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August 10, 2009

**REF: REPORT OF *IN VITRO* SKIN IRRITATION TEST**

Enclosed is the test report regarding the *in vitro* skin irritation of the 100 % Organic Cotton Fabric Material provided by NaturaPura.

The test was carried out using Skinethic reconstituted human epidermis, according to GLP principles and the study plan.

The results from the test show that the material is not irritative to human skin.

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## **Test Report**

### **In vitro Skin Irritation Test of Organic Cotton Fabric Material**

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Sponsor: NaturaPura Ibérica

#### **INTRODUCTION**

As described in the Test protocol, in vitro skin irritation test is a fast and cheap way to test the potential irritative property of fabric material. Following test procedure, results and interpretation were based on acute irritation test of chemical compounds explained in Test protocol.

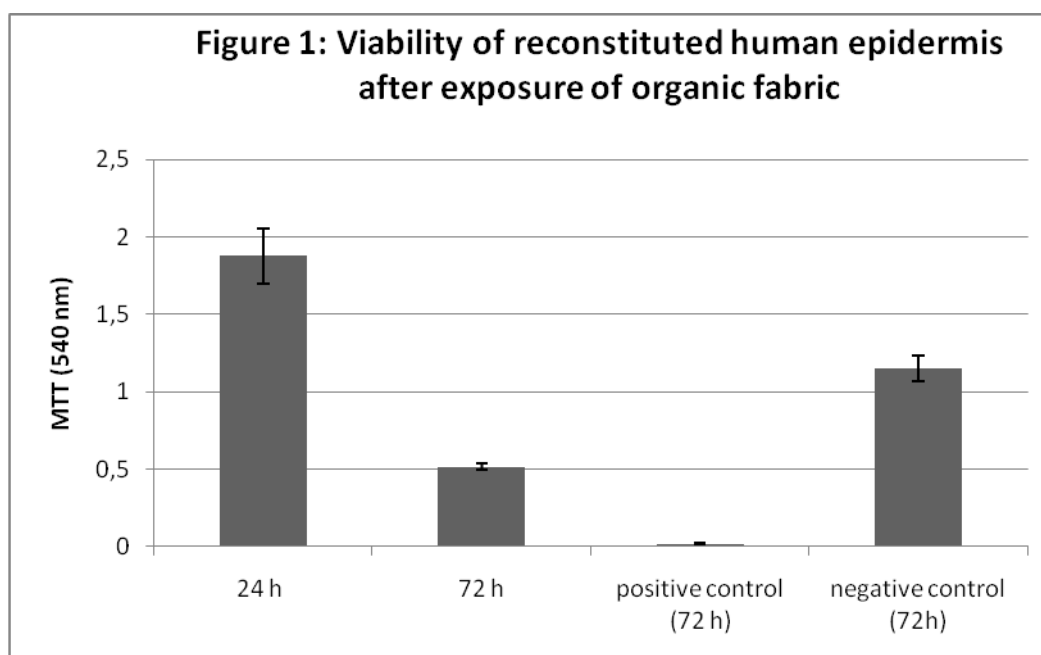
#### **TEST PROCEDURE**

1. 12 RHE tissues (RHE/S/17. Batch no: 09 022A 0704) were pre-incubated with SkinEthic growth medium (batch no: 0907 011J 440) overnight.
2. The test item (0.5 cm<sup>2</sup>) and controls were applied on top of the tissue. Three tissues were used for 1 day test and three for 3 day test. For negative control, Dulbecco's phosphate buffered saline (DPBS, 16 µl) was applied on top of tissues, which was then covered by a nylon mesh (0.5 cm<sup>2</sup>, a known non-irritant). For positive control, 0.8% of sodium dodecyl sulfate (SDS, Sigma L4509, a known irritant) and a nylon mesh were applied as it was done in negative control. Before the test item was applied, it was wetted using growth medium. Three tissues (inserts) were used in each time point and controls. Two of them were used in MTT cell viability test, and one in histology examination. Controls were used for 72 h time point.
3. Tissues were incubated with growth medium in a cell culture incubator at a humidified atmosphere of 37 °C, 5% CO<sub>2</sub> and 95% air for 1 and 3 days.
4. At the end of the incubation time, tissues were washed by DPBS, and then transferred to a 24 well plate.

