

IN VITRO CYTOTOXICITY TEST REPORT

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Client Name: Apron İlaç Med İth. İhr. San. ve Tic. Ltd. Şti.

Client Address: Kozyatağı Mah. Saniye Ermutlu Sok. By Otel No:3/1 Kadıköy/İstanbul

Subject: In Vitro Cytotoxicity Report for Rheutro Product

These results are valid only for the analyzed samples.

Conducted by:

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This report has been prepared in two copies, one for the client and one for the institution's archives, totaling 6 pages.

CONTENTS

1. Title
2. Product Supplier
3. Test Location
4. Guidelines
5. Product
6. Study Dates
7. Cytotoxicity Study
 - 7.1. Materials, Solutions, Equipment
 - 7.2. Experimental Procedure
8. Results

1. Title

In Vitro Cytotoxicity Study for Rheutro Product

The purpose of this report is to present the results obtained from the study, explain how the study was conducted, and describe how the data were collected.

2. Product Supplier

Apron İlaç Med İth. İhr. San. ve Tic. Ltd. Şti.
Kozyatağı Mah. Saniye Ermutlu Sok. By Otel No:3/1 Kadıköy/İstanbul

3. Test Location

Marmara University, Faculty of Pharmacy, Department of Pharmaceutical Biotechnology
Maltepe, Istanbul/Turkey

4. Guidelines

This study was conducted in accordance with the following internationally recognized guidelines:

- EN ISO 10993-5 "Tests for in vitro cytotoxicity"
- EN ISO 10993-12 "Sample preparation and reference materials"

5. Product

Product	Rheutro
Lot	-
Material	Mixture of plant extracts
Formulation	Oral suspension
Number of samples sent	2
Manufacturer/License Holder	Apron İlaç Med İth. İhr. San. ve Tic. Ltd. Şti.
Storage Conditions	Room temperature

6. Study Dates

Sample arrival date: 30.12.2024
Study start date: 06.01.2025
Study end date: 10.01.2025
Report date: 17.01.2025

7. Cytotoxicity Study

The aim of this study was to determine the in vitro cytotoxicity of the tested product on mammalian cells. Cell viability was quantitatively determined using a colorimetric method.

The test principle is based on the metabolic reduction of a soluble dye [MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)] by mitochondrial succinate dehydrogenase in metabolically active cells, converting it into insoluble formazan

crystals. This allows the determination of mitochondrial function in the cultured cells. The number of viable cells is proportional to the amount of formazan produced.

The study was conducted according to the specifications of the EN-ISO10993-5:2009 standard.

7.1. Materials, Solutions, Equipment

Materials:

- 96-well cell culture plates
- T-25 cell culture flasks
- Sterile pipettes and pipette tips
- 1.5 ml centrifuge tubes

Equipment:

- Laminar flow cabinet
- CO2 incubator
- Inverted microscope
- Incubator
- ELISA Reader

Solutions and Reagents:

- L929 cell line (mammalian fibroblast)
- Fetal bovine serum
- DMEM medium
- Sterile PBS buffer (pH: 7.4)
- DMSO (dimethyl sulfoxide)
- Absolute ethanol
- Cell Proliferation Kit I (MTT kit) (Roche)

7.2. Experimental Procedure

- L929 cells were cultured in DMEM medium containing 10% FBS at 37°C with 5% CO2 and seeded into 96-well culture plates at 5×10^3 cells/well, followed by 18-24 hours of incubation.
- The next day, the medium in the wells was removed, fresh medium was added, and the samples were applied to the wells.
- Study groups:
 - Product: final concentration 5% (v/v), 5 µl sample added to 100 µl medium

- Product: final concentration 10% (v/v), 10 µl sample added to 100 µl medium
 - Control: cells only (L929)
 - Positive control: 20% (v/v) DMSO
 - Negative control: PBS
- Each group was tested with n=6 replicates.
- After 48 hours of incubation, the medium in the wells was removed, and the wells were washed with 100 µl PBS. Then, 100 µl of fresh medium was added to each well, followed by 10 µl of MTT solution, and incubated for 4 hours to allow crystal formation.
 - 100 µl of 10% SDS was added to each well, mixed, and incubated at 37°C to dissolve the crystals.
 - Absorbance was measured at 550 nm and 690 nm using an ELISA Reader, and calculations were performed.
 - The percentage of cell viability was calculated using the following formula:
$$\% \text{ viability} = (\text{absorbance of sample} / \text{average absorbance of control group}) \times 100$$
-

8. Results

- At a concentration of 5% (v/v), cell viability was observed to be above 60% compared to the control group.
- At a concentration of 10% (v/v), cell viability was observed to be above 75% compared to the control group.
- Based on the data obtained, the product is not cytotoxic.