ORIGINAL ARTICLE

CORRELATIONS *IN SILICO*/*IN VITRO*/*IN VIVO* REGARDING DETERMINATING ACUTE TOXICITY IN NON-CLINICAL EXPERIMENTAL TRIAL, ACCORDING TO BIOETHIC REGULATIONS INFORCED BY THE EUROPEAN UNION

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Abstract

A non-clinical experimental trial on laboratory animals was conducted in order to determine the acute toxicity of 7 newly synthesized compounds (derivatives of beta phenyl-ethyl-amine) with potential antidiabetic/antiobesity effect. The trial objective was to reduce to minimum, according to European regulations, the number of laboratory animals employed in order to determine the lethal dose 50% (DL50). To this end, alternative methods of toxicity evaluation were used: *in silico* (QSAR method) and *in vitro* (plant cell inhibition), which were applied in consecutively performed *in vivo* trial (white male mice, NMRI strain). The research established a good correlation between the performed tests and may serve as a screening model for active substances with high biological potential.

Rezumat

A fost condus un studiu non-clinic, pentru determinarea toxicităţii acute la animale de laborator, pentru evaluarea a şapte compuşi nou sintetizaţi (structură chimică de beta fenil etilamină), cu potenţial efect antidiabetic/antiobezitate. Obiectivul studiului a fost de a reduce la minim conform reglementărilor europene, numărul de animale de laborator necesar pentru determinarea dozei letale 50% (DL_{50}). În acest scop, am utilizat metode alternative de evaluare a toxicităţii: *in silico* (*metoda QSAR)* şi *in vitro* (inhibiţie pe celula vegetală), pe care le-am utilizat ulterior în testul efectuat *in vivo* (şoareci albi, masculi, suşa NMRI). Cercetarea a stabilit o bună corelaţie între testele utilizate, şi poate servi ca model de *screening* şi pentru alte substanţe active cu potenţial biologic ridicat.

Keywords: $beta_3$ adrenergic agonists, acute toxicity

Introduction

Acute toxicity determination in laboratory animals is a compulsory stage in the research for introduction in therapy of newly synthesized active substances [23]. Determination of this parameter in the classic approach has as an objective determining LD_{50} (lethal dose 50%) in laboratory animals. Nowadays, in numerous European countries there is an increasingly manifest tendency of reducing the use of this test and replacing it with *in vitro* or *in silico* alternatives, an approach which is in line with the "3R" principle (Replacement, Reduction, Refinement) [27, 28].
In determining acute toxicity one must consider that data should be obtained with regard to short-term substance's harmfulness, as administered in single dose, and should contribute to its placement along a toxicity scale: Hodge-Sterner scale [11], Harmonized Global System (GHS) of Classification and Labeling of Chemical Substances (2005).
Recent developmental policies in EU and world-wide are recognizing an even greater importance to using both structure-activity relationships (SARs) and also quantitative structure-activity relationships (QSARs), generically included in QSAR models as baseline methods of "guided synthesis" of potentially active chemical compounds or as "directed" pharmacological trials.
In order to minimize the number of laboratory animals, alternative procedures were developed, presented *in extenso* in the OECD (Organization for Economic Cooperation and Development) guideline: „up-and-down" [3] and „fixed dose" method [23].

In the present paper the objective was to determine acute toxicity in laboratory animals for seven newly synthesized compounds (A1-A7) with potential antidiabetic/anti-obesity activity [9, 10]. To this end the researchers tried to employ a restrictive number of animals for complying with European regulations currently in force [25, 26] by using both the data provided from a QSAR model, previously selected for *in silico* prediction of compound toxicity and also the data on toxicity obtained *in vitro* on plant cells [5].

For the newly synthesized beta phenyl-ethyl amine derivatives an assumption was made that a potential for antidiabetic activity existed, starting from the proven major implication of $beta_3$ adrenergic receptors in glucose metabolism and obesity [1, 2, 18]. Numerous non-clinical studies have demonstrated that $beta_3$ agonists administration has reduced glucose and lipids plasma concentrations in diabetic rats specifically modified genetically for this purpose (kk, C57BL/KsJ-db/db: according to genetic models for antidiabetic drug trials, animals are to be hyperglycemic, hyperinsulinemic rat with rapid development of obesity after weaning) or in rats with experimentally induced diabetes [7, 12].

