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CASE STUDY

Assessment of cytotoxicity, skin and oral irritation of medical devices with hydrating properties: A case study

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ABSTRACT

Ensuring the biocompatibility of medical devices (MDs) is essential for regulatory compliance and user safety. Under the evolving requirements of the European Medical Device Regulation (EU MDR 2017/745), products that come into contact with mucosal surfaces – such as those intended for oral use – must be supported by robust biocompatibility data. This study evaluated the ***in vitro*** cytotoxicity and irritation potential of the medical device hydropastilky in comparison with a benchmark product of similar composition and intended use, following ISO 10993 guidelines. Hydropastilky are designed to moisturise, protect, and support the regeneration of the oral and throat mucosa. Cytotoxicity was assessed using the Neutral Red Uptake (NRU) assay (ISO 10993-5). Hydro pastilky maintained cell viability above 50% at an extract concentration of up to 34.5%. Cytotoxic effects observed at higher concentrations were attributed to the strong hydrating and water-binding properties of the product, which posed challenges during extract preparation and likely impacted cell osmolarity as well. The benchmark product yielded comparable results. To generate more physiologically relevant data, irritation testing was performed using 3D reconstructed human epidermal (EpiDerm™) and oral mucosal (EpiOral™) models (ISO 10993-23). Both products demonstrated tissue viabilities above 50%, with most values exceeding 75%, confirming their non-irritant classification after prolonged exposure of 18 hours. These findings support the biocompatibility as well as biosimilarity of hydropastilky and the benchmark product and highlight the role of new approach methodologies (NAMs) in meeting modern regulatory expectations.

KEY WORDS: hydropastilky; cytotoxicity; irritation; sensitisation; medical devices; ISO 10993

Introduction

Ensuring the biocompatibility of medical devices intended for dermal or mucosal application is critical for regulatory approval and user safety. The ISO 10993 series provides internationally recognised standards for assessing biological risks, including cytotoxicity (Part 5), sensitisation (Part 10), and irritation (Part 23). These standards have increasingly been adopted to support the ethical evaluation of medical devices using scientifically validated *in vitro* approaches. With the implementation of the European Medical Device Regulation (EU MDR 2017/745), manufacturers face stricter requirements to demonstrate the safety and performance of products that come into contact with human tissues. In particular, the MDR emphasises the need for robust biological evaluation data addressing the so-called "big three" endpoints: cytotoxicity, irritation, and sensitisation (Kandarova *et al.*, 2024). For products with mucosal contact - such as lozenges or pastilles - additional experimental evidence is required under the modernised EU MDR to support claims of biocompatibility. As part of this process, manufacturers actively seek scientifically sound, animal-free testing strategies that meet both regulatory expectations and societal demands for responsible innovation. In response to both regulatory pressure and the global shift toward non-animal methods, new approach methodologies (NAMs) are increasingly used to replace traditional *in vivo* testing. When scientifically justified,

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in vitro methods described in ISO 10993-5:2009 and 10993-23:2021 offer reliable, reproducible, and ethically responsible alternatives for evaluating biological safety. This study aimed to evaluate the cytotoxicity and irritation potential of *hydropastilky*, a moisturising throat lozenge intended for oral mucosa application, using validated *in vitro* models under ISO 10993-5 and ISO 10993-23. A benchmark product of similar composition and intended use was included in the study to assess biosimilarity and support a comparative evaluation of biocompatibility.
In addition to the experimental evaluation of cytotoxicity and irritation, a literature-based review was conducted to assess other toxicological endpoints of the individual components of *hydropastilky*, including acute toxicity, irritation, and sensitisation potential. This assessment aimed to strengthen the overall biocompatibility profile of the product and ensure alignment with current regulatory expectations. The results generated in this study contribute to the body of evidence required under the MDR framework and support the broader implementation of NAMs in medical device safety assessment.