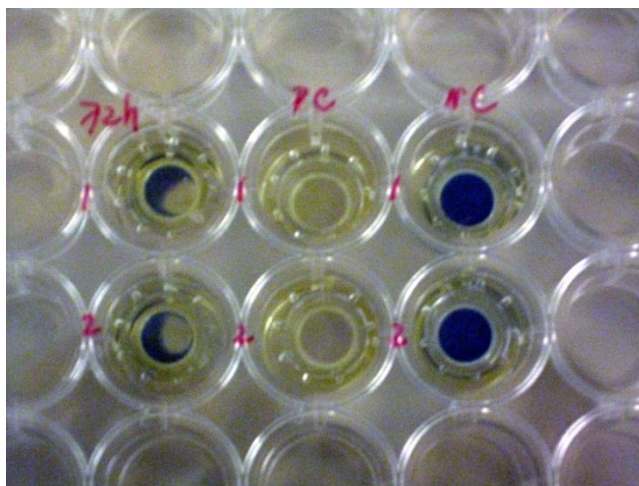
5. Then tissues were incubated with 300 µl MTT (1 mg/ml diluted in Skinethic maintenance medium. Batch 0907 011J 439) for 3 hours at 37 °C, 5% CO<sub>2</sub> and 95% humidified atmosphere.
6. At the end of incubation, tissues were transferred to a new 24 well plate pre-filled with 800 µl isopropanol per well, and then a further 700 µl isopropanol was added on the top of tissue (inside insert), and incubated for 2 hours at room temperature with gentle agitation (150 rpm).
7. At the end of incubation, 3 x 200 µl extraction solution per well were pipetted (= 3 wells per tissue i.e. 2 replicates per tissue) into a 96-well plate. Isopropanol was used as blank control.
8. The optical density (OD) of isopropanol extraction was read using a 96-well plate spectrophotometer (Hidex Chameleon V multiplate reader) at wavelength of 540 nm.
9. Data will be recorded and printed using software MicroWin 2000.
10. For histological examination, tissues were fixed in 4% formalin. Paraffin sections were made according to a routine procedure. Paraffin section at 5 µm thick were cut, dehydrated and stained with hematoxylin-eosin (HE). Histological examination of the tissues was carried out using a microscope (Olympus BX60).

## RESULTS AND DISCUSSION

The results of cell viability measured by MTT assay is shown in Figure 1. From the figure, we can see that MTT measurement at 24 h is significant higher than those of 72 h and positive control, even that of negative control at 72 h. This suggests the test item was not irritative at



that time point. The MTT measurement at 72 h is lower than that of negative control, somehow indicating the irritative property of test item at this time point. However, when the MTT staining pattern was examined, it is clear that this is not true. From Figure 2, we can see the tissues exposed to test items (left two inserts) at 72 h had only partially stained with MTT.



*Figure 2. The MTT staining pattern of tissues exposed to test item at 72 h.*

Nearly half of tissue was not stained at all (comparable to that of positive control, PC) indicating that part of tissue was dead. The exact reason for this phenomenon is not known. However, since two tissues exhibited exact same pattern, it excludes the possibility of bubbles or other operational errors. The most plausible explanation for this is the relative thick fabric blocked air or oxygen to reach tissues, causing partial death of the tissues. That phenomenon did not happen in negative control because of much thinner of nylon material used. The part of tissues stained with MTT have the almost same intensity as that in negative control, indicating if tissues do get enough air or oxygen, their viability is as good as negative control. As a result the staining pattern showed in figure 2 indicate that test item at 72 h is not irritative.

The histology examination of tissues exposed to test item and controls are shown in figures 3A, 3B, 3C and 3D. The results of histological examination confirm the conclusion from MTT assay. The histological and cellular structure of epidermis exposed to test item for 24 h (Figure 3A) is almost the same as observed in negative control (Figure 3C). At 72 h (Figure 3B), degeneration of cells were seen. For positive control (Figure 3D), almost no alive cells were observed.