Recently published literature data highlighted further actions for $beta_3$ adrenergic receptors, like cardio-protective effect [6, 14, 16] and modulatory effect on arterial pressure (positively or negatively, depending on selectivity for adrenergic receptors) [20].

The rationale for the non-clinical pharmacological and toxicological research in the present work relies on the high biological potential of these newly synthesized beta phenyl-ethyl amine derivatives.

Materials and Methods

QSAR method

As common knowledge, the essential of QSAR domain (Quantitative chemical Structure–biological Activity Relationships) can be summarized as follows: the biological activity, like any other physical-chemical propriety, is a function attached to the micromolecular structure which enters an organism [4, 15]. A micromolecule is defined as any molecule which is not a biological compound (protein, nucleic acid, etc.) that acts at a receptor level (biomicromolecule), yielding a certain biological effect. QSAR determines the mathematical form of the function that describes the above-mentioned functional relationship, as both the biological activity and also the molecular chemical structure are numerically described (represented by numbers) [8, 19].

A specific QSAR model was chosen for *in silico* toxicity prediction for newly synthesized substances (National Institute for Chemical-Pharmaceutical Development, Bucharest, Romania - INDCF), supposed to have antidiabetic/anti-obesity activity. The QSAR model was assembled starting from several standard adrenergic agonists, then validated and tested experimentally for the seven newly synthesized compounds, generically designated A1 – A7. The experiment also consisted in using the software application BIOINFOQSAR (original software which predicts pharmacological properties in correlation with quantum-molecular properties, developed by the research group within the Department of Physical and colloidal chemistry, UMF "Carol Davila", Bucharest, Faculty of Pharmacy) [29]. The obtained results have proven application's capability of providing pertinent information concerning biological activities of substances based on interpretation of their chemical structure.

In vitro toxicological trials for newly synthesized compounds – phyto-biological testing (Triticum test)

Triticum test [5] consists in determining the maximal active dilution of the researched substance, which influences radicular elongation and caryokinetic film depending on exposure period.

Caryopses of *Triticum vulgare* Mill, cultivar *Dropia*, *Poaceae* family were employed.

10^{-4} molar solutions of the tested substances were prepared by dissolving in 100 mL chloroform the following quantities: 0.0357 g A1; 0.0371 g A2; 0.0387 g A3; 0.0357 g A4; 0.0426 g A5; 0.0387 g A6; 0.0391 g A7. From the respective solutions, the following volumes for each compound were pipetted in Petri dishes: 15 mL; 1 mL; 0.1 mL. The dishes were brought to dry on water bath then maintained in a sterilization oven at 60°C until the chloroformic odour disappeared. Over the obtained residue, in each Petri dish 15 mL of distilled water was added, resulting 3 dilutions samples for each compound (Table I).

Table I

The protocol for obtaining dilutions

Sample	Sample volumes in chloroform (brought to dry)	Distilled water	Concentration (mol/100 mL)
A	15 mL sample	15 ml	10^{-4}
B	1 mL sample	15 ml	0.67×10^{-5}
C	0,1 mL sample	15 ml	5.6×10^{-6}
M (control)	15 mL chloroform	15 ml	-

Wheat caryopses, selected as homogenous as possible, were soaked in distilled water for 24 hours and placed in Petri dishes (200 - 250 mm diameter) on filter paper, previously wetted in distilled water. After 24 hours the caryopses with the main root of about 1 cm were selected and randomized for testing. For each dilution, 10 caryopses per one Petri dish of 90 mm diameter were used and testing solutions were added. The experiment was carried out under constant temperature (25ºC) and humidity (85%) in a MLR-35H germinating chamber.

Evaluation of radicular elongation was performed by a linear measurement, from initial treatment (day 1) at 24 hrs. interval and at 5 days, considered the most active phase of growth.

Method of in vivo determination of toxicity

Starting from previous data provided by the QSAR model selected for *in silico* toxicity prediction for the testes substances, a conclusion was drawn that LD_{50} is obtained for all tested compounds at doses over 900 mg/kg bw, orally. By correlating these data with experimental results from plant cell, progressive tests of toxicity for each active substance were initiated in groups of 2 animals (white male mice, NMRI strain) by administering a dose equal to a tenth of the lethal dose (100 mg/kg bw) theoretically determined *in silico*.

