; and SOD3, a distinct Cu2+/Zn2+ enzyme [*Che et al., 2016*].

Last, catalase is another of the most important antioxidant enzymes. It is present in almost all aerobic organisms. Catalase breaks down two hydrogen peroxide molecules into one molecule of oxygen and two molecules of water in a two-step reaction [*Von Ossowski et al., 1993*]. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS), since it has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of hydrogen peroxide molecules to water and oxygen each second [*Goodsell, 2004*].

10. METHODOLOGY

10.1 Hypothesis and experimental design

Hypothesis: The test product stimulates the expression of *SOD1 (Superoxide dismutase 1), CAT (Catalase)*, and *NFE2L2 (Nuclear factor erythroid 2-related factor 2)*, in *HaCaT* keratinocytes.

Experimental design: Application of the tested product for 6 hours, in order to stimulate the gene expression of *SOD1, CAT, and NFE2L2*.

10.2 Materials

REAGENTS: Dulbecco's Eagle's Modified Medium (DMEM) 4.5 g/L glucose + GlutaMAX™ (Gibco), Heat-inactivated fetal bovine serum (FBS, Gibco), Penicillin/Streptomycin 10000 U/mL (Gibco), Phosphate buffered saline (PBS, Corning), Trypan Blue 0.4 % (Sigma-Aldrich), Trypsin-EDTA 0.5 % (Gibco), MTT (Fisher Scientific), DMSO (Sigma-Aldrich), RNeasy Mini Kit (Qiagen), RNase free DNase kit (Qiagen), PrimeScript™ RT reagent Kit (Perfect Real Time, Takara Bio), Premix Ex Taq ™(Probe qPCR, Takara Bio), pre-designed PrimeTime qPCR Probe Assays with FAM-conjugated, ZEN/IBFQ-quenched probes specific for ACTB (Hs.PT.39a.22214847), NFE2L2 (Hs.PT.58.28159373), SOD1 (Hs.PT.58.20593019), GPX1 (Hs.PT.58.39247474.g) and CAT (Hs.PT.56a.25069031) (Integrated DNA Technologies).

EQUIPMENT: Steri-Cycle CO2 incubator (Thermo Scientific), Bio II Advance Laminar Flow Hood (Telstar) Primovert Inverted Microscope (Zeiss), Shaking incubator, SI500 (Stuart Equipment), , Sigma 1-16 microcentrifuge (Sigma Laborzentrifugen), Nanodrop 2000 spectrophotometer (Invitrogen), Ultrafreezer -86ºC Serie 89000 (Thermo Scientific), Fisherbrand™ Isotemp™ Heating Block (Fisher Scientific), StepOnePlus™ Real-Time PCR System (Applied Biosystems).

10.3 Procedure

10.3.1 Extraction of actives from bracelets

The sample presentation was a solid bracelet device format by the sponsor (Tanka LTD). As cells in culture can only be exposed to active ingredients dissolved in medium, an extraction procedure was applied to obtain the actives contained in the bracelets. Prior

to extraction, the bracelets were sterilized with 3 consecutive washes in PBS containing penicillin/streptomycin 100 U/mL. The bracelets were cut into small fragments of approximately 0.5 cm size and placed into 50 ml centrifuge tubes (see image below). Then, bracelet pieces were covered with 12 ml of serum-free DMEM + 1 g/L glucose.

Bracelet pieces in extraction medium were incubated at 60 ºC for 72 h with shaking. After this period, the T-Flex supernatants were recovered and filtered through a 0.22 µm filter, aliquoted and stored at -80ºC prior to use.

