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TEST REPORT 23 45 00056

NOVASIN

INTRADERMA

IN VITRO WOUND HEALING ASSESMENT

Conclusion

The test item “NOVASIN” exhibits significant wound healing activity at the concentration range 0.05 mg/ml – 10 mg/ml.

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FINALREPORT

In vitro wound healing assessment of

Novasin

ID:23 45 00056

STUDY No.:2023/WH001

STUDY DIRECTOR: CHARA ALMPANI

STUDY COMPLETED ON 05.07.2023

SPONSOR

QACS Ltd

Quality Assurance and Control Systems

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TEST FACILITY

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CONTENTS

1 STUDY DETAILS

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Study Schedule

Study Initiation Date:	02.06.2023
Experimental Starting Date:	19.06.2023
Experiment Completion Date:	30.06.2023
Study Completion Date:	05.07.2023

2 SUMMARY

Wound healing is a complex process that involves various cellular and molecular events. Impaired wound healing can lead to chronic wounds, which can have a significant impact on the quality of life of patients.

The test item, "Novasin", is a novel product that has been developed for the treatment of wounds. The scratch assay was conducted to evaluate the wound-healing potential of the test item compared to control groups. The assay measures the migratory capacity of cells across a scratch-induced wound *in vitro*, providing valuable insights into the potential therapeutic effects of the test item.

A consistent scratch was created across each monolayer of BALB/c 3T3 cells using a sterile pipette tip. The treated groups included Novasin-treated, positive control, and negative control groups. Images of the scratch were captured at regular intervals (0, 12 and 24 hours) and analyzed using image analysis software. The area of the scratch was measured to assess wound closure.

Novasin demonstrated a significant *in vitro* wound healing potential at all tested concentrations. The results suggest that Novasin could be a promising product for the treatment of wounds. Further studies are needed to evaluate the efficacy of Novasin *in vivo*. The findings of this study provide a basis for further research and development of Novasin as a wound healing product.

3 GUIDELINE

Currently there is no specific ISO (International Organization for Standardization) or OECD (Organisation for Economic Co-operation and Development) guideline that is dedicated to the *in vitro* scratch assay for wound healing. The protocol has been established based on literature [1-5].

4 MATERIALS AND METHODS

4.1 Test item information

(As furnished by the Sponsor)

Product Amount:	100 ml
Product type:	liquid
Description:	**Wound healing agent**

4.2 Identity of the test item

The characterisation detail of test item is provided by the study Sponsor. This will be included in the raw data. The responsibility for the correct identity and purity of the test item rests with the Sponsor. The test item will not be authenticated at the test facility.

4.3 Test Medium and Chemicals

Analytical or better grade chemicals were used in the study.

1.

Name: **Bovine Calf Serum**

Lot: 80914215

Bottle #: 0246

Supplier: ATCC (MANASSAS, VA)

2.

Name: **Trypsin/EDTA (0.05% / 0.02%) in PBS**

Lot no: 1830222

Supplier: PAN BIOTECH, Germany

3.

Name: **DMEM high glucose** with stable Glutamine with Sodium pyruvate with 3.7 g/L $NaHCO_3$, Sterile filtered

Lot:9880822

Supplier: PAN BIOTECH, Germany

4.

Name: **DPBS (10x) with Calcium and Magnesium**

Lot: 5220321

Bottle #: 0246

Supplier: PAN BIOTECH, Germany

5.

Name: **Penicillin – Streptomycin solution**, sterile filtered

Lot: M10I056

UN-NR: 2811

Supplier: PAN BIOTECH, Germany

4.4 Test system

Cell line	**BALB/3T3 clone A31**
Organism:	*Mus musculus,* mouse
Cell type:	Fibroblast
Tissue:	Embryo
Supplier:	ATCC
Passage number:	80 - 85

4.5 Test Item Preparation

The test item was treated according to ISO 10993-12:2012, (Biological evaluation of medical devices - Part 12: Sample preparation and reference materials) by the process of dilution in DMEM enriched with bovine calf serum (BCS) (1%). Diluting the test item in DMEM with low levels of serum provides a suitable culture medium that supports cell growth, maintains cell phenotype, and minimizes potential interference, enabling the accurate evaluation of cell migration and wound healing processes.

