Software/web server article

SAEDC: Development of a technological solution for exploratory data analysis and statistics in cytotoxicity

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ABSTRACT

Introduction: The intergovernmental organizations Organisation for Economic Co-operation and Development (OECD) and Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) have developed guidelines for the use of *in vitro* models for toxicological evaluation of chemicals. However, the presence of manual steps and the requirement of multiple tools for data analysis, apart from being costly and time-consuming, can inadvertently introduce errors by researchers. Objectives: We have developed the SAEDC platform (Technological Solution for Exploratory Data Analysis and Statistics for Cytotoxicity, in Portuguese), which enables analysis of cytotoxicity data from assays following OECD Guideline No. 129. Methodology: *In vitro* experimental data were used to compare with the analysis methodology suggested in the Guideline. We analyzed 117 data sets covering chemicals from Category I to Unclassified according to GHS classification. Results: The four-parameters of non-linear regression (4PL) calculated by the SAEDC platform showed no significant differences compared to standard methodology in any of the data sets ($p > 0.05$). The coefficient of determination (R-squared) also demonstrated not only a good fit of the 4PL model to the data but also significant similarity to values obtained by the conventional methodology. Finally, the SAEDC platform predicted LD_{50} values for the chemicals from IC_{50}, using the Registry of Cytotoxicity (RC) regression models. Conclusion: The comparison with the standard data analysis methodology revealed that SAEDC platform fulfills the requirements for cytotoxicity data analysis, generating reliable and accurate results with fewer steps performed by researchers. The use of SAEDC platform for obtaining toxicity values can reduce analysis time compared to the standard methodology proposed by regulatory agencies. Thus, automation of the analysis using the SAEDC platform has the potential to save time and resources for cytotoxicity researchers and laboratories while generating reliable results.

1. Introduction

Centuries ago, the Renaissance physician Paracelsus (1493–1541) stated: "What is there that is not poison? All things are poisonous, and nothing is without poison. Only the dose determines that something is not a poison." Paracelsus was one of the pioneers of toxicology and may be considered the father of this branch of science [1,2]. At the heart of this field lies the property of "toxicity." Although there is still no consensus regarding its definition to this day, it could be thought of as being "the degree to which a substance (a toxin or poison) can harm a living organism" [3].

More than five centuries have passed since the pioneering studies of Paracelsus, but interest in this area has not receded. On the contrary, it

can be said that this is one of the most important topics in drug development [1,4–6]. Several models have been and are being developed to measure the harmfulness of a chemical to the human body. So important is this concept that the drug approval process for entry into the market takes approximately 12 years and costs billions of dollars [7].

There are currently three main categories of toxicity models in preclinical research to minimize the risks associated with adverse effects: *in silico, in vitro,* and *in vivo* [3]. However, with the rapid development and technical advancement in the pharmaceutical industry, performing more effective drug screening and toxicity testing has become more urgent. Furthermore, although animal experiments are still required for this process, *in vitro* assays are becoming increasingly important [8].

In the face of this great advancement and development of

increasingly assertive methods [9–11], *in vitro* approaches have been adopted, initially in the field of genotoxicity and later in the evaluation of topical toxicity, by regulatory toxicology agencies [12,13]. The current trends are for broader swathes of toxicology to become subject to these methods [14].

The Organization for Economic Cooperation and Development (OECD) thus published guidance documents for using *in vitro* models to predict chemical toxicity [15]. With these guidance documents, other laboratories were able to develop their own versions of assays [9, 16–19]. However, the analysis methodology remains the same [15]:

"Perform a Hill function analysis of the replicate cell viability data for each concentration using statistical software (e.g., GraphPad PRISM® http://www.graphpad.com/prism/Prism.htm) to calculate the IC$_{50}$ for each test substance. The Hill function is recommended because all the dose-response information, rather than a few points around the IC$_{50}$, is used. The Hill function also provides the slope of the dose-response […]."

Even though it is recommended, and indeed common practice, to use non-linear models for biological data, the data analysis methodology recommended by the OECD in its guidance document requires many manual steps to be performed. Another one of its disadvantages is the need for multiple platforms (i.e., software programs) to evaluate cytotoxicity assays [15]. In addition to being expensive and time-consuming, this process could lead to accidental user errors, increasing the probability that a drug will be misclassified as either safe or unsafe during the initial stages of preclinical phases [3].

In response to these challenges, we developed the SAEDC web application ("Technological Solution for Exploratory Data Analysis and Statistics in Cytotoxicity" in Portuguese). This user-friendly tool uses a single platform capable of analyzing cytotoxicity data obtained from the *OECD Guidance Document Number 129* [15]. Our main goals included not just generating reliable data but also obtaining a reduction in the number of user steps required, giving faster results, and reducing the probability of making accidental errors during data manipulation.

2. Methodology

2.1. Obtaining experimental data

Previous data developed by our research group [20] were utilized to assess the potential of the SAEDC platform. The assays adhered to the recommendations of ICCVAM [13] and OECD [15], employing the neutral red uptake methodology. Twelve reference substances with well-known toxic effects were selected, as described in the classification of the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Raw data has been deposited in the Institutional Repository of the Oswaldo Cruz Foundation (Fiocruz) – ARCA and is accessible for retrieval and download through the following link: doi: https://doi.org/10.35078/KS5IGG.