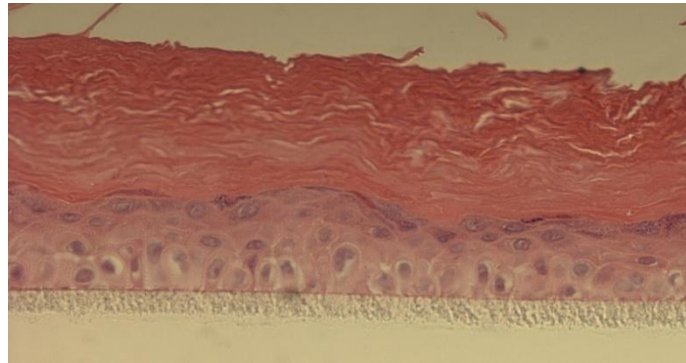


Figure 3A: reconstituted human epidermis exposed to test item for 24h.

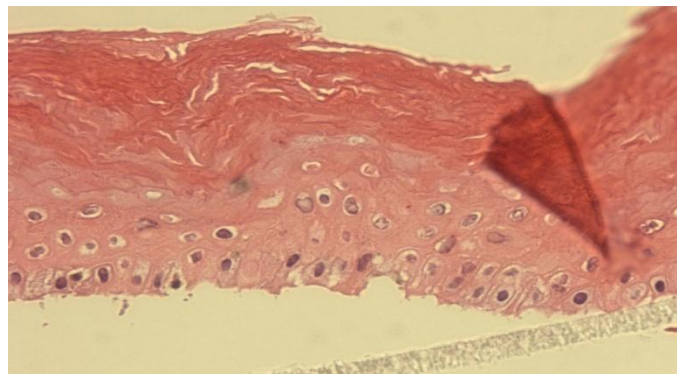


Figure 3B: reconstituted human epidermis exposed to test item for 72h.

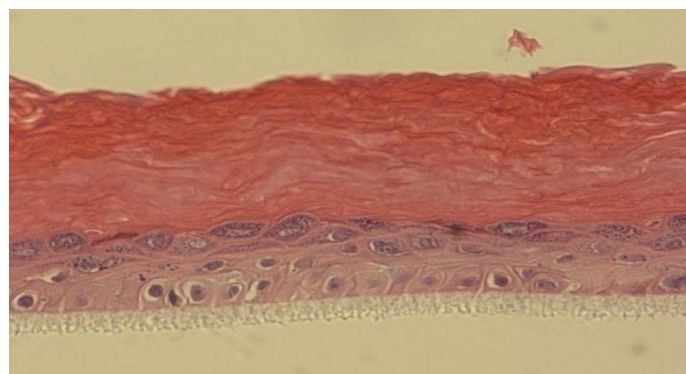


Figure 3C: reconstituted human epidermis, negative control (DPBS).

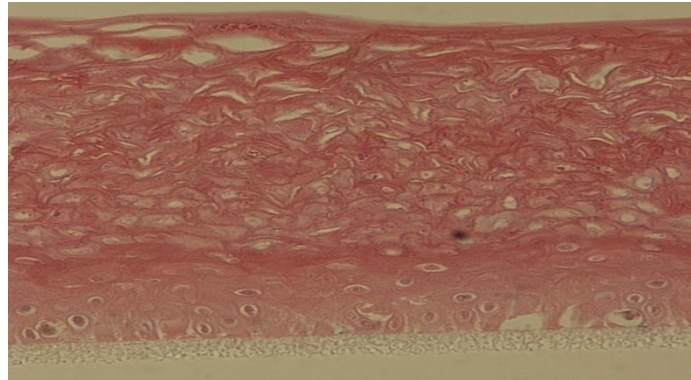


Figure 3D: reconstituted human epidermis, positive control (0.8% SDS).

## CONCLUSION

The 100% organic cotton fabric provided by NaturaPura is not irritative to human skin, tested using Skinethic reconstituted human epidermis.

## REFERENCES

1. Skinethic skin irritation test<sup>-42bis</sup> Standard Operating Procedure (SOP). SkinEthic Laboratories, January 2009, INVITTOX Version 1.0
2. A. de Brugerolle de Fraissinette et al: Productivity of an *in vitro* model for acute and chronic skin irritation (SkinEthic) applied to the testing of topical vehicles. Cell Biology and Toxicology, 1999; 15: 121-135