

Research Article

Development and validation of *Triticum* phytobiological method as an alternative procedure for investigating in vivo acute toxicity on mice

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Abstract

The goal of this study was to validate an alternative method for determining in vivo acute toxicity using vegetal material instead of laboratory animals, starting from the phytobiological method known also as the *Triticum* technique. We set out to demonstrate that vegetal cells have similar sensitivity to some toxic agents as animal cells, in which case a statistical correlation could be established. A series of new compounds synthesized by the Romanian National Institute for Chemical Pharmaceutical Research and Development as potential β_3 adrenergic receptors agonists were tested for their acute toxicity using classic animal exposure models, before investigating possible anti-diabetic and anti-obesity effects. We then determined whether similar conclusions might be reached exposing vegetal material to the same agents. We successfully demonstrated that plants are affected in a very similar way as animals when exposed to some potentially toxic agents, providing new possibilities for ending unethical animal experiments.

Keywords: bioethics, *triticum* phytobiological method, alternative method, acute toxicity, mice



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Introduction

Toxicity studies are an essential part of any research meant to develop new drugs. At present no study on toxicity can be undertaken without animal testing, and in order to achieve statistical significance, it may be necessary to use a relatively high number of individuals. Bioethical considerations, however, emphasize the need to use as few animals as possible in any given test. Thus emerges a conflict between the need for statistics validation and the bioethics constraints of any experimental study. Solving this ongoing dilemma will require new methods for determining toxicity without the use of lab animals. A series of *in silico*/ *in vitro*/ *in vivo* correlations have already been established regarding toxicity tests by our research team (1, 2).

Generally, the humane use of animals in research is governed by three principles: replace, reduce, refine. Replacement refers to the use of non-sentient animals or materials instead of conscious live animals. Reduction involves decreasing the number of animals used in a specific procedure or experiment. Refinement implies the use of advanced techniques, able to decrease the magnitude and incidence of animal pain and distress. These three principles were established in 1959 based on the writings of Russell and Burch, with the intention of advancing a more ethical and humane perspective regarding the use of animals in experimental studies (3).

We developed the Triticum phytobiological method as an alternative method to determine *in vivo* acute toxicity by introducing a new and original quantitative parameter: the inhibitory concentration 50% (IC_{50}) calculated by the graphic method of the regression curves. This *in vitro* parameter was intended as an alternative to the *in vivo* parameter – lethal dose 50% (LD_{50}) (4).

Materials and methods

The Triticum phytobiological method consists of exposing wheat seeds to 6 molar dilutions of a water soluble compound and measuring radicular elongation of the germinating seeds for five consecutive days. Microscopic cellular changes are also observed. IC_{50} is meant to show the correlation between the radicular growth and the concentration of the substance in contact with the seeds.

The *in vivo* acute toxicity in mice was determined for 2 new series of compounds, potentially β_3 adrenergic receptor agonists by using the regression curve method. Taking into account the observed LD_{50} , compounds were classified using the 1956 Hodge – Sterner toxicity scale.

The new method was developed by observing the correlation between the phytobiologic toxicity (IC_{50}) and *in vivo* acute toxicity in mice (LD_{50}) using compounds C₁, C₂, C₃. The validation of the Triticum method was accomplished using compounds belonging to series A (A₁-A₈) (5-7).

Statistical evaluation of the results was performed using special software, GraphPad Prism version 5.01. This software analyzes two group populations, either with normal distributions using the Student t test, or with skewed distributions using the Mann-Whitney test. More than 2 groups are analyzed using ANOVA. D`Agostino – Pearson test was used to determine whether the population is distributed normally.

All experimental procedures were carried out in accordance with the European Directive 2010/63/UE of 22nd September 2010, and The Romanian Government Ordinance 37/30.01.2002 regarding the protection of animals used for experimental and other scientific purposes. All experimental procedures were approved by the Ethical Committee of the Faculty of Pharmacy Bucharest.

Results

The LD₅₀ values were determined using regression curves. The classification of the compounds belonging to series C, in accordance to the Hodge – Sterner toxicity scale, is shown in table 1. In the case of compounds belonging to series A, LD1 could not be determined because it was higher than the maximum dose that can be administrated as a suspension in mice (1000 mg/kg).