Additionally, validation data were obtained from the study titled *In Vitro* Cytotoxicity Test Methods for Estimating Acute Oral Systemic Toxicity [21], conducted by The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), through the contribution of Dr. Nicole Kleinstreuer, Director of NICEATM and Executive Director of ICCVAM and Dr. David Allen, Principal Investigator at Inotiv, providing support to NICEATM, to whom requests of the raw data can be made by email (niceatm@niehs.nih.gov). For the analysis of method equivalence, 200 randomly selected datasets were utilized, comprising 65 chemicals with known toxic effects.

2.2. Data analysis

The SAEDC platform was developed following the ICCVAM [13] and OECD [15] guidance documents. Two data analysis methods were used to validate the platform: the first followed the recommendations of the

guidance documents, and the second used the SAEDC platform. The values obtained from each of these methods were then compared to each other. The ICCVAM [13] and OECD [15] guidance documents suggest using Microsoft Office Excel to determine cell viability and convert it to a control percentage. The data should then be transferred to the GraphPad Prism® statistical software program in order to calculate the IC$_{50}$ value, where a four-parameter sigmoidal dose-response curve (bottom, top, HillSlope, and EC$_{50}$) is applied. When the Bottom parameter is not equal to 0, the EC$_{50}$ reported by the Hill function differs from the IC$_{50}$, as the Hill function defines EC$_{50}$ as the concentration-value that corresponds to the response which is midway between Top and Bottom. Therefore, the calculation of the Hill function in the Prism® software needs to be rearranged to obtain the concentration corresponding to the IC$_{50}$. For clarity, this method, which follows the ICCVAM/OECD recommendations [13,15], will be referred to as "Manual analysis.".

The SAEDC platform is independent of external software. The raw data obtained from the Synergy H1 Multi-Mode reader was directly imported into the application, which performed the procedures to convert cell viability relative to the positive control (100 %). A four-parameter non-linear regression model (4PL) was used to analyze the dose-response curve and the bottom, top, HillSlope, and EC$_{50}$ parameters (Equation 1).

Equation 1 - Non-linear regression model.

$$f(x) = bottom + \frac{top - bottom}{1 + \left(\frac{EC50}{x}\right)^{HillSlope}}$$

From the EC$_{50}$ parameter of the 4PL model, the rearranged Hill function was applied to obtain the IC$_{50}$ concentration (Equation 2).

Equation 2 – Rearranged Hill function for IC$_{50}$ correction.

$$\log IC50 = \log EC50 - \frac{\log\left(\frac{top-bottom}{Y-bottom} - 1\right)}{HillSlope}$$

where $Y = 50$.

2.3. Goodness of Fit

The model fit was calculated using the R-squared coefficient of determination (Equation 3), which assesses the adequacy of the dose-response curve to the analyzed data, considering the difference between the observed and expected data.

Equation 3 – Coefficient of Determination (R-squared)

$$R^2 = 1 - \frac{\sum (y_i - \widehat{y}_i)^2}{\sum (y_i - \overline{y})^2}$$

2.4. Prediction of LD$_{50}$

IC$_{50}$ values can be used to predict the LD$_{50}$ and the GHS category of a given chemical. To achieve this, the Registry of Cytotoxicity (RC) rat-only weight (Equation 4) and millimole regressions (Equation 5) were applied using IC$_{50}$ values to estimate LD$_{50}$ values [13]. The RC rat-only weight regression equation is typically used when molarity information is unknown, and test substances are combined with other mixtures, whereas the RC rat-only millimole regression equation is typically used for pure substances [13]. Both equations are valid for LD$_{50}$ prediction.

Equation 4 - Registry of Cytotoxicity rat-only weight regression equation.

$$LD50\left(\frac{mg}{kg}\right) = 10^{0,372 \times \log IC50\left(\frac{ug}{mL}\right) + 2,024}$$

Equation 5 - Registry of Cytotoxicity rat-only millimole regression equation.

$$LD50\left(\frac{mmol}{kg}\right) = 10^{0,439\times\log IC50(mM)+0,621}$$

scatter plot and a regression line, accompanied by a regression equation where the intercept represents a constant, and the slope indicates a proportional measurement error [22].

2.5. Comparison between analytics platforms

The bottom, top, HillSlope, and EC_{50} parameters obtained from the two analysis models (SAEDC vs. Manual analysis) were compared using a paired t-test (two-tailed, alpha=0.05). The goodness of fit of the 4PL model to the data was determined using the correlation coefficient (r), and the comparison between the parameters was presented as the mean of differences (SAEDC – Manual analysis) ± standard deviation of differences. For the assessment of equivalence between the analysis methodologies, the Passing-Bablok test was applied to the IC_{50} values derived from the NICEATM study. The results are presented through a

