

***In vitro* Skin Irritation Test of Honeypolis using Human Skin Model**

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(Received 14 November 2018; Revised 28 November 2018; Accepted 28 November 2018)

Abstract

Ethanol extracted propolis (EEP) was mixed with honey (honeypolis) to dissolve well in water and *in vitro* skin irritation test was conducted. *In vitro* method is designed to predict and classify the skin irritation potential of a chemical by assessment of its effect on EpiDerm™, a reconstituted three-dimensional human *epidermis* model. Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity measured by formazan production from MTT after a 60 min exposure period. In this study under the given conditions honeypolis showed no irritant effects. Honeypolis meets acceptance criteria if: mean absolute OD 570 nm of the three negative control tissues is ≥ 0.8 and ≤ 2.8 , mean relative tissue viability of the three positive control tissues is $\leq 20\%$, standard deviation of relative tissue viability obtained from each three concurrently tested tissues is $\leq 18\%$. Honeypolis is therefore classified as "non-irritant" in accordance with UN GHS "No Category".

Key words: Propolis, *In vitro* skin irritation test, Human skin model

INTRODUCTION

Propolis is the substance produced by honeybees with the resin of the plant and the enzymes of the salivary gland. They are considered natural antibiotics. Propolis has a large amount of active ingredients as flavonoids and phenolic compounds, it comes from plants. There are a number of studies on this and antioxidative effects (Bors *et al.*, 1990; Heim *et al.*, 2002; Russo *et al.*, 2002; Kumazawa *et al.*, 2004; Woo *et al.*, 2013), it has been reported that the synergistic effect is caused by the combined action of the phenolic compound and the resinous material (Burdock, 1998; Markham *et al.*, 1996). Propolis contains a lot of water insoluble substances, mainly it was extracted with ethyl alcohol (Woo *et al.*, 2015). Ethanol extracted propolis is turbid when mixed with water, and substances that do

not dissolve in water are lumpy. To solve this problem, honeypolis was prepared by mixing honey and ethanol extracted propolis, it has loosens well in water (Woo *et al.*, 2017).

Acute irritation is a local, reversible inflammatory response of normal living skin to direct injury caused by the application of an irritant substance for up to 4 hours. The potential to induce skin irritation is an important consideration included in procedures for the safe handling, packing and transports of chemicals (UN, 2015). Current guidelines include OECD guideline 404 (OECD, 2015a) for acute dermal irritation and corrosion of chemicals. This guideline is based on the method described by Draize (Draize, 1944), and generally involves the rabbit as the experimental animal. In order to replace *in vivo* testing on skin irritation validation studies on alternative *in vitro*

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methods were conducted under the auspices of ECVAM (Fentem *et al.*, 2001; Faller *et al.*, 2002; Cotovio *et al.*, 2005; Zuang *et al.*, 2002). It was concluded that the modified *in vitro* EpiDerm™, Skin Irritation Test (EPI-200-SIT) showed evidence of being a reliable and relevant stand-alone replacement test for *in vivo* skin irritation testing (EURL-ECVAM, 2008) or a partial replacement test with a testing strategy (OECD, 2014).

In vitro test may be used for the hazard identification of irritant chemicals in accordance with UN GHS “Category 2”. It does not allow the classification of chemicals to the optional UN GHS “Category 3” (mild irritants). Therefore all remaining substances will not be classified, i.e. UN GHS “No Category” (UN, 2015; EURL-ECVAM, 2009; EC, 2008). *In vitro* method is designed to predict and classify the skin irritation potential of a chemical by assessment of its effect on EpiDerm™, a reconstituted three-dimensional human *epidermis* model. Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity measured by formazan production from MTT after a 60 min exposure period.

In this study to use honeypolis as skin material, *in vitro* skin irritation test was performed instead of *in vivo*.