Table 1. Correlation between the in vivo acute toxicity (LD_{50%}) and the phytobiologic toxicity (IC_{50%}). Hodge-Sterner classification of the compounds from series C

Comp.	Toxicity	LD ₅₀ in mice after oral administrati on (mg/kg)	IC ₅₀ (moli/100ml) 3rd day
C ₁	Moderate (LD ₅₀ = 50 - 500 mg/kg)	210.29	0.002359746
C ₂	Low (LD ₅₀ = 0,5- 1g/kg)	672.72	0.004574164
C ₃	Very low (LD ₅₀ = 1- 5g/kg)	1023.98	0.008080654

Regression curves for the IC₅₀ determination were linear for a domain of 3-4 concentrations and the regression factors generally have values higher than 0.8. The regression curve for compound C₂ is shown in the following Figure 1.

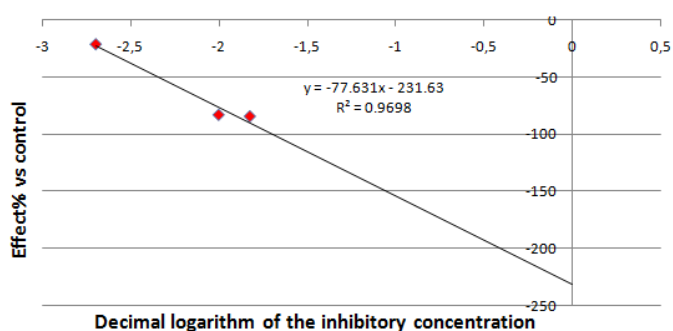


Figure 1. C₂ regression curve expressing the influence of the inhibitory concentration on radicular growth in the 3rd day of measurement

The regression curve for compound C₃ is shown in Figure 2.

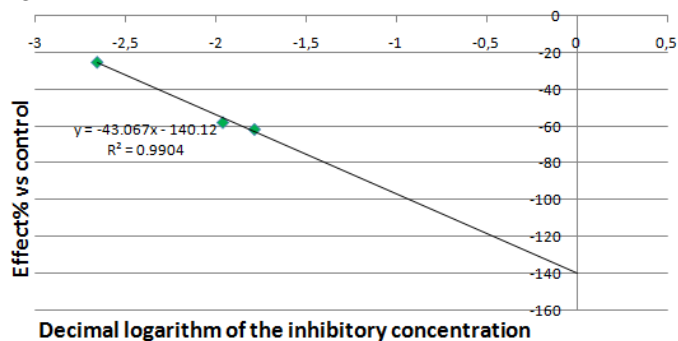


Figure 2. C₃ regression curve expressing the influence of the inhibitory concentration on radicular growth in the 3rd day of measurement

Using the regression equation, the value of IC₅₀ was determined as illustrated for compound C₂ and C₃:

C₂	C₃
$y = -77.631x - 231.63$	$y = -43.067x - 140.12$
$-50 = -77.631 \lg IC_{50} - 231.63$	$-50 = -43.067 \lg IC_{50} - 140.12$
$-50 + 231.63 = -77.631 \lg IC_{50}$	$-50 + 140.12 = -43.067 \lg IC_{50}$
$181.63 = -77.631 \lg IC_{50}$	$90.12 = -43.067 \lg IC_{50}$
$\lg IC_{50} = -181.63 / 77.63 = -2.34$	$\lg IC_{50} = -90.12 / 43.067 = -2.093$
IC₅₀ = 10^{-2.34} = 0.004574164	IC₅₀ = 10^{-2.093} = 0.008080654

The calculated value of this innovative parameter is consistent with a low level of toxicity, confirmed by the vivo tests on mice. The statistical significance of radicular elongation variations for the groups treated with different concentrations of compound C₂ in the 3rd day of measurements is shown in Table 2.

Table 2. Statistical significance of radicular elongation variation under treatment with C₂ in 3rd day of measurements

Group	M	C2A	C2B	C2C	C2D	C2E	C2F
M ± SD	6.69 ± 1.39	1.00 ± 0	1.02 ± 0.04	1.04 ± 0.07	1.13 ± 0.12	5.3 ± 1.68	7.48 ± 1.34
Normal distribution (D'Agostino – Pearson test)	YES	NO	NO	NO	YES	NO	YES
Effect% vs control (M)	-	-85.05%	-84.75%	-84.45%	-83.11%	-20.78%	+11.81%
t test (p) vs M	-	-	-	-	p < 0.0001	-	p > 0.05
Mann-Whitney test (p) vs M	-	p < 0.01	p < 0.01	p < 0.01	-	p > 0.05	-

The statistical significance of radicular elongation concentrations of compound C₃ in the 3rd day of variations for the groups treated with different measurements is shown in Table 3.

