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CYTOTOXIC EFFECTS OF *SALVIA OFFICINALIS* EXTRACT ON FISH GILL CELLS: A PRELIMINARY STUDY USING RTGILL-W1

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Introduction

The growing demand for ethical and cost-effective ecotoxicological testing has led laboratories to adopt *in vitro* alternatives that reduce the use of live vertebrate animals. The RTgill-W1 method offers key ethical benefits over traditional *in vivo* fish toxicity tests by minimizing animal use, aligned with the 3R principles (replacement, reduction, refinement). The method uses a permanent cell line from rainbow trout gill tissue, providing strong predictive power, as the correlation between *in vitro* EC50 and *in vivo* LC50 is validated for many chemicals. Using three fluorescent markers, it evaluates contaminant mechanisms by measuring metabolic activity, membrane integrity, and lysosomal effects. RTgill-W1 is faster and less costly than tests on live fish, suitable for chemical screening and environmental monitoring. Its international validation shows consistent, transferable results even without prior expertise. However, results require careful interpretation and should be supported by *in vivo* data for full ecotoxicological risk assessment. The method is not yet universally accepted in regulations like REACH, limiting its current practical use. Scientific literature increasingly recognizes RTgill-W1 as a popular *in vitro* ecotoxicology model, especially for chemical toxicity and contaminant evaluation. In recent years, publications in fields such as environment, agriculture, pharmacology, biochemistry, immunology, microbiology, and chemistry have grown. About 24% of environmental studies use RTgill-W1 to study pollutant biotransformation, bioaccumulation, oxidative stress, xenobiotic metabolism, gene expression and toxicity of emerging pollutants (PFAS, microplastics, biocides). This study focuses on the application of toxicological testing method using fish cell lines, specifically the RTgill-W1 line derived from the gill epithelium of rainbow trout (*Oncorhynchus mykiss*), in accordance with OECD Test Guideline 249:2021.

Materials and methods

Fresh sage leaves *Salvia officinalis* were pretreated with cellulase and pectinase (1:1) in acetate buffer (pH 4) at 40 °C for 60 min, then subjected to hydrodistillation. Afterwards, the essential oil was collected.

The cytotoxicity methodology involved exposing RTgill-W1 cells cultured in 24-well plates to *Salvia officinalis* oil extract for 24 hours, followed by assessment of cell viability using fluorescent markers (Alamar Blue, CFDA-AM, Neutral-red) that indicated membrane integrity and metabolic activity. The toxic effect was quantified

by calculating the EC50 value, which represented the concentration causing a 50% reduction in cell viability. The laboratory conditions were described in the OECD 249 standard. Cell viability was calculated as a percentage based on fluorescence units in exposed wells relative to control wells: Cell viability (%) = (fluorescence in exposed wells / fluorescence in control) × 100%. Acceptability criteria for validation included variability in fluorescence below 20% in cell-free control wells, less than 10% difference in fluorescence between negative and solvent controls, and the positive control with 3,4-Dichloroaniline had to fall within reference ranges specific to each fluorescent marker.

Results and conclusions

RTgill-W1 cells were exposed to 9 concentrations of oil extract in 0.25 to 200 mg/L domain. After intoxication, preliminary results estimate toxicity levels >100 mg/L plant extract (depending on fluorescent markers: Alamar Blue, CFDA-AM, Neutral-red), indicating non-toxic effect according to REACH classification. Cell viability decreased as the test concentration increased (Figure 1). *Salvia* extract showed no relevant toxicity on metabolic activity and cell membrane integrity up to 50 mg/L. At 100 and 200 mg/L the cytotoxic effects are registered 28-45 to 90%.

Fluorescent markers	<i>Salvia officinalis</i> oil extract	Reference test 3,4-Dichloroaniline
	EC50-24h (mg/L)	
Alamar Blue	148.86 ± 26.78	78.80 ± 11.96
CFDA-AM	139.22 ± 8.46	93.64 ± 3.60
Neutral-red	135.30 ± 2.32	71.95 ± 0.95

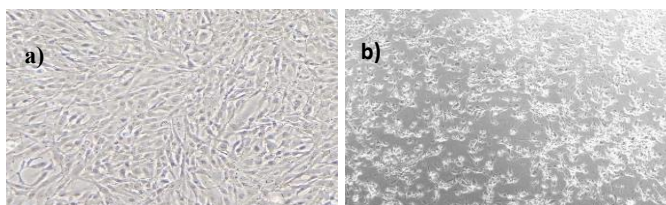


Figure 1. Aspect of RTgill-W1 at 24h for exposure to *Salvia Officinalis* oil extract, a) control, b) 200 mg/L, 10x (Olympus System Microscope)

This approach offers a promising alternative to reduce animal testing in ecotoxicology and also to evaluate the toxicity of plant extracts on aquatic organisms, ensuring environmental safety.

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