3. Results

The results from the assessment of drugs belonging to different GHS toxicity categories were compared using SAEDC and Manual analysis methodologies. For category I, II, III, IV, and V chemicals, the experiments were effectively paired (r > 0.9075; p ≤ 0.0004), and no significant differences were found (p > 0.05). Additionally, unclassified chemicals were evaluated, and similar findings were observed, with no significant differences between experiments involving glycerol and ethylene glycol (p > 0.05). Figs. 1–3 show the results.
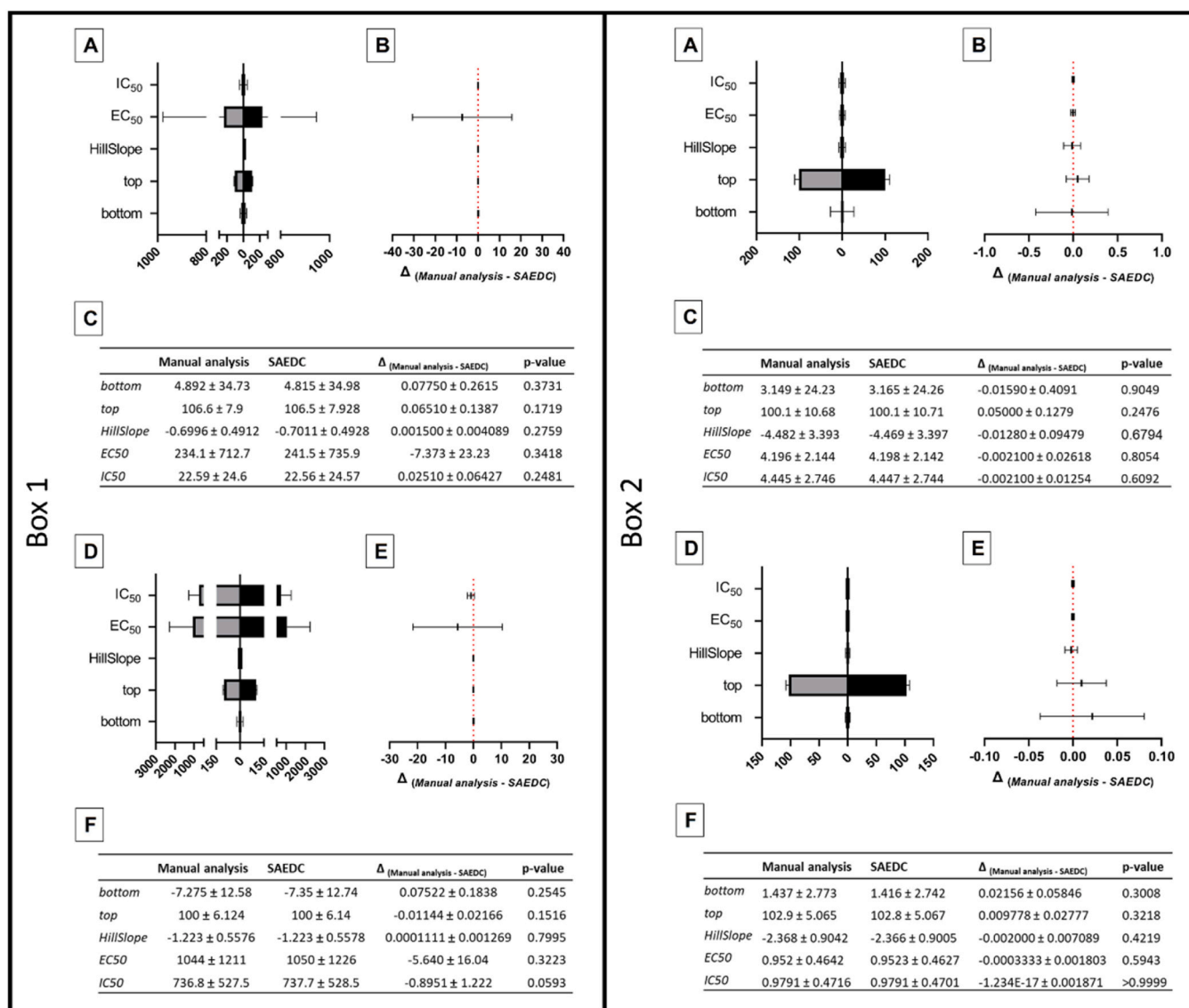