Table 3. Statistical significance of radicular elongation variation under treatment with C₃ in the 3rd day of measurements

Group	M	C3A	C3B	C3C	C3D	C3E	C3F
M ± SD	6.69 ± 1.39	2.38 ± 0.26	2.03 ± 0.74	2.57 ± 0.51	2.82 ± 0.40	5 ± 0.58	6.13 ± 1.88
Normal distribution (D'Agostino–Pearson test)	YES	NO	YES	YES	YES	YES	YES
Effect% vs control (M)	-	-64.42%	-69.66%	-61.58%	-57.85%	-25.26%	-8.37%
t test (p) vs M	-	-	p<0.0001	p<0.0001	p<0.0001	p< 0.01	p>0.05
Mann-Whitney test (p) vs M	-	p< 0.01	-	-	-	-	-

The influence of various concentrations of compound C₂ on radicular elongation is illustrated in Figure 3:

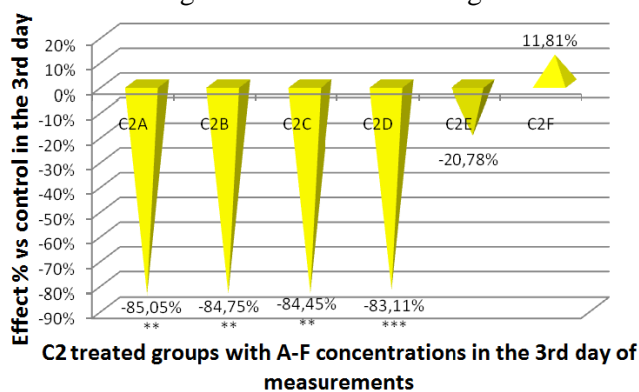


Figure 3. Evolution of radicular elongation versus control, under the influence of compound C₂ in various concentrations (A – the highest, F – the lowest)

The influence of various concentrations of compound C₃ on radicular elongation is illustrated in Figure 4:

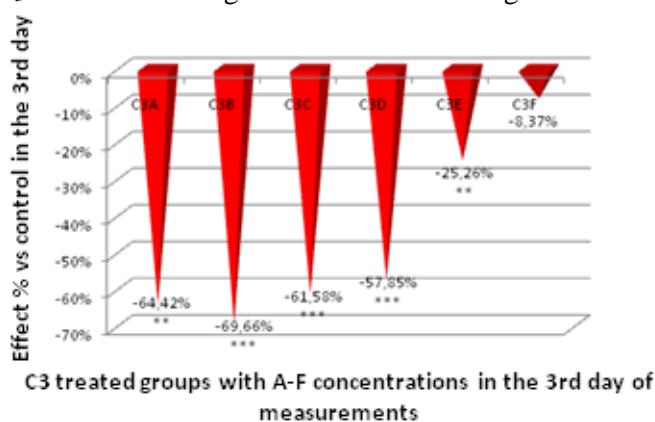


Figure 4. Evolution of radicular elongation versus control, under the influence of compound C₃ in various concentrations (A – the highest, F – the lowest)

The validation of the Triticum method as a reliable alternative to the classic in vivo toxicity tests was accomplished by applying the technique to the compounds of the series A (A₁–A₈). The regression curve for compound A₂ is shown in Figure 5.

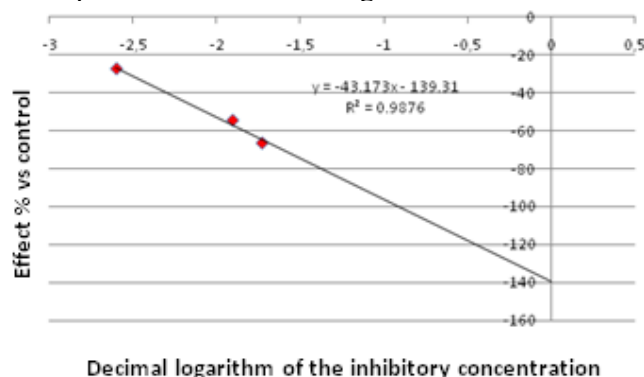


Figure 5. A₂ regression curve expressing the influence of the inhibitory concentration on radicular growth in the 3rd day of measurement

The regression curve for compound A₅ is shown in Figure 6.