10.3.2 Gene expression assessment

Confluent HaCaT cells cultured on a 75 cm2 Nunc™ EasYFlask™ (Thermo Fisher Scientific) were detached by incubation with Trypsin-EDTA 0.5 % without phenol red (Gibco) during 2 min at 37ºC, 5 % CO2. Trypsin was then inactivated by adding 4 volumes of DMEM 4.5 g/L glucose (Gibco) supplemented with 10 % FBS (Gibco) (hereafter D10 medium) and mixing thoroughly. The obtained cell suspension was mixed 1:1 with trypan blue 0.4 % and incubated during 30 s at room temperature to allow the penetration of the dye inside the non-viable cells. Cell counting of the trypan blue-diluted cell suspension was performed in a Countess II Automated Cell Counter (Thermo Fisher Scientific). Accordingly, cells were conveniently diluted in D10 medium to a final density of 2.4·105 cells/ml. Cells were then seeded in a 24-well Clear Flat Bottom TC-treated Culture Microplate (Falcon) by adding 0.5 mL/well of the aforementioned cell suspension (1.2·105 cells/well). Cells were allowed to attach to the cell culture plastic by incubation overnight at 37ºC, 5 % CO2.

After the attachment period, D10 medium was removed and replaced by fresh D10 with or without the test products. Different experimental conditions were set:

- Control: cells belonging to this group were kept in D10 with no additional components.
- Treatment with the tested sample: this experimental condition involved exposure to T-Flex extract at 1 % and 10 %.

For all the experimental conditions, 0.5 mL of culture medium supplemented as indicated above were added per well. Cells were incubated in the presence of treatments for 24 h prior to RNA isolation.

After the incubation period, cell culture medium was completely removed from wells and cells were rinsed with 0.5 mL PBS/well. Then, PBS was also discarded and 350 µL RLT

buffer from RNeasy Mini Kit were added per well. Tissue culture plates were subjected to a freeze-thaw cycle at -80ºC to allow the formation of crystals that helped disrupt cells and RNA was isolated from cell extracts with RNeasy Mini Kit as per manufacturer's instructions. RNA was eluted from columns in a final volume of 30 µL of nuclease-free from the RNeasy Mini Kit.

Total RNA concentration from samples was quantified through absorbance measurements at 260 nm in a Nanodrop 2000 spectrophotometer. RNA purity was also assessed with 260/280 nm measurements.

Next, RNA was reverse transcribed into complementary DNA (cDNA) with PrimeScript™ RT reagent Kit as per manufacturer's instructions with a template input of 350 ng (6 H) in a 10 µL reaction volume (final concentration: 35 ng/µL).

Finally, relative gene expression was assessed by quantitative polymerase chain reaction (qPCR) using the Premix Ex Taq ™ enzyme and mastermix according to supplier's guidelines. Briefly, 20 ng of input cDNA were loaded in a final reaction volume of 10 µL, with primers/probe concentrations of 500 nM/125 nM.

10.4 Data processing

For each technical replicate, the relative gene expression for the different experimental conditions versus the Control was calculated by the $2^{-\Delta\Delta Ct}$ Method *[Livak and Schmittgen, 2001]*, with *ACTB* used as housekeeping gene, and expressed as a percentage (%) versus Control.

Data are represented normalized to the untreated Control, in order to detect the capacity of the tested product to induce the expression of the corresponding genes. Data are represented in bar graphs as Mean ± Standard Error of the Mean (SEM).

10.5 Statistical analysis

GraphPad Prism V9 software was used for the statistical analysis. Data were analyzed using ordinary one-way ANOVA test followed by Dunnett's post hoc multiple comparisons test (data normalized to Control). Statistical significance was set at $p < 0.05$.

One-way ANOVA, also called one-factor ANOVA, determines how a response is affected by one factor. In this experiment, we measure the response to one test product at 2

different concentrations. The conditions are the factor, and it is said to have 3 different levels, one for each experimental condition. One-way ANOVA is used when there are three or more levels, as some mathematic adjustments need to be applied to reduce the possibility of finding false positive results.

If there are only two levels of one factor (only two test conditions), then we should use an unpaired Student's t-test (Welch's correction, equal SDs are not assumed), although the underlying math is the same for a t-test and one-way ANOVA with two groups.

ROUT (Robust regression and Outlier removal) method was used to identify outliers in the raw data, with a coefficient Q of 1 %. The value of Q determined how aggressively the method defines the outlier and unless specific elsewhere, it is recommended to stick to 1 % in this type of research experiments [*Motulsky and Brown, 2006*].