Fig. 1. SAEDC and Manual Analysis Methodologies show Similarity in Calculating Non-Linear Regression Model Parameters for Categories 1 and 2 GHS. No statistically significant (paired t-test) difference was observed between the proposed methodology (SAEDC) and the conventional methodology (Manual analysis) for the calculation of non-linear regression model parameters (bottom, top, HillSlope, EC50), and IC50 values for in vitro cytotoxicity data of GHS Category 1 (Box 1) chemicals Cycloheximide (A,B,C) and Indomethacin (D,E,F) and GHS Category 2 (Box 2) chemicals Sodium arsenite (A,B,C) and Sodium dichromate dihydrate (D,E,F). The gray bars represent the values calculated by the Manual analysis methodology (A,D). The black bars represent the values calculated by the SAEDC methodology (A,D). Average difference between predicted values by the SAEDC and Manual analysis methodologies (B,E). Tables (C,F) present the mean ± standard deviation values. The values on the bar chart (A,D) are represented in absolute value (modulus) for visualization purposes. The bottom and top values are expressed in % relative to the control. The HillSlope value has no unit of measurement. The EC50 and IC50 values are expressed in μg/mL. **The values on the bar chart are represented in absolute value (modulus) for visualization purposes.**
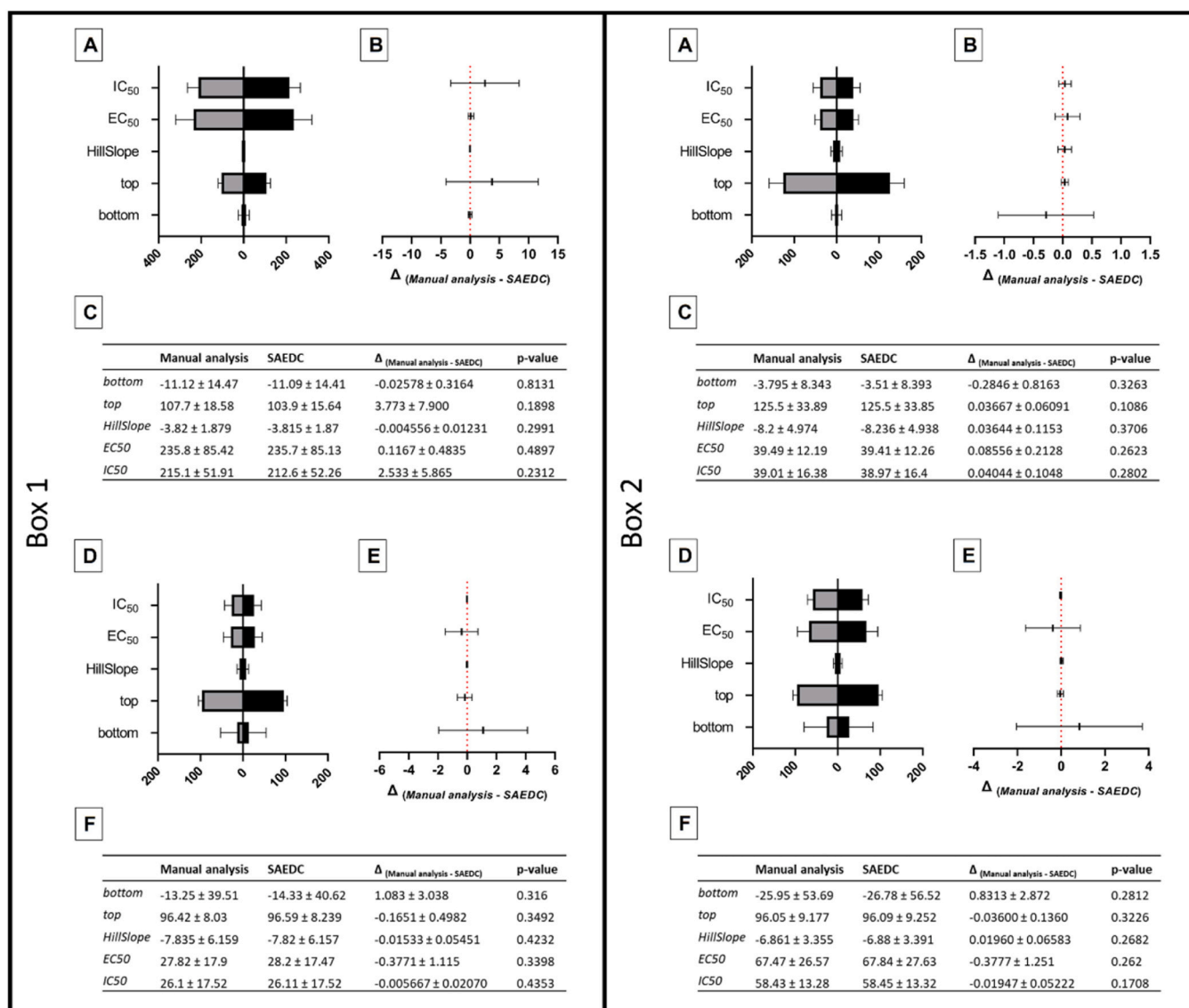
Fig. 2. - SAEDC and Manual Analysis Methodologies show Similarity in Calculating Non-Linear Regression Model Parameters for Categories 3 and 4 GHS. No statistically significant (paired t-test) difference was observed between the proposed methodology (SAEDC) and the conventional methodology (Manual analysis) for the calculation of non-linear regression model parameters (bottom, top, HillSlope, EC50), and IC50 values for in vitro cytotoxicity data of GHS Category 3 (Box 1) chemicals Sodium oxalate (A,B,C) and Hexachlorophene (D,E,F) and GHS Category 4 (Box 2) chemicals Propranolol HCl (A,B,C) and Sodium dodecyl sulfate (D,E,F). The gray bars represent the values calculated by the Manual analysis methodology (A,D). The black bars represent the values calculated by the SAEDC methodology (A,D). Average difference between predicted values by the SAEDC and Manual analysis methodologies (B,E). Tables (C,F) present the mean ± standard deviation values. The values on the bar chart (A,D) are represented in absolute value (modulus) for visualization purposes. The bottom and top values are expressed in % relative to the control. The HillSlope value has no unit of measurement. The EC50 and IC50 values are expressed in μg/mL. **The values on the bar chart are represented in absolute value (modulus) for visualization purposes.**