Following this observation, a collectivity of 56 white male mice (mean weight: 24.2 ± 1.43 g), NMRI strain (from the Biobase of UMF "Carol Davila", Bucharest) was selected and animals were randomized to groups of 8 animals: seven groups were treated with a single dose of 1,000 mg/kg bw, p.o. from the substances A1- A7.

The parameters observed were: lethality, body weight, modification in outer aspect and in motor behaviour, for 14 days [23].

Animals were housed in Plexiglas cages, with free access to food and water. Throughout the experiment, temperatures between 21-24°C and relative humidity between 45-60% were maintained.

All procedures were performed observing the bioethical regulations in animal research for scientific purpose, according to Directive 86/609/EEC from November 24, 1986 and to Romanian Governmental Ordonnance no. 37/01.30.2002. The study was approved by the Ethics Committee of the "Carol Davila" University of Medicine and Pharmacy of Bucharest.

Statistical analysis

The results of *in vitro* and *in vivo* tests were processed statistically using software application GraphPad Prism version 5.01.

Testing of normality of response distribution in population was performed by D`Agostino – Pearson test, as recommended by the software. Result comparison used the effects on radicular elongation for the tested compounds, the initial length of the radicles and the growth in control group.

In order to establish the statistical significance two tests were employed: t Student (compares two sets of results with normal distribution) or Wilcoxon (compares two sets of results with abnormal distribution). The degree of statistical significance is conventionally noted as follows: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; ns = non-significant.

The results that evaluated the body weight of the animals treated with the tested compounds were statistically processed by using GraphPad Prism software version 5.01.

Results and Discussion

Acute toxicity determined by QSAR method

The software application BIOINFOQSAR has calculated the specific molecular descriptors for each substance starting from an entry data set (training-set), based on which a predictive mathematical model was constructed for the tracked biological property.

A total of 14 substances were employed, running a series of analysis and computation of molecular descriptors in order to establish the equation-model QSAR of prediction for the chosen biological activity: LD_{50} (Table II).

The first set of newly synthesized compounds, with beta phenyl ethylamine nucleus, were generically nominated by letter C and indices 1 to 5 with different radicals substitutes to the nucleus (compound structure was not disclosed as the compounds are already patented).

Table II

The set of analysed substances (training set)

Crt. no.	Substance	LD_{50} mg/kg bw [reference]
1	Salbutamol	2,707 [22]
2	Salmeterol	300 [30]
3	Terbutaline	205 [31]
4	Fenoterol	1,990 [22]
5	Buphenine	4,800 [32]
6	Orciprenaline	42 [31]
7	Ritodrine	540 [31]
8	Pindolol	263 [31]
9	C1	210.29 [17]
10	C2	672.72 [17]
11	C3	1,023.98 [17]
12	C4	1,586.52 [17]
13	C5	500 [17]
14	C6	913.34 [17]

Equation-predictive model is a multilinear regression model, as follows:

LOG10 (DL 50) = $-0.150*C_1SP_3 + 7.060*VC\text{-}4 + 1.086*VP\text{-}3 + 2.80*VP\text{-}4 - 9.11*VP\text{-}6$

in which the terms (molecular descriptors) are:

- $^1SP_3$ – index of molecular connectivity to carbon atom, in terms of hybridization (constitutive molecular descriptor);
- VC-3 - Kier - Hall index of Chi-Cluster type of third order (conformational molecular descriptor);
- VP-3, VP-4, VP-6 - Kier- Hall index of Chi-Path type, of 3rd, 4th, 6th order [4, 13].

QSAR equation of prediction was validated through a series of specific statistical tests, whose values are presented below:

- coefficient of square mean deviation R2 = 0.936;
- square standard deviation S2 = 0.0316;
- Fisher statistical test F = 59.4089;
- AKAIKE test AIC = -6.3416.

The high predictive capability of the QSAR model (equation 1) is presented graphically by plotting the predicted value together with the one calculated from the experimentally determined value for the analysed LD50 property (Figure 1).