Asterisks (*) over column bars in the graphs of results indicate statistical significance between the corresponding comparisons. Statistical significance is indicated with asterisks as * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, and *** for $p < 0.0001$.

10.6 Protocol deviations

Not applicable.

11. RESULTS

Results showed the treatment with T-Flex extract at 10 % during 6 hours significantly activated the expression of *SOD1*, *CAT*, and *NFE2L2* by 23.6 %, 41.0 %, and 106.3 %, compared to the untreated control. When the sample was applied at 1 %, a significant activation of *CAT* (+35.6 %) was also obtained, whereas no significant neither relevant results were obtained for *SOD1* nor *NFE2L2* (Figure 4, Table S1).

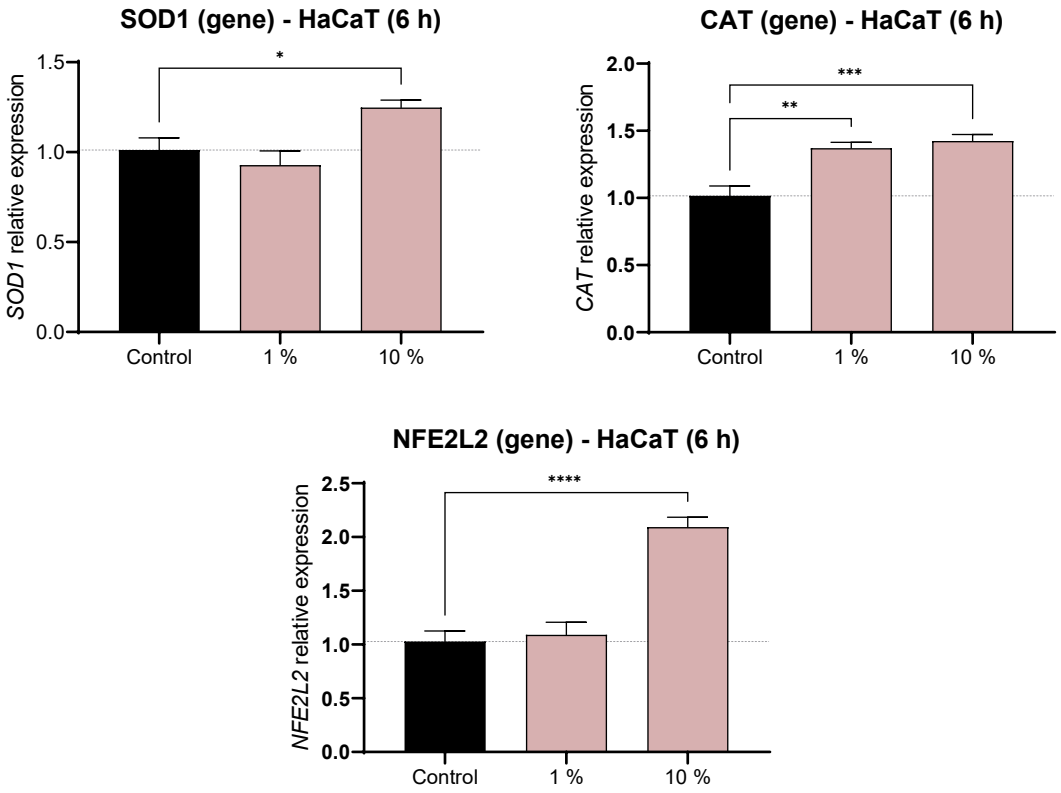

Figure 4. Effects in the gene expression of SOD1, CAT, and NFE2L2*. Bar graph represents relative gene expression of SOD1 (Superoxide dismutase 1), CAT (Catalase), and NFE2L2 (Nuclear factor erythroid 2-related factor 2), after 6 hours of treatment in human HaCaT keratinocytes with Bracelet Tanka T-Flex Extract (1 % and 10 %). Asterisks (*) over bars indicate statistical significance vs Control (ordinary one-way ANOVA test followed by Dunnett's multiple comparisons test).*

12. CONCLUSION AND SIGNATURES

The purpose of this project was to evaluate the antioxidant capabilities of "Bracelet Tanka T-Flex Extract" after *in vitro* treatment in human HaCaT keratinocytes during 6 hours.