MATERIALS AND METHODS

Ethanol extracted propolis (EEP) solution

Ethanol extracted propolis (EEP) solution was prepared by extracting Korean propolis. Raw propolis 1kg was extracted with 80% ethanol 3.5 L (Woo *et al.*, 2012). EEP solution was filtered with Whatman No. 2 filter paper and then concentrated to 18% concentration.

Honey

Acacia honey was purchased from Korean Beekeeping agricultural cooperative. The moisture content was 18.5%.

Honeypolis

Honeypolis was made with honey and EEP solution. We

prepared 1kg acacia honey in beaker, poured 100ml EEP solution, and stirred with stirrer (Hei-torque200, Germany) over 1 hour. It was used as the test material.

Controls

Controls were set up in parallel to honeypolis in order to confirm the validity of the test. Negative Control was performed with Dulbecco's phosphate buffered saline (DPBS; Gibco, USA), for the positive Control, 5% sodium dodecyl sulfate (TC-SDS-5%; MatTek, USA) was used.

Test System

The test was carried out with the reconstituted three-dimensional human skin model EpiDerm™ (MatTek, USA). This skin model consists of normal human epidermal keratinocytes (NHEK) which have been cultured to form a multilayered, highly differentiated model of the human *epidermis*. The NHEK are cultured on chemically modified, collagen-coated cell culture inserts (Millicell®, USA). The EpiDerm™ *epidermis* model exhibits *in vivo* like morphological and growth characteristics which are uniform and highly reproducible. It consists of organised basal, spinous and granular layers and a multi-layered *stratum corneum* analogous to patterns found *in vivo*.

Pre-Experiments

To check the non-specific MTT-reducing capability of 30µL of honeypolis were mixed per 1mL MTT medium and incubated for 60 min at $37 \pm 1^\circ\text{C}$ in the incubator.

To check the colouring potential of 30µL of honeypolis were mixed per 300µL aqua dest, and per 300µL isopropanol each in a transparent recipient and incubated at $37 \pm 1^\circ\text{C}$ for 60 min.

Experimental Procedure

Upon receipt of the EpiDerm™, the tissues were inspected visually and transferred into 6-well plates containing 0.9mL assay medium per well. The surface was dried using a sterile cotton tip and the plates were incubated in a humidified incubator at $37 \pm 1^\circ\text{C}$, 5.0% CO₂

Table 1. Tissue viabilities of negative control, positive control and honeypolis

Name	Negative Control	Positive Control	Honeypolis
Total mean and SD OD ₅₇₀ of 3 replicate tissues	1.734*±0.051	0.049±0.017	1.492±0.009
Mean and SD relative tissue viability [%]	100.0±2.9***	2.8**±1.0	86.0±0.5
CV [% Viabilities]	2.9	34.5	0.6

*Blank-corrected mean OD_{570nm} of the negative control corresponds to 100% absolute tissue viability.

**Mean relative tissue viability of the positive control tissues is ≤ 20%.

***Standard deviation (SD) obtained from the three concurrently tested tissues is ≤ 18%.

Table 2. Tissue viabilities of the NSMTT control

NSMTT	KU	KT	NK
Mean and SD OD ₅₇₀	0.037±0.004	0.097±0.015	1.864±0.048
NSMTT [%]		3.22	-
Mean and SD relative tissue viability [%]		-	100.0±2.6
CV [% Viabilities]		-	2.6

KT: honeypolis treated killed tissues.

KU: killed tissue was left untreated as a control.

NK: negative control of living tissues.

NSMTT: Non-specific reduction of MTT.

NSMTT [%] = [(OD_{KT}-OD_{KU})/OD_{NK}]*100.

for 60±5 min. Subsequently the tissues were transferred into new wells containing 0.9mL pre-warmed assay medium per well and were incubated for 18±3 h in a humidified incubator at 37±1°C, 5.0% CO₂.