3.1. Methodological Concordance Assessment

The results of the Passing-Bablok statistical test revealed a significant agreement between the evaluated analysis methodologies. From the sample set, four assays were excluded from the analysis due to GraphPad Prism's inability to determine the IC$_{50}$ value through the 4PL regression. The scatter plot (Fig. 4A) and the regression line (Fig. 4B) exhibit a consistent trend, indicating a linear relationship between the measurements obtained by the methods under investigation. The regression equation, with the intercept representing the constant and the slope indicating proportional measurement error, substantiates the stability of this relationship. Furthermore, the analysis of 95 % confidence interval estimates for the intercept (–0.0009884 to 0.008212) and slope (0.9998 to 1.0008) demonstrated that their values do not significantly differ from zero and one, respectively. This finding reinforces the robustness of

the agreement between the methodologies, enhancing confidence in the application and comparability of the obtained results.

3.2. Prediction of LD$_{50}$

Finally, the IC$_{50}$ values from each experiment were used to predict LD$_{50}$ values using the Registry of Cytotoxicity (RC) regressions for rat-only weight (Equation 4) and millimolar (Equation 5) (Fig. 5). The values presented in Table 1 represent the mean of experiments, providing a comprehensive overview of the results obtained from the study.

4. Discussion

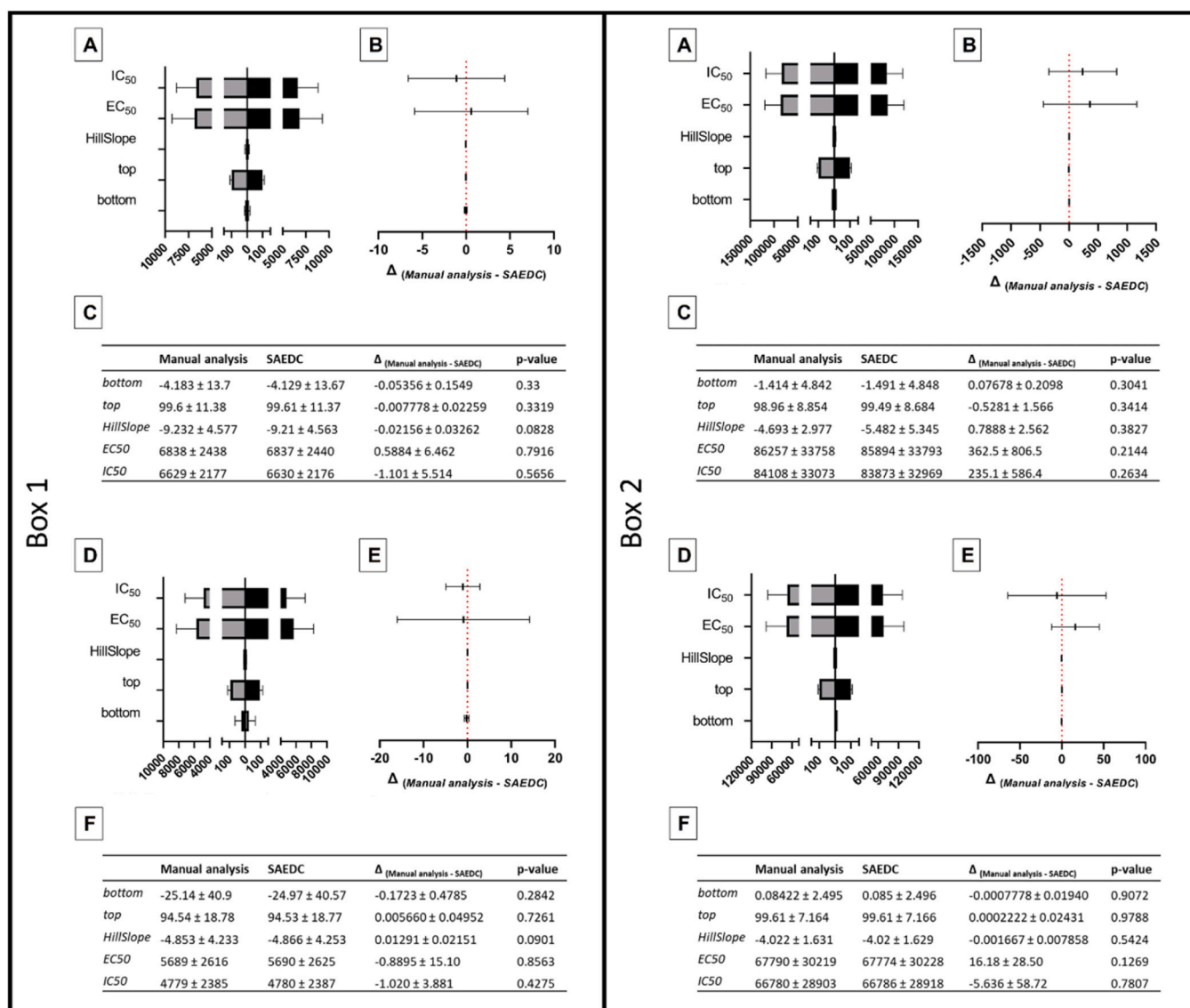The development of the SAEDC platform was guided by the