To achieve this goal, human HaCaT keratinocytes were cultured in the presence of the tested sample during 6 hours at different concentrations (1 % and 10 %) for 6 hours. After the incubation period, total RNA was purified, quantified, and complementary DNA (cDNA) was synthesized. Relative gene expression of *SOD1 (Superoxide dismutase 1), CAT (Catalase), and NFE2L2 (Nuclear factor erythroid 2-related factor 2)* was determined through RT-qPCR using $2^{-\Delta\Delta Ct}$ Method, with *ACTB* used as housekeeping gene.

Results showed the treatment with T-Flex extract at 10 % during 6 hours significantly activated the expression of *SOD1*, *CAT*, and *NFE2L2* by 23.6 %, 41.0 %, and 106.3 %, compared to the untreated control. When the sample was applied at 1 %, a significant activation of *CAT* (+35.6 %) was also obtained, whereas no significant neither relevant results were obtained for *SOD1* nor *NFE2L2*.

In conclusion, the *in vitro* treatment with "**Bracelet Tanka T-Flex Extract**" in human **HaCaT keratinocytes** displays **antioxidant effects, substantiated in a significant upregulation of *SOD1 (Superoxide dismutase 1), CAT (Catalase), and NFE2L2 (Nuclear factor erythroid 2-related factor 2)***, compared to the untreated control.

Study Director

Alejandro Pérez Fernández, PhD **Date:** 01/12/2022

… declares the study was carried out under my responsibility, the results reported in this final report accurately and completely reflect the raw data of the study, and the content of the study report is reliable and takes into account the "Good Laboratory Practices" (February 2004) from Directive 2004/10/EC of the European Parliament and of the Council.

2.5.4.13=Reg:46030 /Hoja:V-200549 /Tomo:11055 /Folio:185 /Fecha:30/09/2021 /Inscripción:1, serialNumber=IDCES-29206074F, givenName=DAVID, sn=GONZALEZ FERNANDEZ, cn=29206074F DAVID GONZALEZ (R: B16909699), 2.5.4.97=VATES-B16909699, o=DERMACLAIM LAB SOCIEDAD LIMITADA, c=ES
2022.12.01 16:32:31 +01'00'

13. ARCHIVING AND DISCLOSURE

All original raw data, including data sheets, laboratory protocols, technical procedures, laboratory notebooks, correspondence files, copies of final reports, and remaining samples, are maintained on the premises of Dermaclaim Lab S.L., in limited access marked storage files. Altogether, including the information provided by the sponsor, information about materials, reagents, or methodology, and all the information generated by Dermaclaim Lab S.L. (statistical analysis, graphical representations, etc.) is considered Confidential, and will not be shared with third parties.

To prevent loss of and protect intellectual property, the final report has been electronically signed using the official signature of Dermaclaim Lab S.L. (VAT: B16909699). Any attempt to remove the signature will irreversibly damage the label and leave an immediate trace, thus invalidating the document.

Only reports containing the Dermaclaim Lab S.L. electronic signature intact, will be recognized by Dermaclaim Lab S.L. as a certified original.

Dermaclaim Lab S.L. represents fully independent testing facilities committed to the highest standards of unbiased testing and reporting. Dermaclaim Lab S.L. is not in partnership, affiliation and/or association, in any way, with any other corporation, company, sole proprietorship, partnership, client, laboratory, and/or any other business entity. Dermaclaim Lab S.L. is not legally responsible or bound to any claim(s) provided by a third party claiming any kind of association with Dermaclaim Lab S.L.

The industrial and intellectual property rights that may arise from the contracted services, as well as the ownership of the results, belongs entirely to the Sponsor, unless expressly stated otherwise in the corresponding budget.

14. CERTIFICATIONS AND REGULATIONS

The study protocol considers the "Good Laboratory Practices" (February 2004) from Directive 2004/10/EC of the European Parliament and of the Council.