Materials and methods

Test materials

The MD product Hydropastilky contains active ingredients that form a so-called hydrogel complex during sucking. This hydrogel complex – composed of carbomer, xanthan gum, and hyaluronic acid – creates a protective, moisturising film over the irritated oral and throat mucosa. This effect is achieved through the high water-binding capacity of xanthan gum (a polysaccharide), the water-retaining properties of carbomer, which stabilises the gel structure in the neutral or slightly alkaline environment of saliva, and the hydrating properties of hyaluronic acid. The irritated mucosa may regenerate more rapidly due to the action of calcium pantothenate, which helps relieve symptoms commonly associated with upper respiratory tract irritation, such as sore throat, scratchiness, burning sensations, hoarseness, and coughing.

The formulation of hydropastilky, as stated in the product's Instructions for use includes:

Active ingredients: calcium pantothenate (the calcium salt of vitamin B5), hyaluronic acid (sodium hyaluronate), carbomer, and xanthan gum.

Excipients: sorbitol, xylitol, anhydrous citric acid, magnesium stearate, gum arabic, and peppermint flavouring.

The product is registered with the Slovak State Institute for Drug Control (abbreviated as ŠÚKL in Slovak) under registration code 98263, European Article Number 8588003769725, and was classified as a Class I medical device until 2024.

The benchmark product used in this study to assess biosimilarity is also supplied in the form of tablets of comparable size. Its formulation contains xanthan gum, carbomer, sodium hyaluronate, and additional excipients. This product is classified as a Class IIa medical device. Both products are available over the counter and were purchased from a local pharmacy.

Extracts preparation

Extracts of the test items were prepared following ISO 10993-12:2021, following the guidance in Table 1 for irregularly shaped solids and semi-solid materials. A concentrated extract was prepared using an extraction ratio of 0.2 g/mL, as recommended for powders, pellets, and similar forms.

For cytotoxicity testing, the extraction medium was Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% bovine serum. For irritation testing using 3D reconstructed tissue models, two extraction vehicles were used: sterile saline and pharmaceutical-grade sesame oil, representing both polar and non-polar conditions. Approximately 1 g of each test item was weighed and placed into 5 mL of extraction medium for irritation testing. All extractions were performed in sterile, closed containers under aseptic conditions, as outlined in ISO 10993-12. Samples were incubated at 37±1 °C for 24±2 hours in a water bath under orbital shaking at 500 RPM to ensure uniform material–vehicle interaction. Following extraction, the level of absorption was assessed visually. If the test item had absorbed a significant portion of the medium, an additional extraction vehicle was added to achieve saturation, with the volume recorded and added to the initially absorbed quantity. This approach complies with the ISO provisions for hygroscopic or water-absorbing test items and was adopted for the cytotoxicity assessment. For the irritation studies in 3D models, we maintained the recommended concentration of 0.2 mg/mL, as 3D tissue models tolerate highly concentrated chemicals and formulations. *Hydropastilky* were relatively well solubilised in an aqueous medium, resulting in a concentrated liquid extract. In contrast, the benchmark product absorbed the vehicle extensively, initially forming a thick, gel-like extract. Both test items exhibited poor solubility in sesame oil, which limited their extractability under lipophilic conditions.

Biological models

Balb/c 3T3 Mouse fibroblast cell line: The adherent, continuous Balb/c 3T3 fibroblast cell line (supplied by Merck from ECACC, UK) was used to assess general cytotoxicity. This cell line is widely used in toxicological studies due to its sensitivity, robustness, and reproducibility in detecting cytotoxic effects. It is also explicitly recommended in ISO 10993-5 (Annexe C) as a validated *in vitro* model for cytotoxicity testing.