After this pre-incubation the tissues were treated with each dose group in triplicate, starting with the negative control. After dosing of all tissues, all plates were transferred to the incubator for 35±1 min. Afterwards all plates were removed from the incubator and placed under the sterile flow for the remaining time until the 60±1 min incubation time of the first dosed tissue was over. Then the tissues were washed by filling and emptying the inserts 15 times with DPBS using a constant stream in about 1.5cm distance from the tissue surface, staggered again in e.g. one-minute intervals. Subsequently, the inserts were completely submerged three times in 150mL DPBS and shaken to remove rests of honeypolis. Finally, the inserts were rinsed once from the inside and the outside with sterile DPBS. Excess DPBS was removed by blotting the bottom with blotting paper. The inserts were placed in prepared new 6-well plates containing 0.9mL pre-warmed

fresh assay medium per well and the tissue surface was dried using a sterile cotton tip. The plates were post-incubated at 37±1°C, 5.0% CO₂, humidified to 95%, for 24±2 h. Following this incubation the tissues were transferred to new wells containing 0.9mL fresh assay medium and incubated for additional 18±2 h.

After this post-incubation period the bottom of the inserts were blotted on sterile blotting paper and the inserts were transferred in a prepared 24-well plate containing 300µL pre-warmed MTT medium. This plate was incubated for 3 h±5 min at 37±1°C, 5.0% CO₂, humidified to 95%.

After the MTT incubation period, the tissues were rinsed three times with DPBS and afterwards placed on blotting paper to dry. The tissues were transferred into 12-well plates and immersed in 2mL isopropanol, sealed to inhibit evaporation. Extraction was carried out protected from light at room temperature at least for 2 h with gentle shaking on a plate shaker.

Before using the extracts, the plate had been shaken for at least 15 min on a plate shaker and the inserts were

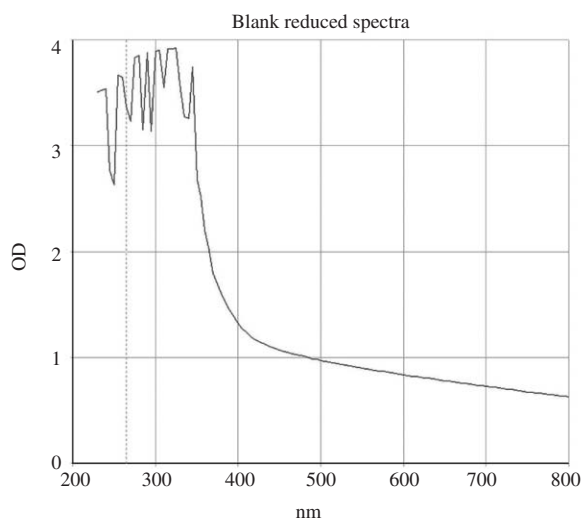


Fig. 1. Absorption spectrum of honeypolis mixed with aqua dest.

pierced with an injection needle. The extract was pipetted up and down 3 times before $2 \times 200\mu\text{L}$ aliquots per each tissue were transferred into a 96-well plate. OD was measured at 570nm with a filter band pass of maximum $\pm 30\text{nm}$ without reference wavelength in a plate spectrophotometer using isopropanol as a blank.

Data Analysis

Irritant potential of honeypolis was predicted from the relative mean tissue viabilities compared to the negative control tissues concurrently treated with DPBS. honeypolis is considered to be irritant to skin in accordance with regulation EC 1272/2008 (UN GHS “Category 2”) (EURL-ECVAM, 2009; EC, 2008), if the tissue viability after exposure and post-incubation is less or equal to 50%. Further testing is required to resolve between UN GHS categories 1 and 2 and decide on the final classification of the test substance (OECD, 2014). The test substance may be considered as non-irritant to skin in accordance with UN GHS “No Category” if the tissue viability after exposure and post-treatment incubation is more than 50%.