The whole process involving this assay was performed following UNE-EN-ISO 9001/2015 Quality Management System guidelines, certified on August 5th, 2022 (reference code, EC-10984/22).

14.1 SUBSTANTIATION OF CLAIMS (EU Regulation 655/2013)

Cosmetic claims must comply with EU Regulation 655/2013 that provides the Common Criteria to ensure that the information conveyed to the end-users through claims is useful, understandable, and reliable so that consumers can make informed decisions.

Claims for cosmetic products, whether explicit or implicit, shall be supported by adequate and verifiable evidence regardless of the types of evidential support used to substantiate them, including where appropriate expert assessments. Evidence for claim substantiation shall consider the state-of-the-art practices.

Products may bear claims that relate to the nature of experimental studies. Consumer expectations regarding these claims may vary depending upon the presentation of the claim and its specific context. However, in all circumstances, consumers will expect that such claims are made only when the effects tested are favorable.

Studies must be relevant and comprised of methods which are reliable and reproducible. The studies should follow a well-designed and scientifically valid methodology according to good practices. The criteria used for evaluation of product performances should be defined with accuracy and chosen in compliance with the aim of the test.

The experimental aspect of studies calls for reliance on knowledge and awareness of statistical principles in the design and analysis of the study. This is necessary to ensure that the studies achieve scientifically and statistically valid conclusions.

It is important that the person conducting the study has the appropriate qualifications, has training and experience in the field of the proposed study, and has professional integrity and ethical standards.

15. REFERENCES

Alfadda, A.A.; Sallam, R.M. Reactive Oxygen Species in Health and Disease. J. Biomed. Biotechnol. 2012, 2012, 936486.

Boukamp, Petra; Petrussevska, Rule T.; Breitkreutz, Dirk; Hornung, Jiirgen; Markham, Alex; Fusenig, Norbert E. (1 March 1988). "Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line". The Journal of Cell Biology. 106 (3): 761–771. doi:10.1083/jcb.106.3.761. PMC 2115116. PMID 2450098.

Casares L, García V, Garrido-Rodríguez M, Millán E, Collado JA, García-Martín A, Peñarando J, Calzado MA, de la Vega L, Muñoz E. Cannabidiol induces antioxidant pathways in keratinocytes by targeting BACH1. Redox Biol. 2020 Jan;28:101321. doi: 10.1016/j.redox.2019.101321. Epub 2019 Sep 5. PMID: 31518892; PMCID: PMC6742916.

Che, M.; Wang, R.; Li, X.; Wang, H.-Y.; Zheng, X.S. Expanding roles of superoxide dismutases in cell regulation and cancer. Drug Discov. Today 2016, 21, 143–149.

Chen Q., Ames B. N. Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells. Proceedings of the National Academy of Sciences of the United States of America. 1994;91(10):4130–4134. doi: 10.1073/pnas.91.10.4130.

Colombo I, Sangiovanni E, Maggio R, et al. HaCaT Cells as a Reliable In Vitro Differentiation Model to Dissect the Inflammatory/Repair Response of Human Keratinocytes. Mediators Inflamm. 2017;2017:7435621. doi:10.1155/2017/7435621

de Vries HE, Witte M, Hondius D, Rozemuller AJ, Drukarch B, Hoozemans J, van Horssen J. Nrf2-induced antioxidant protection: A promising target to counteract ROS-mediated damage in neurodegenerative disease. Free Radic Biol Med. 2008;45:1375–1383.

Finkel T. Signal transduction by reactive oxygen species. J. Cell Biol. 2011;194(1):7–15.

Goodsell DS (2004-09-01). "Catalase". Molecule of the Month. RCSB Protein Data Bank. Retrieved 2016-08-23.

I. Von Ossowski, G. Hausner, and P. C. Loewen, “Molecular evolutionary analysis based on the amino acid sequence of catalase,” Journal of Molecular Evolution, vol. 37, no. 1, pp. 71–76, 1993.