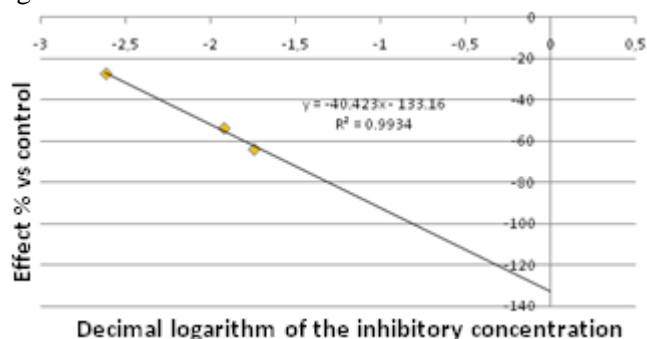


Figure 6. A₅ regression curve expressing the influence of the inhibitory concentration on radicular growth in the 3rd day of measurement

Using the regression equation, the value of IC_{50} was determined as illustrated for compound A₂:

$$y = -43.173x - 139.31$$

$$-50 = -43.173lgIC_{50} - 139.31$$

$$-50 + 139.31 = -43.173lgIC_{50}$$

$$89.31 = -43.173lgIC_{50}$$

$$lgIC_{50} = -89.31/43,173 = -2.069$$

$$IC_{50} = 10^{-2.069} = 0.0085378$$

The calculated value of the inhibitory concentration 50% (IC_{50}) is consistent with a low level of toxicity confirmed by in vivo tests on mice and reflected through a high lethal dose 50%.

Using the regression equation, the value of IC_{50} was determined as illustrated for compound A₅:

$$y = -40.423x - 133.16$$

$$-50 = -40.423lgIC_{50} - 133.16$$

$$-50 + 133.16 = -40.423lgIC_{50}$$

$$83.16 = -40.423lgIC_{50}$$

$$lgIC_{50} = -83.16/40.423 = -2.057$$

$$IC_{50} = 10^{-2.057} = 0.008765069$$

The statistical significance of radicular elongation variations for the groups treated with different concentrations of compound A₂ on the 3rd day of measurements is shown in Table 4:

Table 4. Statistical significance of radicular elongation variation under treatment with A₂ in the 3rd day of measurements

Group	M	A2A	A2B	A2C	A2D	A2E	A2F
M ± SD	7.789 ± 1.43	1.011 ± 0.033	1.322 ± 0.589	2.611 ± 0.679	3.544 ± 0.654	5.656 ± 0.664	9.433 ± 1.247
Normal distribution (D'Agostino – Pearson test)	YES	NO	NO	YES	YES	YES	YES
Effect% vs control (M)	-	-87.02%	-83.03%	-66.48%	-54.5%	-27.38%	+21.11%
t test (p) vs M	-	-	-	p<0.0001	p<0.0001	p<0.0001	p<0.0001
Mann-Whitney test (p) vs M	-	p<0.01	p<0.01	-	-	-	-

The statistical significance of radicular elongation variations for the groups treated with different concentrations of compound A₅ in the 3rd day of measurements is shown in Table 5:

Table 5. Statistical significance of radicular elongation variation under treatment with A₅ in the 3rd day of measurements

Group	M	A5A	A5B	A5C	A5D	A5E	A5F
M ±SD	8.75 ± 1.94	2.40 ± 0.74	2.50 ± 0.39	3.16 ± 0.87	4.05 ± 1.20	6.34 ± 1.48	8.69 ± 1.91
Normal distribution (D'Agostino – Pearson test)	YES	YES	YES	YES	YES	NO	NO
Effect% vs control(M)	-	-72.59%	-71.45%	-63.83%	-53.68%	-27.55%	-6.7%
t test (p) vs M	-	p<0.0001	p<0.0001	p<0.0001	p<0.0001	-	-
Mann-Whitneytest (p) vs M	-	-	-	-	-	p<0.01	p>0.05

The influence of various concentrations of compound A2 on radicular elongation is illustrated in Figure 7:

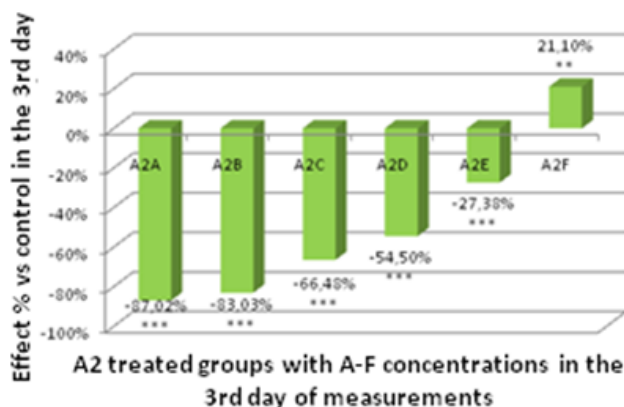


Figure 7. Evolution of radicular elongation versus control, under the influence of compound A2 in various concentrations (A – the highest, F – the lowest)

The influence of various concentrations of compound A5 on radicular elongation is illustrated in Figure 8:

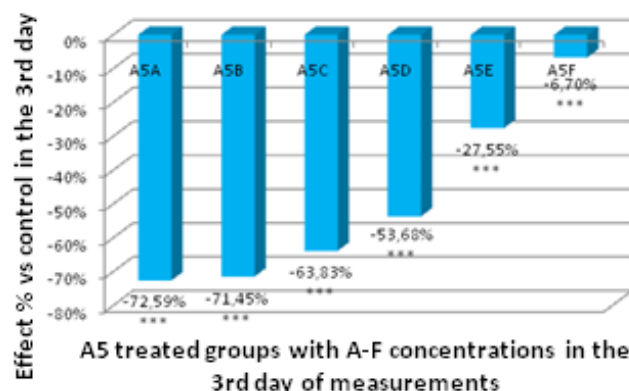


Figure 8. Evolution of radicular elongation versus control, under the influence of compound A5 in various concentrations (A – the highest, F – the lowest)

A strong correlation between IC_{50} and LD_{50} was observed in all studied compounds.

Discussion

Using the phytobiological method known also as the Triticum technique, this study investigated an alternative method for determining *in vivo* acute toxicity using vegetal material instead of laboratory animals. We set

out to demonstrate that vegetal cells have similar sensitivity to some toxic agents as animal cells, in which case, a statistical correlation could be established. A series of new compounds synthesized by the Romanian National Institute for Chemical – Pharmaceutical Research and Development as potential β_3 adrenergic receptors agonists were tested for their acute toxicity using classical animal exposure models, before investigating possible anti-diabetic and anti-obesity effects (5-7).

The validation of the phytobiological method as an alternative for preliminary toxicological evaluations is an original contribution toward identifying bioethical ways to assess the toxicity of new substances without using laboratory animals. In order to perfect this method, we have used 2 new series of compounds with potential β_3 adrenergic properties. The method proved to be reliable and reproducible. The improved Triticum technique has confirmed the level of toxicity attributed to most of the studied compounds by the classic *in vivo* acute toxicity tests performed on mice (8, 9).

Only in case of compounds A1, A3, and A8 was the correlation between the Triticum method and the *in vivo* toxicity test weaker, probably due to a significant difference between the *in vivo* bioavailability after oral administration and the diffusion process through the membranes of vegetal cells.

The ideal observation day in the Triticum test proved to be the 3rd day, as it showed an evolution of the IC_{50} in a 2:1 geometric ratio for compounds belonging to different toxicity classes, similar to sodium fluoride administration (10).

A good correlation was observed between the radicular elongation and the cellular multiplication, as well as between the toxicity of compounds C2, C3 expressed by LD_{50} in mice and that expressed by IC_{50} in the Triticum test (11).

The regression curves for the IC₅₀ determination were linear for a domain of 3-4 concentrations and regression coefficients generally had values higher than 0.8 (12, 13).

Conclusions

Experimental studies may involve a potential conflict between two distinct but interrelated specialties, represented by statistical reliability (requiring a large number of cases) and bioethics (suggesting the need to reduce the number of cases to as few as possible). Solving this ongoing dilemma requires developing new methods for determining toxicity without the use of lab animals.

In this study we presented an alternative for determining in vivo acute toxicity using vegetal material instead of laboratory animals, starting from the phytobiological method known also as the Triticum technique. This technique successfully showed that plants are affected in a very similar way as animals when exposed to some potentially toxic agents, thus identifying a new possibility for limiting or ending unethical animal experiments.

Although anatomical and morphological differences between animal and vegetal cells limits, to some extent, the use of the Triticum method for determining the toxicity level of new compounds, it nevertheless offers new directions in the field of toxicological research.

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