Fig. 3. - SAEDC and Manual Analysis Methodologies show Similarity in Calculating Non-Linear Regression Model Parameters for Categories 5 and Unclassified GHS. No statistically significant (paired t-test) difference was observed between the proposed methodology (SAEDC) and the conventional methodology (Manual analysis) for the calculation of non-linear regression model parameters (bottom, top, HillSlope, EC50), and IC50 values for in vitro cytotoxicity data of GHS Category 5 (Box 1) chemicals Potassium chloride (A,B,C) and Trichloroacetic acid (D,E,F) and GHS Unclassified (Box 2) chemicals Glycerol (A,B,C) and Ethylene glycol (D,E,F). The gray bars represent the values calculated by the Manual analysis methodology (A,D). The black bars represent the values calculated by the SAEDC methodology (A,D). Average difference between predicted values by the SAEDC and Manual analysis methodologies (B,E). Tables (C,F) present the mean ± standard deviation values. The values on the bar chart (A,D) are represented in absolute value (modulus) for visualization purposes. The bottom and top values are expressed in % relative to the control. The HillSlope value has no unit of measurement. The EC50 and IC50 values are expressed in µg/mL. **The values on the bar chart are represented in absolute value (modulus) for visualization purposes.**

recommendations of the ICCVAM [13] and OECD [15] for the elaboration of *in vitro* toxicity tests. Comparison with the standard data analysis methods showed that the developed platform meets expectations, generating reliable and accurate results in fewer steps.

The four-parameter logistic equation, also known as the Hill equation (Equation 1), is commonly used to describe the non-linear relationship frequently observed between dose and response. The equation estimates four parameters (*bottom, top, EC_{50}/IC_{50}, and HillSlope*) and has been employed in many areas, such as quantitative pharmacology [23]. The four parameters are usually enough to characterize the dose-response shapes [24], giving us a useful framework for describing the interaction between a test substance and a living organism and allowing for the estimation of the effective dose required to elicit a given response [23,25].

Defining the initial points is a crucial step for performing non-linear

regressions, as these values are used as a starting point for parameter optimization and provide an approximate model estimation. The GraphPad PRISM® and SAEDC platforms adopt different rules for defining the initial points. Both platforms define the bottom and top as the minimum and maximum values of the data, respectively, for the initial parameters. However, they define the EC_{50}/IC_{50} and *HillSlope* parameters differently. The GraphPad PRISM® platform defines EC_{50}/IC_{50} as the value of X (concentration) that corresponds to the response which is midpoint of Y and *HillSlope* equal to –1, while the SAEDC platform defines EC_{50}/IC_{50} as the average of the concentrations closest to the midpoint between *bottom* and *top,* and *HillSlope* equal to 1.5 since the value is expected to vary between 0.5 and 2.5 [26].

The bottom and top parameter constraints can assist in analyzing data with more than one possible fit and enable comparing different dose-response curves [27,28]. Although not assessed in this study, the
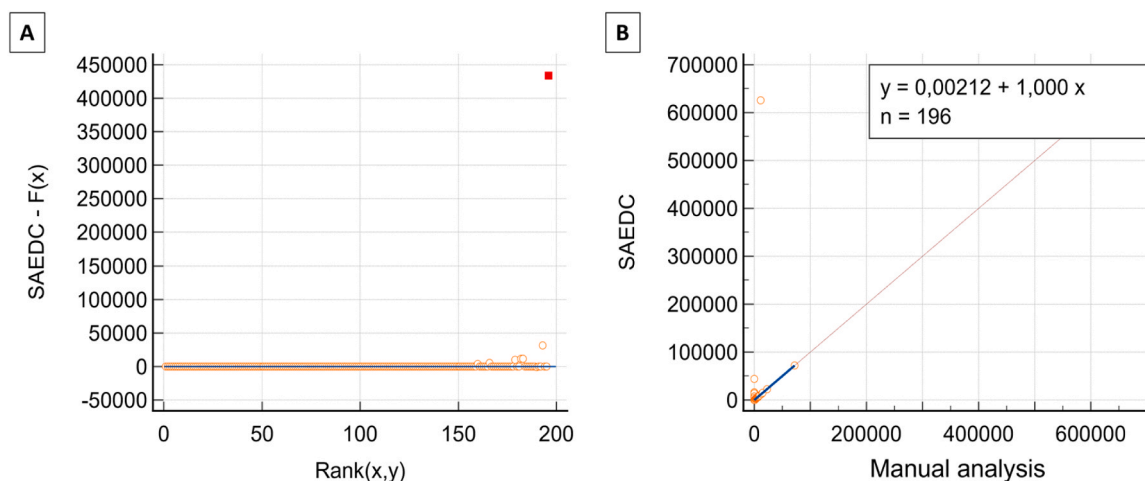
Fig. 4. - Methodological Concordance Assessment: Passing-Bablok Test Results and Evaluation of IC_{50} Determination. Statistically significant (*Passing-Bablok test*) similarity was observed between the proposed methodology (SAEDC) and the conventional methodology (Manual analysis) for the calculation of IC_{50}. The scatter plot (A) and the corresponding regression line (B) demonstrate a coherent pattern, suggesting a linear association between the measurements acquired through the investigated methods.