EpiDerm™ (EPI-200): A three-dimensional reconstructed human epidermal model manufactured by MatTek In Vitro Life Science Laboratories (Bratislava, Slovakia) under GMP-compliant conditions. The model is composed of non-transformed human keratinocytes and closely replicates the structure and function of the human epidermis, including a functional stratum corneum. EpiDerm™ has been officially adopted in ISO 10993-23 (2021) as a validated *in vitro* model for assessing skin

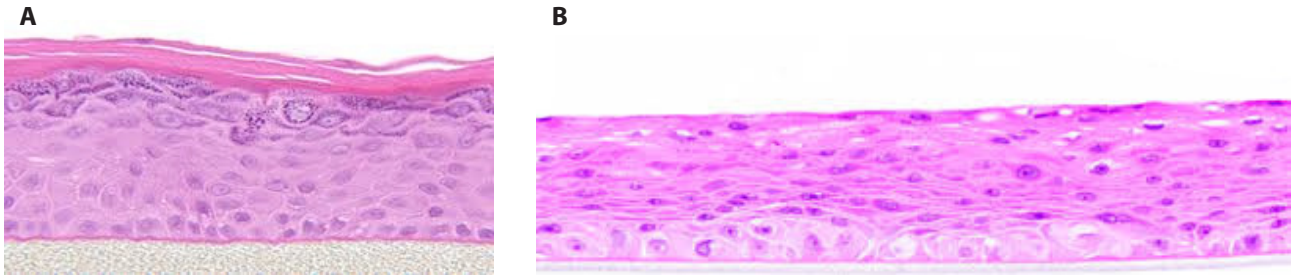

Figure 1. Histological structure of reconstructed human tissue models used for irritation testing (H&E staining, cross-sections). **(A)** EpiDerm™: Reconstructed human epidermis composed of normal human keratinocytes forming a stratified, keratinised epithelium with a basal layer, spinous and granular layers, and a well-developed stratum corneum. This structure mimics native human skin and is accepted under ISO 10993-23:2021 for in vitro skin irritation testing. Structurally, it also resembles the histology of gingival tissues in the oral cavity. **(B)** EpiOral™: Reconstructed human oral epithelium, composed of non-keratinised human keratinocytes forming a stratified squamous epithelium resembling the architecture of native oral mucosa. The absence of a stratum corneum reflects its physiological relevance for evaluating mucosal irritation under ISO 10993-23:2021. Source of the pictures: www.mattek.com

(intracutaneous) irritation. Each tissue batch is quality-controlled by the manufacturer for viability, barrier function, and the absence of microbial contamination.

EpiOral™ (ORL-200): A reconstructed human oral epithelium model mimicking the architecture and functionality of the oral mucosa (commercially available from MatTek). It is composed of non-transformed human keratinocytes and is validated for use in irritation testing for mucosal applications. The tissues are tested by the manufacturer for the absence of pathogens and to ensure standard barrier properties. Following ISO 10993-23:2021, which permits the use of tissue-specific models for evaluating irritation of non-cutaneous application sites, the EpiOral™ reconstructed human oral epithelium model was used to assess mucosal irritation and biosimilarity of *hydropastilky* and benchmark product. This model is physiologically relevant for evaluating medical devices intended for oral cavity applications.

Cytotoxicity Testing

The cytotoxic potential of the test items was assessed using the Neutral Red Uptake (NRU) assay, in accordance with ISO 10993-5:2009. The assay was conducted with Balb/c 3T3 mouse fibroblast cells, as advised in Annex C of ISO 10993-5.

Cytotoxicity testing was conducted in 96-well plates using semi-confluent 3T3 monolayers (seeding density 1×10^5 cells/mL). Cells were exposed to serial dilutions of the test extracts for 24±2 hours. After treatment, cell viability was assessed by quantifying the uptake of Neutral Red dye, which accumulates in the lysosomes of viable cells. Optical density (OD) was measured at 540 nm using a microplate reader against a blank.

The assay included a negative control (medium-treated cells) and a positive control, Sodium lauryl sulphate (CAS# 151-21-3), in four concentrations specified by ISO 10993-5 (0.05 mg/mL; 0.1 mg/mL; 0.15 mg/mL; 0.2 mg/mL). Cell viability was calculated as a percentage of the negative control. Extracts were classified as non-cytotoxic if they maintained viability ≥70%. IC_{50} values (concentration reducing viability by 50%) were calculated for each test item to allow direct comparison.