Kim, Eun-Kyung & Jang, MinGyeong & Song, Min-Jeong & Kim, Dongwoo & Kim, Yosup & Jang, Ho Hee. (2019). Redox-Mediated Mechanism of Chemoresistance in Cancer Cells. Antioxidants. 8. 471. 10.3390/antiox8100471.

Kundu JK, Surh YJ. Nrf2-Keap1 signaling as a potential target for chemoprevention of inflammation-associated carcinogenesis. Pharm Res. 2010;27:999–1013.

Lin TK, Zhong L, Santiago JL. Anti-Inflammatory and Skin Barrier Repair Effects of Topical Application of Some Plant Oils. Int J Mol Sci. 2017 Dec 27;19(1):70. doi: 10.3390/ijms19010070.

Motulsky HM and Brown RE, Detecting outliers when fitting data with nonlinear regression – a new method based on robust nonlinear regression and the false discovery rate, BMC Bioinformatics 2006, 7:123.

Schoop, Veronika M.; Fusenig, Norbert E.; Mirancea, Nicolae (March 1999). Epidermal Organization and Differentiation of HaCaT Keratinocytes in Organotypic Coculture with Human Dermal Fibroblasts. Journal of Investigative Dermatology. 112 (3): 343–353. doi:10.1046/j.1523-1747.1999.00524.x. PMID 10084313.

Spector A. Review: oxidative stress and disease. Journal of Ocular Pharmacology and Therapeutics. 2000;16(2):193–201. doi: 10.1089/jop.2000.16.193.

Stowe, D.F.; Camara, A.K.S. Mitochondrial Reactive Oxygen Species Production in Excitable Cells: Modulators of Mitochondrial and Cell Function. Antioxid. Redox Signal. 2009, 11, 1373–1414.

Sykiotis GP, Bohmann D. Stress-activated cap'n'collar transcription factors in aging and human disease. Sci Signal. 2010;3:re3.

16. ATTACHMENTS

16.1 Attachment 1. Raw data qPCR

Condition	Replicate	Ct				ΔCt			ΔΔCt			RQ		
		ACTB	SOD1	NFE2L2	CAT	SOD1	NFE2L2	CAT	SOD1	NFE2L2	CAT	SOD1	NFE2L2	CAT
Control	1	15,51752	19,28648	22,97415	23,4819	3,592688	7,280353	7,788104	-0,06057794	-0,1617386	-0,11644559	1,042883	1,118634	1,084061
	2	15,26874	19,0582	22,82679	23,3403	3,364409	7,132991	7,646505	-0,28885657	-0,3091004	-0,2580452	1,221672	1,238935	1,195857
	3	16,26452	19,48487	23,18579	23,66028	3,79108	7,491993	7,966488	0,13781446	0,04990175	0,06193775	0,908895	0,966002	0,957977
	4	15,93156	19,72804	22,86101	23,39419	4,03425	7,167215	7,700395	0,38098468	-0,27487666	-0,20415479	0,767913	1,209891	1,152011
	5	15,58709	19,38649	23,05732	24,13093	3,692698	7,363527	8,437137	0,03943287	-0,07856409	0,53258706	0,973037	1,055967	0,691314
	6	15,59334	19,13826	23,91026	23,58246	3,444468	8,216469	7,888671	-0,20879751	0,774378	-0,01587923	1,155724	0,584641	1,011067
T-Flex 1 %	1	15,76146	19,96616	22,78254	23,15921	4,218915	7,035289	7,411967	0,56564977	-0,40680269	-0,49258339	0,675651	1,325744	1,406962
	2	15,45945	19,24291	22,82747	23,03943	3,49566	7,080223	7,292182	-0,1576051	-0,36186808	-0,61236791	1,115434	1,285089	1,528766
	3	15,66543	19,91345	22,83884	23,13254	4,166203	7,091589	7,385289	0,5129373	-0,35050256	-0,51926086	0,700794	1,275005	1,433221
	4	15,98478	19,48965	23,27046	23,24627	3,742405	7,523213	7,499021	0,0891397	0,08112198	-0,40552926	0,940083	0,945322	1,324575
	5	15,73741	19,31733	23,94698	23,27297	3,570081	8,199733	7,525721	-0,0831844	0,75764126	-0,37882841	1,059354	0,591463	1,300285
	6	15,87496	19,29476	23,0214	23,34708	3,547508	7,274153	7,599833	-0,10575733	-0,16793798	-0,30471652	1,076059	1,123452	1,235176
T-Flex 10 %	1	15,87447	19,13903	22,31962	23,35086	3,188437	6,369028	7,400264	-0,46482884	-1,07306367	-0,50428615	1,380154	2,103896	1,418421
	2	15,57626	19,23075	22,07512	23,18615	3,280153	6,12453	7,235556	-0,37311221	-1,317561	-0,66899419	1,295144	2,492444	1,589964
	3	16,02403	19,28675	22,34442	23,2509	3,336157	6,39383	7,300311	-0,31710863	-1,04826149	-0,60423932	1,245831	2,068036	1,520177
	4	15,81012	19,23486	22,44526	23,42298	3,284269	6,494668	7,472383	-0,36899656	-0,94742393	-0,43216651	1,291454	1,928426	1,349258
	5	16,11176	19,48467	22,50941	23,52152	3,534072	6,558818	7,57093	-0,1191932	-0,88327306	-0,33361948	1,086127	1,844555	1,260171
	6	15,93258	19,35684	22,31136	23,35752	3,406245	6,36077	7,40693	-0,24702013	-1,08132132	-0,49761974	1,186753	2,115973	1,411882