SAEDC platform has a parameter constraint option. By adjusting these parameters, one can improve the quality of the model's approximation to real data and interpret these parameters as having some physical meaning [27,29]. This is particularly important for experiments that follow the standard recommended by the OECD, which requires comparing the dose-response curve to a control group that represents the maximum efficacy of the studied drug [15].

The Hill model seeks to identify the best parameter values of a model by minimizing the sum of the squares of the distances of the data points. Thus, initial values for the unknown parameters are necessary for model optimization, which can influence the quality of the response obtained [30]. It was observed that some parameters, classified as ambiguous, obtained with the SAEDC platform presented a slight but not significant variation from expected values. However, numerical differences were observed between the two methods only when applying 4PL regression on the same dataset, and the values prior to this step do not vary or, when they do vary, they can be attributed to the rounding of decimal places. Therefore, it is important to note that repeated analyses of the same data may not always provide the same results, especially in cases involving ambiguity or incomplete curves [28].

Although the 4PL model is indicated for cytotoxicity studies and the evaluation of dose-response curves, when the bottom and top parameters differ from 0 and 100, respectively, the EC_{50} reported by the Hill function is not the same as the 50 % effect since the Hill function defines EC_{50} as the concentration that corresponds to the response which is midpoint between top and bottom [29,31]. Therefore, the EC_{50}/IC_{50} value returned by the model must be rearranged to calculate IC_{50} concentration. Unlike commercial platforms suggested by ICCVAM [13] and OECD [15], the SAEDC platform can correct the EC_{50}/IC_{50} value of the adjusted model, allowing it to match the LD_{50} value of the studied chemical.

The SAEDC platform not only reduces the effort required by researchers when performing cytotoxicity data analysis but also aligns with the principles of the 3 R's philosophy [32], specifically "refinement." The LD_{50} value is remarkably useful when performing subsequent *in vivo* studies with the investigated chemical, as the recommended initial dose to be administered in living organisms is 3.2-fold lower than the calculated LD_{50} value of each chemical. By providing a predicted LD_{50} value, the SAEDC platform can assist in *in vivo* research starting from a known initial point rather than the average concentration of 300 mg/kg, as recommended by the Acute Toxic Class (ATC) method [33]. Abud et al. have shown that estimating the initial dose using knowledge of LD_{50} can reduce the number of animals sacrificed in *in vivo*

toxicity assays [9].

Although this is not the first alternative to widely used commercial software in the field of cytotoxicity [26,34], our platform was not developed to replace other solutions. Instead, it aims to assist researchers in the later stages of *in vitro* data collection and to speed up the generation of EC_{50}/IC_{50} and LD_{50} values while maintaining confidence in the accuracy of the data. A preliminary analysis performed in our laboratory, using in-house data, comparing the methods described here (SAEDC vs. manual analysis) for obtaining the EC_{50}/IC_{50} and LD_{50} values showed a ten- to thirty-fold reduction in analysis time when using the SAEDC platform (data not shown). However, a limitation of our study is that our platform does not provide other statistical analyses. Therefore, we do not encourage the replacement of other platforms with this one, but rather, their integration.

5. Conclusion

The non-linear model is widely used and recommended for analyzing new drugs' dose-response curves. However, the data analysis method recommended by the Organization for Economic Cooperation and Development (OECD) [15] requires several manual steps to be undertaken in its guidance document. The SAEDC platform was developed to simplify data collection for studying chemical toxicity, enabling automated data analysis and reducing the time and number of steps required. This study demonstrated the developed platform's functionality, which can generate reliable data in a short time frame.

The SAEDC platform presents a practical and efficient solution for toxicity data analysis, which can significantly aid studies involving new drugs. By using the SAEDC platform, researchers can save time and resources, as well as obtain accurate and reliable results. Automating the data analysis process can also contribute to a better understanding of chemical toxicity, which is crucial for the safe and effective development of new drugs. The tool is still in the development phase, and its test version can be accessed at the following address: http://login.saedc.online/.