RESULTS AND DISCUSSION

The potential of honeypolis to induce skin irritation was analysed by using the three-dimensional human *epidermis*

model EpiDerm™ (MatTek) comprising a reconstructed epidermis with a functional *stratum corneum*.

The mixture of $30\mu\text{L}$ honeypolis per 1 mL MTT medium showed reduction of MTT compared to the solvent. The mixture turned blue/purple. For quantitative correction of results, two killed tissues were treated with $30\mu\text{L}$ of honeypolis (KT) and two killed tissues were left untreated as a control (KU), respectively (Table 2). NSMTT was $\leq 30\%$ (3.22%) relative to the negative control of living *epidermis*.

The mixture of $30\mu\text{L}$ of honeypolis per $300\mu\text{L}$ aqua dest. showed coloring detectable by unaided eye-assessment. Therefore, the absorption of the chemical in water was measured in the range of $570 \pm 30\text{nm}$. Honeypolis in water absorbed light in the relevant range (Fig. 1). For quantitative correction of results, the non-specific color of additional viable tissues ($\text{NSC}_{\text{living}}$) was determined by using additional viable tissues without MTT-staining and calculated (Table 3). $\text{NSC}_{\text{living}}$ was $\leq 5\%$ relative to the negative control of living *epidermis*, therefore no correction of the results was necessary. Since correction of the results using the $\text{NSC}_{\text{living}}$ control was not necessary, also double correction using the $\text{NSC}_{\text{killed}}$ control (Table 4) was not required. Honeypolis showed no irritant effects.

The mean relative tissue viability (% negative control) was $> 50\%$ (82.5%, NSMTT-corrected) after 60 min treatment and 42 h post-incubation (Table 1).

The controls confirmed the validity of the study. The mean absolute OD_{570} of the negative control tissues was ≥ 0.8 and ≤ 2.8 (1.777). The mean relative tissue viability (% negative control) of the positive control was $\leq 20\%$ (2.8%). Standard deviation (SD) of viability of replicate tissues of all dose groups was $\leq 18\%$ (0.5 ~ 2.9%).

The test meets acceptance criteria if: mean absolute OD 570nm of the three negative control tissues is ≥ 0.8 and ≤ 2.8 , mean relative tissue viability of the three positive control tissues is $\leq 20\%$, standard deviation (SD) of

Table 3. Tissue viabilities of the NSC_{living} control

NSC _{living}	TVT	Negative Control
Total mean and SD OD ₅₇₀	0.008 ± 0.001	1.864* ± 0.048
NSC _{living} [%]	0.41	-
Mean and SD relative tissue viability [%]	-	100.0 ± 2.6
CV [% Viabilities]	-	2.6

NSC_{living}: non-specific color of additional viable tissues.

TVT: additional honeyopolis treated living tissue without MTT staining.

NSC_{living} [%] = [OD_{TVT}/OD_{NK}]*100.

Table 4. Tissue viabilities of the NSC_{killed} control

NSC _{killed}	TVT	Negative Control
Total mean and SD OD ₅₇₀	0.009 ± 0.000	1.864* ± 0.048
NSC _{killed} [%]	0.51	-
Mean and SD relative tissue viability [%]	-	100.0 ± 2.6
CV [% Viabilities]	-	2.6

NSC_{killed}: non-specific color of additional killed tissues.

TKT: additional honeyopolis treated killed tissue without MTT staining.

relative tissue viability obtained from each three concurrently tested tissues is $\leq 18\%$. In this study under the given conditions honeyopolis showed no irritant effects. Honeyopolis is therefore classified as “non-irritant” in accordance with UN GHS “No Category”. Propolis has been proposed that EEP can safely be utilised in the prevention of psoriasis-related inflammatory changes without causing any toxic effect (Orsolich *et al.*, 2014), and as a result, honeyopolis is not a skin irritant and can be used as a skin material.

ACKNOWLEDGEMENTS

This research was funded by RDA agenda research project PJ010912.

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