Irritation Testing

The potential for skin and oral mucosal irritation was evaluated using reconstructed human tissue models, EpiDerm™ and EpiOral™ (MatTek In Vitro Life Science Laboratories), by ISO 10993-23:2021 and the protocols described in studies by Kandarova *et al.* (2018) and Pôbiš *et al.* (2025).

Test item extracts (prepared in sterile saline and pharmaceutical-grade sesame seed oil) were applied topically to the tissue surface in a volume of 100 µL per insert and incubated for 18 ± 0.5 hours under standard culture conditions (37±1 °C, 5% CO_2, ≥95% relative humidity). Each treatment, including test items and controls, was performed in triplicate. The negative control was Dulbecco's phosphate-buffered saline (DPBS), and the positive control was 1% sodium dodecyl sulfate (SDS), prepared in both aqueous and oil-based vehicles.

After exposure, tissue viability was determined using the MTT assay, which quantifies mitochondrial enzymatic activity. The yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; CAS No. 298-93-1) is metabolised by viable cells to produce a blue formazan salt. After a 3-hour incubation with MTT (300 µL per insert, concentration: 1 mg/mL), the formazan was extracted using 2 mL of isopropanol. Optical density was measured in flat-bottom 96-well plates (200 µL per well) at 570 nm, using isopropanol as the blank.

The assay is based on the principle that irritating substances can penetrate tissue, impair cell integrity, and reduce metabolic activity. This mechanism applies to both skin and mucosal tissues. The prediction model used for classification follows the criteria of ISO 10993-23:

- Viability ≤50%: classified as irritant
- Viability >50%: classified as non-irritant
- Literature-based assessment

In addition to the experimental evaluation of cytotoxicity and irritation, a targeted literature review was performed to assess additional toxicological endpoints of the individual components of *hydropastilky*, including acute toxicity, irritation, and sensitisation potential. Toxicological information was sourced from publicly

available, peer-reviewed databases, including PubMed, Cochrane CDSR, Google Scholar, Clinicaltrials .gov, the European Chemicals Agency (ECHA) database, the Cosmetic Ingredient Review (CIR), and CosIng, the European Commission's database of cosmetic substances and ingredients.

Results

Cytotoxicity

The cytotoxic potential of *hydropastilky* and the benchmark product was assessed using the Balb/c 3T3 Neutral Red Uptake (NRU) assay following ISO 10993-5:2009. Serial dilutions of the test item extracts (6.7%, 9.9%, 14.6%, 21.4%, 31.5%, 46.3%, 68.0%, and 100.0%) were applied to subconfluent 3T3 fibroblast monolayers, and cell viability was measured after 24±2 hours of exposure. Sodium dodecyl sulfate (SDS) served as the positive control, producing a dose-dependent decrease in cell viability, thus confirming the assay's validity.

Hydropastilky extracts exhibited a concentration-dependent cytotoxic effect, with cell viability remaining above 70% at concentrations up to 21.4% (Figure 2A). Normal cell morphology was also observed up to this concentration. The extract sustained viability above 50% at a 34.5% concentration of the neat extract, with a calculated IC_{50} of 37.4%, indicating only mild cytotoxic potential at higher concentrations. This effect was attributed to the product's hydrophilic nature and water-binding properties, likely causing local osmotic imbalances rather than direct chemical toxicity.

The benchmark product yielded very similar outcomes, with an IC_{50} of 37.3%, suggesting comparable cytotoxicity profiles and supporting the concept of biosimilarity. At the two highest concentrations, the benchmark product formed a visible semi-gelled ring along the well walls, which artificially increased optical density readings due to the retained Neutral Red dye (Figure 2B).

Both the positive and negative controls were within the acceptable historical range, with an OD of the negative control above 0.3 and an IC_{50} for SDS at 0.03 mg/mL. The overall dose-response trends for both products are presented in Figure 2.

