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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 β3 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 C_1, C_2, C_3. The validation of the Triticum method was accomplished using compounds belonging to series A (A_1-A_8) (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 LD_{50} 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 administration (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 C_2 is shown in the following Figure 1.

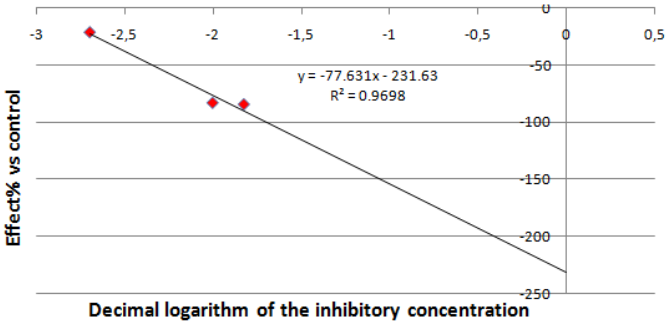

Figure 1. C_2 regression curve expressing the influence of the inhibitory concentration on radicular growth in the 3rd day of measurement

The regression curve for compound C_3 is shown in Figure 2.

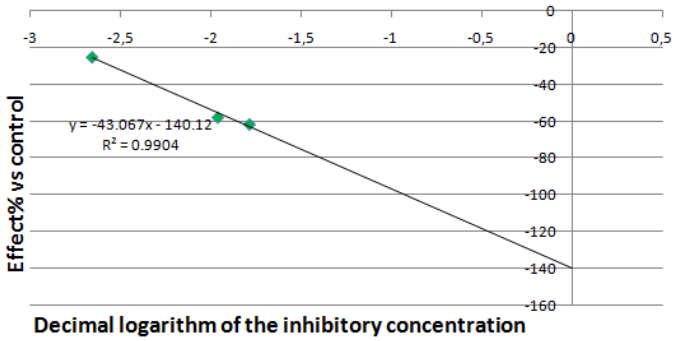

Figure 2. C_3 regression curve expressing the influence of the inhibitory concentration on radicular growth in the 3rd day of measurement

Using the regression equation, the value of IC_{50} was determined as illustrated for compound C_2 and C_3:

C_2	C_3
$y = -77.631x - 231.63$	$y = -43.067x - 140.12$
$-50 = -77.631 lgIC_{50} - 231.63$	$-50 = -43.067 lgIC_{50} - 140.12$
$-50 + 231.63 = -77.631 lgIC_{50}$	$-50 + 140.12 = -43.067 lgIC_{50}$
$181.63 = -77.631 lgIC_{50}$	$90.12 = -43.067 lgIC_{50}$
$lgIC_{50} = -181.63/77.63 = -2.34$	$lgIC_{50} = -90.12/43.067 = -2.093$
$IC_{50} = 10^{-2.34} = 0.004574164$	**$IC_{50} = 10^{-2.093} = 0.008080654$**

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 C_2 in the 3rd day of measurements is shown in Table 2.

Table 2. Statistical significance of radicular elongation variation under treatment with C_2 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 variations for the groups treated with different concentrations of compound C_3 in the 3rd day of measurements is shown in Table 3.

Table 3. Statistical significance of radicular elongation variation under treatment with C_3 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 C_2 on radicular elongation is illustrated in Figure 3:

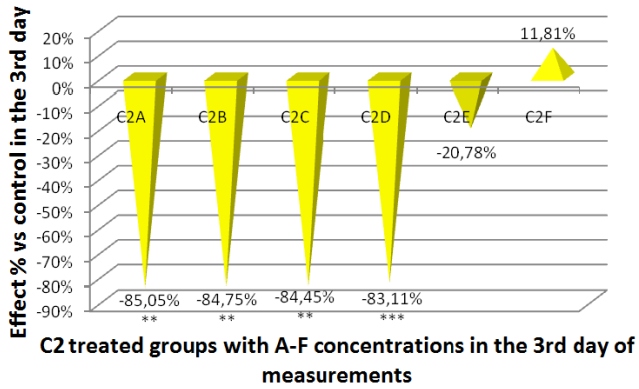

Figure 3. Evolution of radicular elongation versus control, under the influence of compound C_2 in various concentrations (A – the highest, F – the lowest)

The influence of various concentrations of compound C_3 on radicular elongation is illustrated in Figure 4:

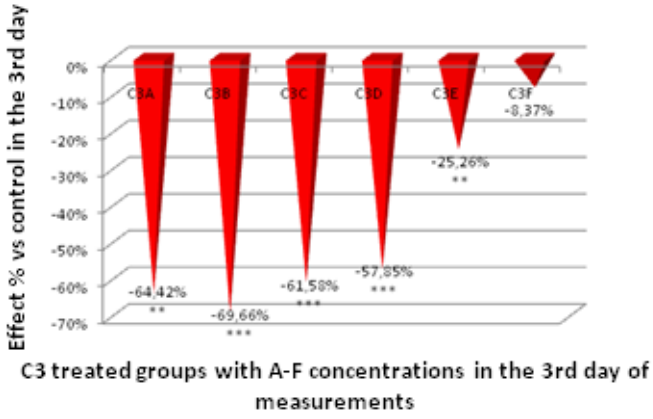

Figure 4. Evolution of radicular elongation versus control, under the influence of compound C_3 in various concentrations (A – the highest, F – the lowest)

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 (A_1-A_8). The regression curve for compound A_2 is shown in Figure 5.