Compliance with ethics requirements

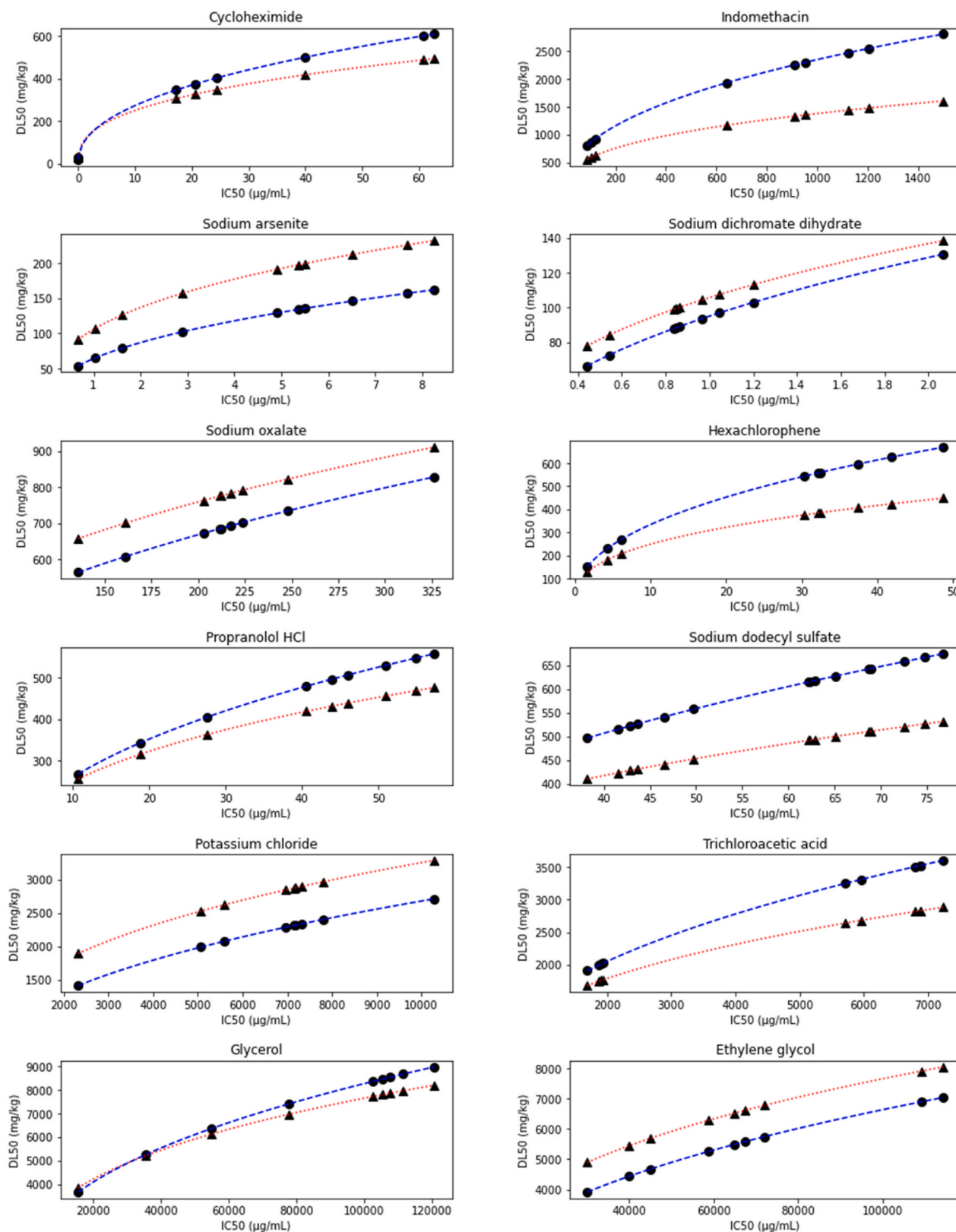This article does not contain any studies with human or animal subjects.

Fig. 5. - Prediction of LD$_{50}$ (rat-only weight) and LD$_{50}$ (rat-only millimole) from IC50, for Class I to VI toxicity chemicals. The SAEDC methodology demonstrated the ability to predict the LD$_{50}$ value based on the IC50 parameter, following the regression curve estimated by the Registry of Cytotoxicity (RC) rat-only weight and millimole. **Row 1 -** Chemicals belonging to toxicity Class I. **Row 2 -** Chemicals belonging to toxicity Class II. **Row 3 -** Chemicals belonging to toxicity Class III. **Row 4 -** Chemicals belonging to toxicity Class IV. **Row 5 -** Chemicals belonging to toxicity Class V. **Row 6 -** Unclassified chemicals. (▲) Predicted LD$_{50}$ values by RC rat-only weight regression. (—) Predictive curve of the RC rat-only weight regression model. (●) Predicted LD$_{50}$ values by RC rat-only millimole regression. (.) Predictive curve of the RC rat-only millimole regression model.

Table 1

Estimation of LD50 from IC50 value in in vitro assays.

Test substances	IC50 (µg/mL)	Registry of Cytotoxicity (RC) regression	
		LD50 (rat-only weight) (mg/kg)	LD50 (rat-only millimole) (mg/kg)
Cycloheximide	22,562	249,455	291,702
Indomethacin	737,686	1.129,129	1.877,757
Sodium arsenite	4447	173,918	116,343
Sodium dichromate dihydrate	0979	102,654	92,021
Sodium oxalate	215,481	774,982	685,155
Hexachlorophene	26,108	326,781	467,237
Propranolol HCl	38,965	402,647	459,492
Sodium dodecyl sulfate	58,447	477,129	594,177
Potassium chloride	6.630,185	2.751,081	2.201,635
Trichloroacetic acid	4.689,602	2.362,264	2.865,651
Glycerol	81.255,632	6.857,847	7.305,248
Ethylene glycol	66.785,856	6.461,280	5.444,263

Legend: Values are presented as mean.

CRediT authorship contribution statement

Bernardo Zoehler: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing – Original Draft, Writing – Review & Editing, Visualization. **Alessandra Melo de Aguiar:** Conceptualization, Writing – Review & Editing, Supervision. **Guilherme Ferreira Silveira:** Conceptualization, Methodology, Software, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors have declared no conflict of interest.

Acknowledgments

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