Skin and oral mucosa irritation

The irritation potential of hydropastilky and the benchmark product was assessed using reconstructed human tissue models, EpiDerm™ and EpiOral™, following ISO 10993-23:2021. Extracts of the test items were prepared in polar (saline) and non-polar (pharmaceutical-grade sesame oil) vehicles, with 100 µL of each extract applied topically to the tissue surface. The tissues were incubated for 18±0.5 hours under standard culture conditions. Cell viability was subsequently determined using the MTT assay, and all validity criteria for the test systems were met, including appropriate responses from negative (DPBS) and positive (1% SDS) controls.

The skin irritation assessment using the EpiDerm™ model showed that hydropastilky extracts maintained tissue viability well above the 50% threshold for classification as a non-irritant. Tissue viability for *hydropastilky* ranged from 75.6±2.9% (extract in sesame oil) to 84.4±2.9% (Extract in saline), while *Benchmark* viabilities ranged from 78.1±2.4% (extract in saline) to 78.6±5.1% (extract in sesame oil). Both products were classified as non-irritating for skin. The positive control (1% SDS) reduced viability to below 3%, confirming test sensitivity, while the negative control maintained viability at 100%. The data are summarised in Figure 3.

The oral mucosal irritation potential was further evaluated using the EpiOral™. Tissue viability following exposure to hydropastilky extracts ranged from 56.9±12.5% (sesame oil extract) to 84.5±5.7% (saline extract). The benchmark product showed viabilities of 70.5±2.8% (saline extract) and 76.7±2.9% (sesame oil extract). Despite a moderate reduction in viability with the sesame oil extract of hydropastilky, all values remained above the 50% threshold, indicating a non-irritant classification for both products. No visible tissue damage or assay interference was observed. The test was validated by appropriate responses of the negative control (DPBS,

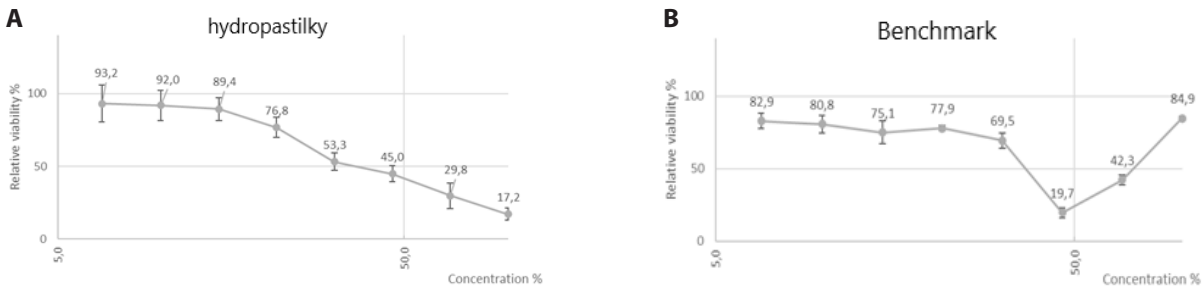

Figure 2. Cytotoxicity of *hydropastilky* and the benchmark product in the 3T3 NRU assay. The figure presents dose-response curves for *hydropastilky* **(A)** and the benchmark product **(B)**, respectively, based on the viability of Balb/c 3T3 cells exposed to serial dilutions of test item extracts (6.7%, 9.9%, 14.6%, 21.4%, 31.5%, 46.3%, 68.0%, and 100.0%). A marked reduction in viability was observed at concentrations ≥46.3% for both products, with calculated IC_{50} values of 37.4% for *hydropastilky* and 37.3% for the benchmark product. Both samples maintained viability above the ISO 10993-5 cytotoxicity threshold (70%) at lower concentrations. At high concentrations, viability was impacted, likely due to osmolarity-related effects. Additionally, the benchmark product formed a gel-like residue that retained traces of the Neutral Red dye, potentially interfering with the OD readings at the highest two concentrations.

100% viability) and the positive control (1% SDS), which consistently reduced viability to below 3%. The data are summarised in Figure 4.