Eight dilution series concentrations were tested: 0,05 mg/mL, 0,1mg/mL, 0,5 mg/mL 1mg/mL, 5mg/mL, 10mg/mL, 50mg/mL and 100mg/mL. Each concentration was tested in triplicates in two separate plates.

4.5.1 Method

The *in vitro* scratch assay, commonly known as the wound healing assay, is predicated upon the controlled generation of a cell-free gap within a cultured cell monolayer. The main objective of this assay is to investigate cellular migration and wound healing processes in an experimental setting. The procedure commences with the establishment of a confluent cell monolayer on the surface of a culture. Subsequently, a precise and linear scratch is created within the monolayer, achieved using a sterile tool, typically a pipette tip. Following the incision, the culture dish is returned to the incubator to facilitate cell migration and gap closure. The progress of cell migration is meticulously monitored and recorded at predefined intervals using microscopy. Through the assessment of the extent of gap closure over time, this assay furnishes valuable insights into cell migration rates, proliferation, and the impact of various stimuli or interventions on wound healing processes *in vitro*.

4.5.2 Test Conditions

Cells from frozen stock cultures were seeded in culture medium at an appropriate density at 37°C and 8% CO_2 ($InCO_2$ Incubator, Memmert), in Dulbecco's modified Eagle's medium (DMEM;high glucose) supplemented with 10% Bovine Calf Serum (BCS) and 1% penicillin-streptomycin (10,000 unit /ml). and subcultured at least twice before use in the assay. Cells yielded from 100 mm culture dishes (Greiner, Germany) grown to 90% cell confluency (~2.8–3.0 × 10^6) were seeded at a density of 5 × 10^4 per well in four 24-well plates (SPL, Korea) and cultivated for approximately 24 h until confluency. Culture medium was aspirated and replaced with PBS. A linear scratch of consistent width was created across the center of each well using a yellow pipette tip, exerting gentle pressure. Following the scratch, the wells were gently washed with culture medium to remove any detached cells or debris. Subsequently, 8 test item concentrations diluted in culture medium supplemented with BCS (1%) were added to the designated wells. A negative control consisting of DMEM supplemented with BCS

(1%) and a positive control consisting of DMEM supplemented with BCS (15%) were added in triplicate to each plate. The plates were incubated until wound closure (24h). Cell migration was evaluated at 12h and 24h using a microscope (Axiovert 25 ZEISS, Switzerland) and ImageJ software.

5 DATA COMPILATION

5.1 Qualitative evaluation

Cells were examined microscopically. Changes in general morphology, vacuolization, detachment, cell lysis and membrane integrity were recorded. Cell migration was evaluated and specific timepoints were selected for acquiring images for quantitative evaluation (0h, 12h, 24h) **(Annex I).**

5.2 Quantitative evaluation

The wound area (A) of each well has been quantified in pixels with ImageJ software for the three timepoints. Wound closure rate has been calculated for the two-time intervals (0h – 12h, 12h-24h) according to the following formula:

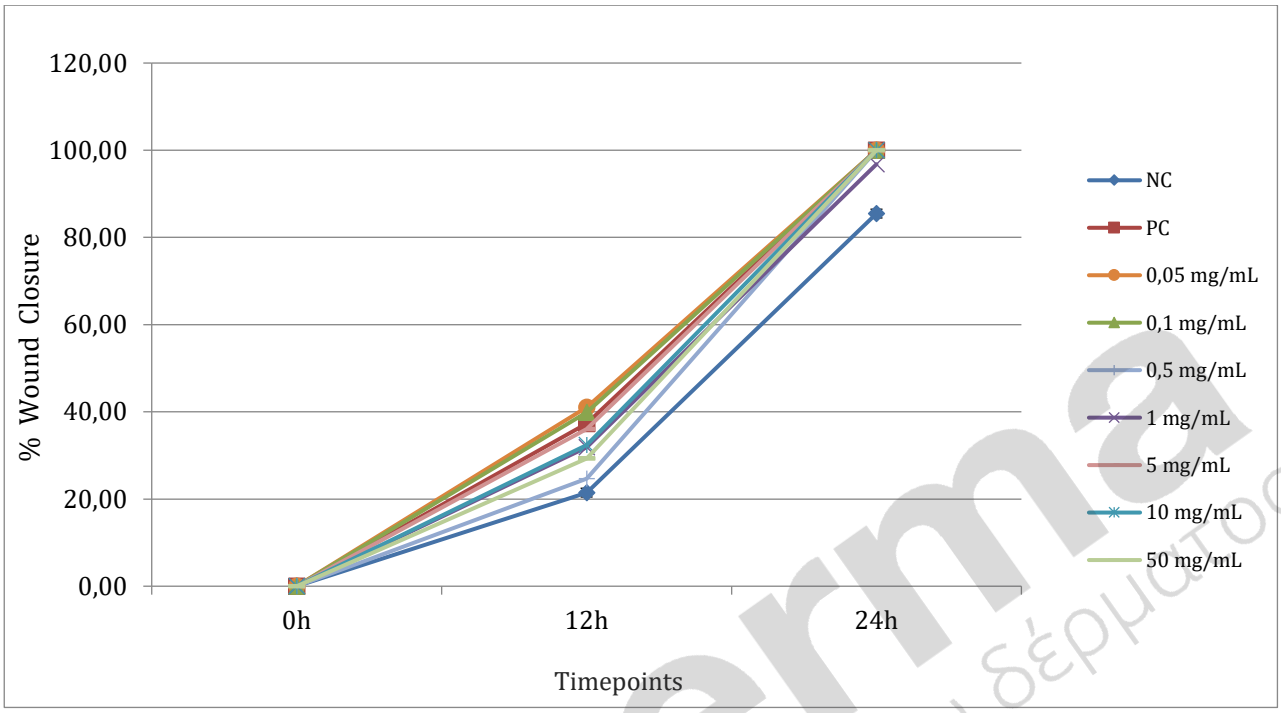
Wound Closure % = (A_{n-1} - A_n/ A_{n-1}) *100

where n: 0h, 12h, 24h

Mean and Standard Deviation have been calculated for the wound closure rates for each tested concentration, the negative and the positive control **(Annex II).**

6 RESULTS AND DISCUSSION

A.



B.