16.2 Attachment 2. Statistical analysis

Gene Expression (qPCR) – SOD1 – Ordinary one-way ANOVA test								
Dunnett's multiple comparisons test	**Mean Diff,**	**95,00% CI of diff,**	**Below threshold?**	**Summary**	**Adjusted P Value**	**?-B**		
1 % vs. Control	-0,08379	-0,2903 to 0,1227	No	ns	0,6749	D	1 %	
10 % vs. Control	0,2359	0,02939 to 0,4424	Yes	*	0,0218	E	10 %	
Test details	**Mean 1**	**Mean 2**	**Mean Diff,**	**SE of diff,**	**n1**	**n2**	**q**	**DF**
1 % vs. Control	0,9279	1,012	-0,08379	0,07921	6	6	1,058	25
10 % vs. Control	1,248	1,012	0,2359	0,07921	6	6	2,978	25
Gene Expression (qPCR) – CAT – Ordinary one-way ANOVA test								
Dunnett's multiple comparisons test	**Mean Diff,**	**95,00% CI of diff,**	**Below threshold?**	**Summary**	**Adjusted P Value**	**?-B**		
1 % vs. Control	0,3561	0,1231 to 0,5892	Yes	**	0,0019	D	1 %	
10 % vs. Control	0,4096	0,1765 to 0,6427	Yes	***	0,0004	E	10 %	
Test details	**Mean 1**	**Mean 2**	**Mean Diff,**	**SE of diff,**	**n1**	**n2**	**q**	**DF**
1 % vs. Control	1,371	1,015	0,3561	0,08940	6	6	3,984	25
10 % vs. Control	1,425	1,015	0,4096	0,08940	6	6	4,582	25
Gene Expression (qPCR) – NFE2L2 – Ordinary one-way ANOVA test								
Dunnett's multiple comparisons test	**Mean Diff,**	**95,00% CI of diff,**	**Below threshold?**	**Summary**	**Adjusted P Value**	**?-B**		
1 % vs. Control	0,06200	-0,2486 to 0,3726	No	ns	0,9581	D	1 %	
10 % vs. Control	1,063	0,7526 to 1,374	Yes	****	<0,0001	E	10 %	
Test details	**Mean 1**	**Mean 2**	**Mean Diff,**	**SE of diff,**	**n1**	**n2**	**q**	**DF**
1 % vs. Control	1,091	1,029	0,06200	0,1191	6	6	0,5204	25
10 % vs. Control	2,092	1,029	1,063	0,1191	6	6	8,924	25

Table S1. *Statistical analysis (ordinary one-way ANOVA test followed by Dunnett's post hoc multiple comparisons test) of results shown in Figure 4.*