Discussion

With the implementation of the European Medical Device Regulation (EU MDR 2017/745), manufacturers are required to provide comprehensive biological safety data to support the intended use of their products. This includes for almost all MDs evidence for the so-called "big three" endpoints: cytotoxicity, irritation, and sensitisation (Kandarova *et al.*, 2024). For products intended for application to mucosal membranes, such as *Hydro Pastilky*, these evaluations are mandatory to demonstrate safety under clinically relevant exposure conditions. Traditionally addressed through *in vivo* testing, these endpoints can now be assessed using New Approach Methodologies (NAMs) that are scientifically robust, faster, and more cost-effective. The use of validated *in vitro* models, compliant with the ISO 10993 series, provides a streamlined and ethical alternative that aligns with modern regulatory and societal expectations.

In this study, *hydropastilky* and a benchmark product were assessed using a combination of *in vitro* and literature-based approaches. The 3T3 NRU cytotoxicity assay, performed per ISO 10993-5, demonstrated a concentration-dependent reduction in cell viability for both products, with calculated IC_{50} values of 37.4% and 37.3%, respectively. These nearly identical results support the biosimilarity of the formulations and indicate mild cytotoxic effects only at high extract concentrations. The observed effects are likely attributable to osmotic imbalances caused by the high water-binding capacity of the ingredients rather than inherent cytotoxicity. Furthermore, in the benchmark product, gel-like residues at high concentrations retained Neutral Red dye, which mildly interfered with optical density readings. This artefact highlights the importance of careful handling of extracts, particularly for hydrophilic, hygroscopic, or viscous materials that are common among topically applied MDs.

The irritation assessment, conducted following ISO 10993-23 using the EpiDerm™ (epidermis) and EpiOral™

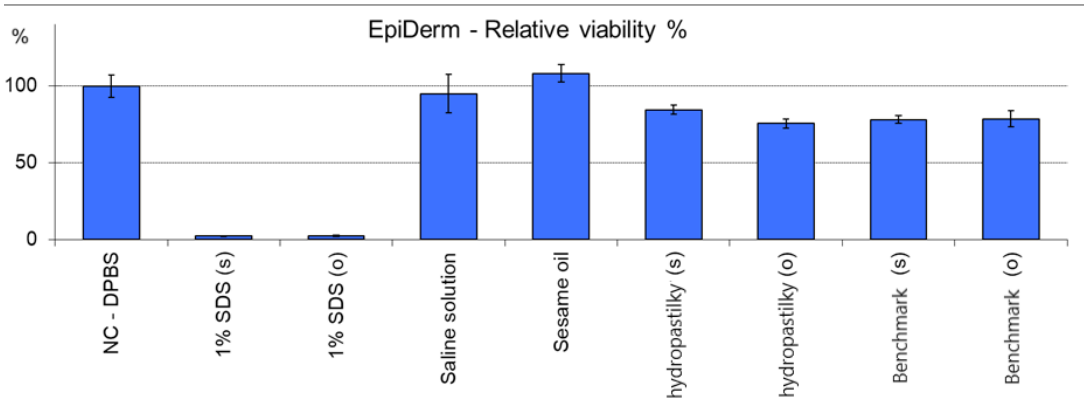

Figure 3. Skin irritation assessment using the EpiDerm™ reconstructed human epidermis model. Viability of EpiDerm™ tissues following exposure to hydropastilky and benchmark product extracts prepared in sterile saline and pharmaceutical-grade sesame seed oil. Data are presented as % viability mean ± SD (n=3 replicates per treatment). Based on the prediction model of ISO 10993-23, both products were classified as non-irritating to skin under all test conditions. No significant differences in tissue viability were observed between the test items or extraction vehicles.