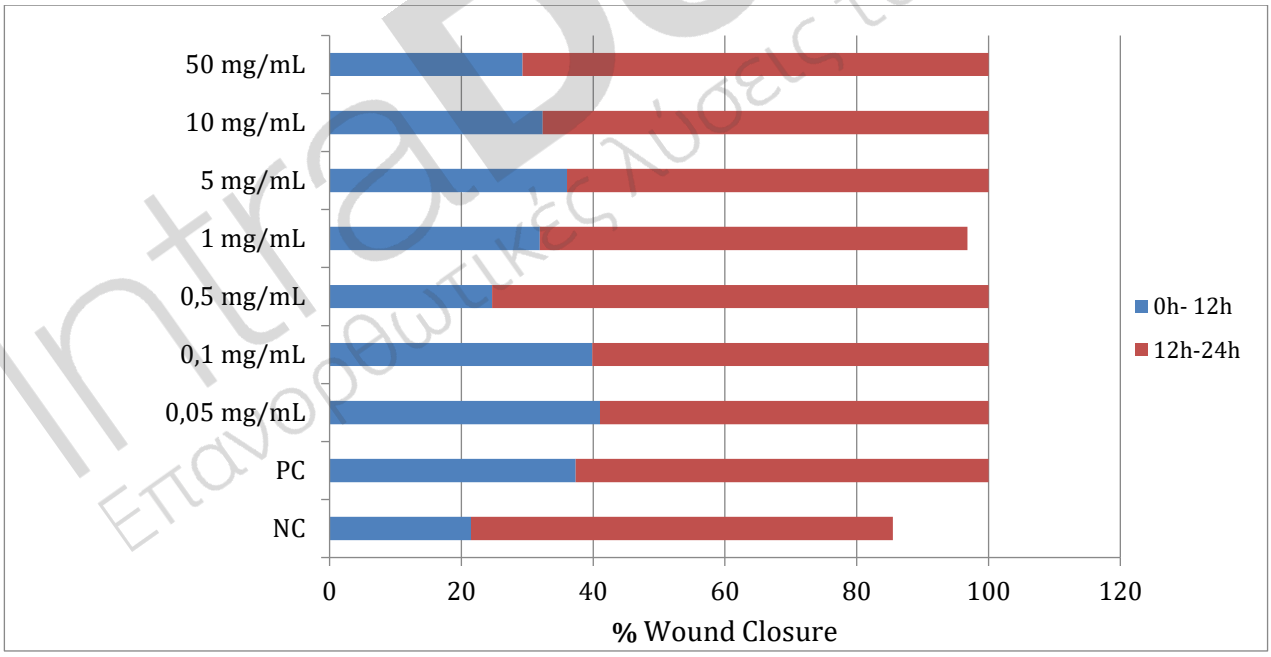


Figure 1: Wound closure rates for each test item concentration, positive (PC) and negative control (NC) for two-time intervals (0h-12h, 12h-24h). Each wound area has been measured in pixels using ImageJ software. Six wells have been assessed per concentration and controls.

Figure 1 indicates that test item significantly increased the rate of wound closure in all tested concentrations. More specifically, at the time interval (0h -12 h) most potent regarding migration rates are the concentrations 0.05 mg/ml and 0.1 mg/ml, exhibiting 41% and 40% respectively. In comparison, wound closure rates were 21% for the negative and 37% for the positive control.

Regarding the remaining concentrations, all exceed the negative control wound rate at this time interval. After 24 hours of incubation, all tested concentrations were effective in promoting wound closure, whereas negative control exhibited 85% wound closure.

Caution should be exerted regarding the concentrations (50 mg/ml) and (100 mg/ml). Due to the observed cytotoxicity, the wound healing activity of the test item at these concentrations could not be accurately assessed or appreciated. Regarding (50 mg/ml), the wound closure rate has been assessed, though alterations to morphology, vacuolization and detachment could be observed. On the contrary, the wound closure rate for the concentration (100 mg/ml) was not possible to be determined and for that reason is not presented in Fig.1.

7 CONCLUSIONS

Test item

NOVASIN (ID: 23 45 00056)

Exhibits significant wound healing activity at the concentration range **0.05 mg/ml – 10 mg/ml.**

The most pronounced effect was observed at the two lowest concentrations tested (0.05 mg/ml & 0.01 mg/ml).

Further research is necessary to determine the optimal dose *in vivo*, to ensure the overall efficacy and tolerability of **Novasin** as a wound healing agent.

8 ARCHIVING

In the archives of Kapodistriaki Dermatofarmakeutiki P.C the following documentation will be archived for 10 years:

- raw data, secondary data
- final report with any amendment

Unused test item will be kept at the testing facility for 90 days after submission of the final report, unless otherwise specified by the sponsor.