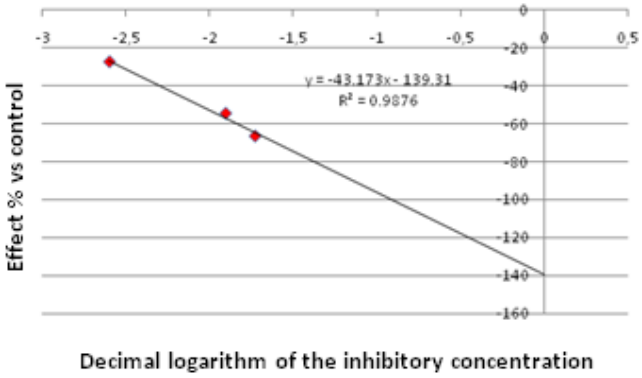

Figure 5. A_2 regression curve expressing the influence of the inhibitory concentration on radicular growthin the 3rd day of measurement

The regression curve for compound A_5 is shown in Figure 6.

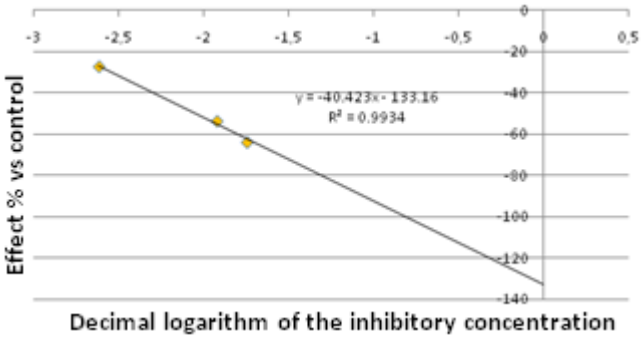

Figure 6. A_5 regression curve expressing the influence of the inhibitory concentration on radicular growthin the 3rd day of measurement

Using the regression equation, the value of IC_{50} was determined as illustrated for compound A_2:

y = -43.173x – 139.31
-50 = -43.173lgIC50 – 139.31
-50 + 139.31 = -43.173lgIC50
89.31 = -43.173lgIC50
lgIC50 = -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 A_5:

y = -40.423x – 133.16
-50 = -40.423lgIC50 – 133.16
-50 +133.16 = -40.423lgIC50
83.16 = -40.423lgIC50
lgIC50 = -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 A_2 on the 3rd day of measurements is shown in Table 4:

Table 4. Statistical significance of radicular elongation variation under treatment with A_2 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 A_5 in the 3rd day of measurements is shown in Table 5:

Table 5. Statistical significance of radicular elongation variation under treatment with A_5 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:

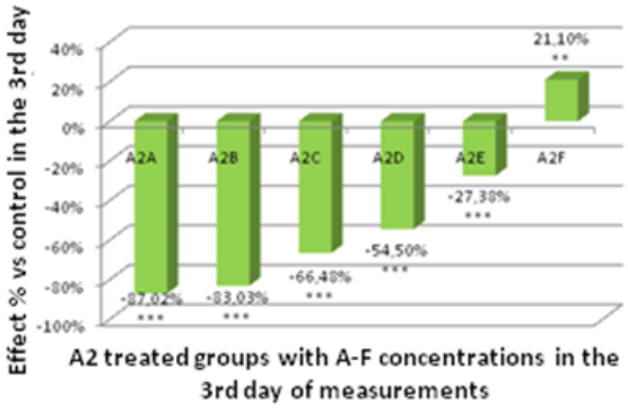

Figure 7. Evolution of radicular elongation versus control, under the influence of compound A2 in various concentrations (A – the highest, F – the lowest)

The influence of various concentrations of compound A_5 on radicular elongation is illustrated in Figure 8:

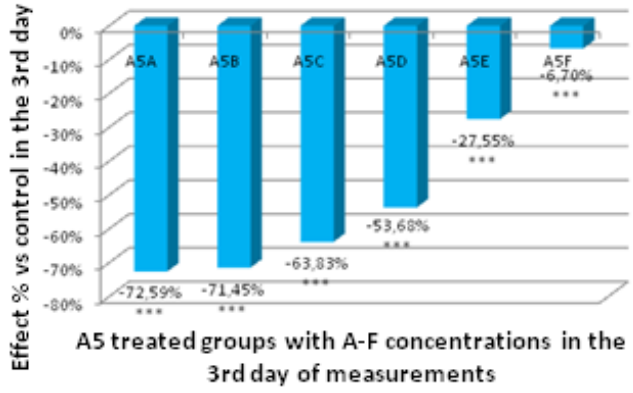

Figure 8. Evolution of radicular elongation versus control, under the influence of compound A_5 in various concentrations (A – the highest, F – the lowest)

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 investigatedan 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 A_1, A_3, and A_8 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, smilar 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 C_2, C_3 expressed by LD_{50} in mice and that expressed by IC_{50} in the Triticum test (11).

The regression curves for the IC_{50} 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 casesto 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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