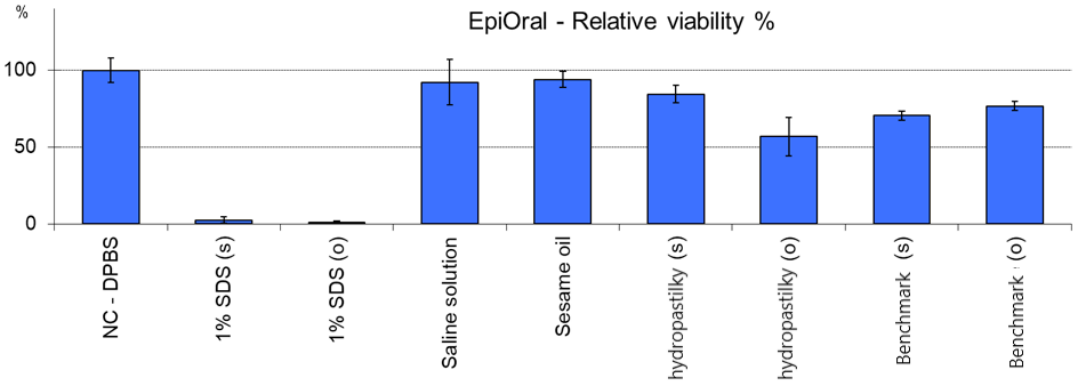

Figure 4. Oral mucosa irritation assessment using the EpiOral™ reconstructed human oral epithelial model. Viability of EpiOral™ tissues following exposure to hydropastilky and benchmark product extracts prepared in sterile saline and pharmaceutical-grade sesame seed oil. Data are presented as mean ± SD (n=3 replicates per treatment). A moderate reduction in viability was observed with the sesame oil extract of Hydro Pastilky, attributed to a single lower replicate value without visible tissue damage or assay interference. Nevertheless, all viability values remained above the 50% threshold, supporting a non-irritant classification for both products according to ISO 10993-23. Test validity was confirmed by appropriate responses of the negative (DPBS) and positive (1% SDS) controls.

(oral mucosa) models, confirmed that both products are non-irritants. The EpiOral™ model, employed for evaluating mucosal compatibility, was selected in line with ISO 10993-23, allowing the use of tissue-specific reconstructed models for non-cutaneous exposure sites (Pôbiš *et al.*, 2025). Viability values across all test conditions and both tissue models remained substantially above the 50% threshold. These results provide robust evidence that the formulations are well-tolerated under prolonged contact conditions that mirror realistic clinical use.

To complement the experimental work, a targeted literature review was conducted to evaluate additional toxicological endpoints, particularly the sensitisation potential of individual ingredients. Searches of PubMed, the European Chemicals Agency (ECHA) database, the Cosmetic Ingredient Review (CIR), and CosIng revealed no sensitising or acutely toxic properties for the main ingredients - sodium hyaluronate, calcium pantothenate, xanthan gum, and carbomer - all of which have established safety records in cosmetics and medical applications. The combined use of *in vitro* testing and literature-based assessment provides a comprehensive, animal-free strategy that aligns with EU MDR and international regulatory expectations.

This study also highlights the practical utility of NAMs not only for initial safety evaluation but also in supporting biosimilarity assessments, reformulation decisions, or extensions of market claims. It underscores the importance of adapting extraction protocols (as described in ISO 10993-12) for products with non-standard physical properties, such as hydrating or gel-forming pastilles.

Conclusion

This study demonstrated that *hydropastilky* and a selected benchmark product from Class IIa exhibit comparable biocompatibility profiles when evaluated using validated *in vitro* methods, as outlined in ISO 10993-5 and ISO 10993-23. Both formulations produced similar cytotoxicity profiles and were found to be non-irritant in highly sensitive reconstructed tissue models of human skin and oral mucosa under prolonged exposure conditions. The results were further supported by a literature search of toxicological data, which confirmed the absence of sensitisation or acute toxicity concerns for the individual ingredients. These findings provide robust evidence of product safety and highlight the value of integrating New Approach Methodologies (NAMs) into medical device biocompatibility and biosimilarity evaluations. This integrated testing approach provides a relevant example of how *in vitro* strategies can replace traditional animal testing in the safety evaluation of medical devices that come into contact with skin and mucosal tissues.

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