9 REPORT DISTRIBUTION

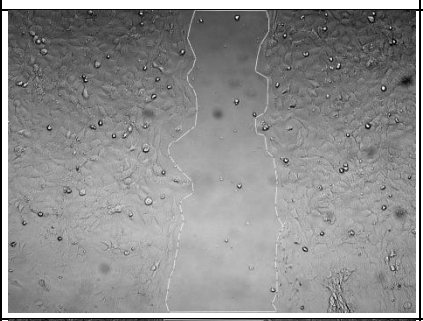
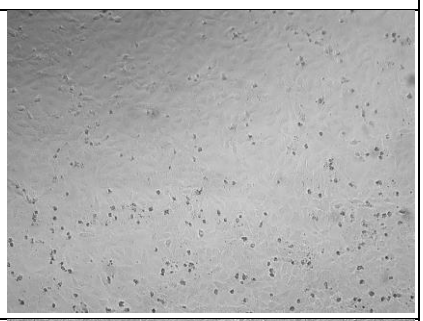
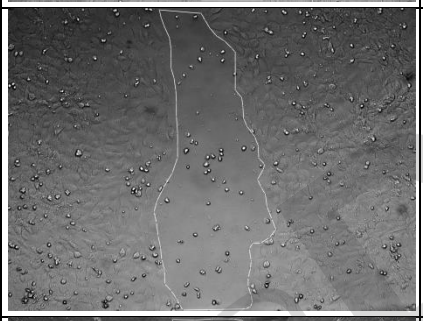
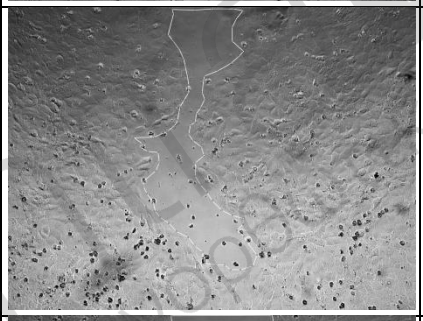
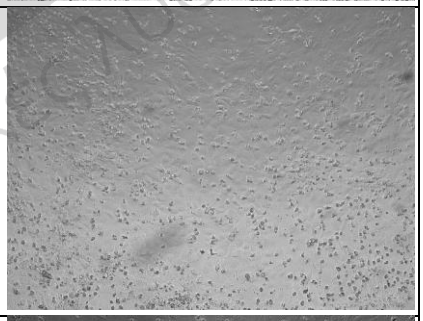
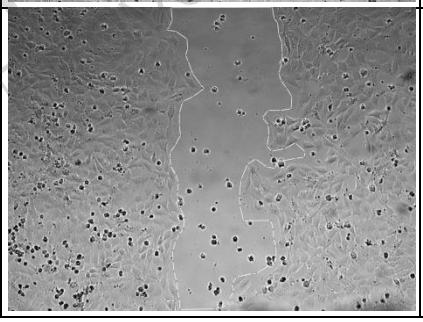
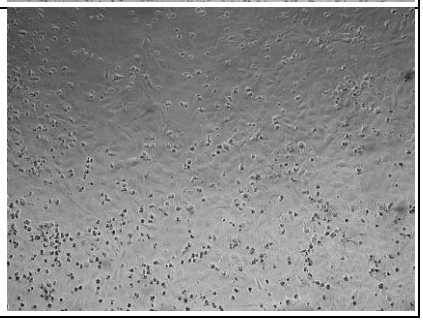
The final report (two original copies) will be distributed as follows:

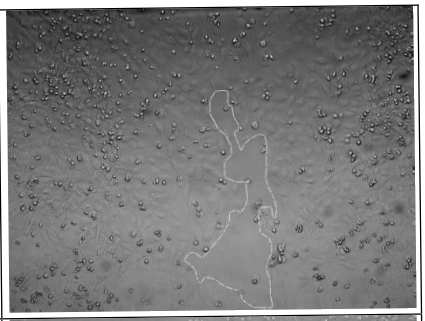
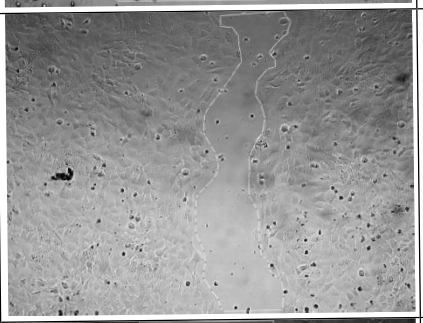
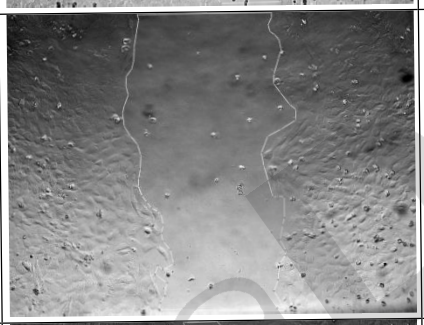
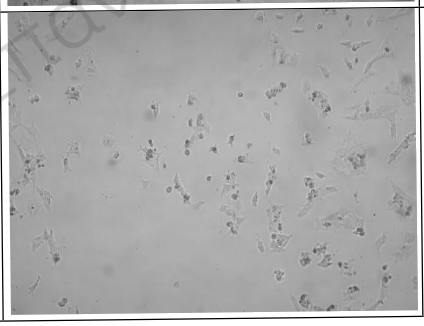
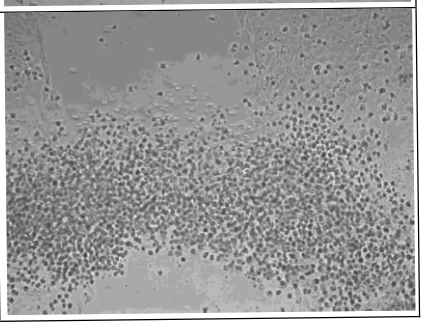
Test Facility Archive:	Copy of the signed final report
Sponsor:	One signed final report in original

10 REFERENCES

1. Liang, C. C., Park, A. Y., & Guan, J. L. (2007). In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nature Protocols, 2(2), 329-333.
2. Gaggioli, C., Hooper, S., Hidalgo-Carcedo, C., Grosse, R., Marshall, J. F., Harrington, K., & Sahai, E. (2007). Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nature Cell Biology, 9(12), 1392-1400.
3. Li, H. J., Reinhardt, F., Herschman, H. R., & Weinberg, R. A. (2012). Cancer-stimulated mesenchymal stem cells create a carcinoma stem cell niche via prostaglandin E2 signaling. Cancer Discovery, 2(9), 840-855.
4. Friedl, P., & Wolf, K. (2010). Plasticity of cell migration: a multiscale tuning model. The Journal of Cell Biology, 188(1), 11-19.
5. Abu-Ghazaleh, R., Kabir, J., Jia, H., Lobo, M., & Zachary, I. (2001). Src mediates stimulation by vascular endothelial growth factor of the phosphorylation of focal adhesion kinase at tyrosine 861, and migration and anti-apoptosis in endothelial cells. Biochemical Journal, 360(1), 255-264.

ANNEX I

	12h	24h
NEGATIVE CONTROL		
POSITIVE CONTROL		
0,05 mg/mL		
0,1 mg/mL		
0,5 mg/mL		

1 mg/mL		
5 mg/mL		
10 mg/mL		
50 mg/mL		
100 mg/mL		

ANNEX II

Wound Closure Rate (%)

	MEAN			STD		
	0h	**12h**	**24h**	**0h**	**12h**	**24h**
Negative Control	0,00	21,47	85,48	-	12,33	9,07
Positive Control	0,00	37,33	100,00	-	10,56	0,00
0,05 mg/mL	0,00	41,06	100,00	-	17,65	0,00
0,1 mg/mL	0,00	39,89	100,00	-	17,93	0,00
0,5 mg/mL	0,00	24,67	100,00	-	8,98	0,00
1 mg/mL	0,00	31,89	96,80	-	13,51	1,40
5 mg/mL	0,00	36,03	100,00	-	13,76	0,00
10 mg/mL	0,00	32,35	100,00	-	15,44	0,00
50 mg/mL	0,00	29,29	100,00	-	17,80	0,00
100 mg/mL	-	-	-	-	-	-