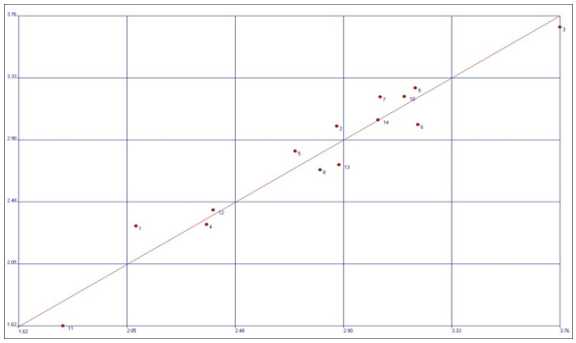

Figure 1.
Graphic representation (Q-Q plot) of observed values (observed logIC50) *versus* calculated values (calculated logIC50)

For the newly synthesized compounds, the subroutines of BIOINFOQSAR software used the molecular descriptors, with emphasis on the ones present in the predictive equation. The following LD_{50} doses were obtained:

- A1: log LD_{50} = 3.21 and LD_{50} = 1621.81 mg/kg bw;
- A2: log LD_{50} = 2.98 and LD_{50} = 955.00 mg/kg bw;
- A3: log LD_{50} = 2,991 and LD_{50} = 981 mg/kg bw;
- A4: log LD_{50} = 2.997 and LD_{50} = 994 mg/kg bw;
- A5: log LD_{50} = 2.969 and LD_{50} = 932mg/kg bw;
- A6: log LD_{50} = 2.964 and LD_{50} = 921 mg/kg bw;
- A7: log LD_{50} = 2.989 and LD_{50} = 976 mg/kg bw.

In vitro toxicity in Triticum test

The radicular elongation from baseline (day 0) was calculated for all samples, according to the following formula:

Effect%/baseline= (M1,5 – M0)/M0 x 100, where:

M0 – mean (for sample) of radicle length in day zero;
M1,5 – mean (for sample) of radicle length when assessed in day 1 and 5, respectively.

The effect of the tested substances on the radicle length as compared with the control sample was calculated according to the following formula:

Effect% $_{A1...\ A7}$/Control= Efect%/baseline$_{A1...\ A7}$ – Efect%/baseline$_M$, where:

Effect%/baseline $_{A1...\ A7}$ – is the change in radicle length as compared with the baseline values, induced by the test substances, for each dilution (A, B, C), determined in days 1 and 5, respectively.

Effect%/baseline$_M$ – is the change in radicle length as compared with the baseline values of the control, calculated according to the above equation;

The experimental results allowed to place the tested compounds in the category of low toxicity due to the fact that, in all tested dilutions, an increase in radicular elongation was registered (Tables III - IV).

It may be seen that in comparison with the control group, the lowest dilution (highest concentration, dilution A, 10^{-4} M), the test substances show a smaller radicle elongation (Figure 2).

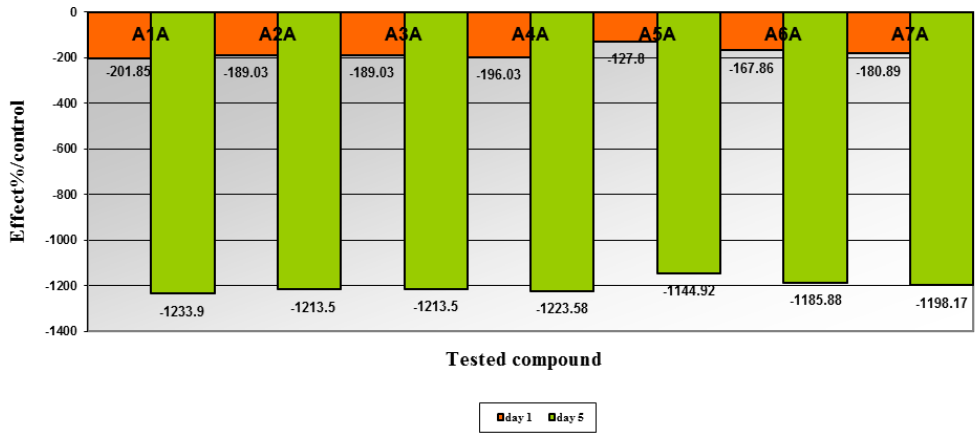

Figure 2.
Modifications in radicle elongation induced by compounds A1 – A7, compared to control group (days 1, 5), dilution A

Table III

Evolution of radicle elongation for the compounds: Control, A1, A2, A3, A4

A1					
Group/Dilution	**Day**	**M ± SD**	**p/initial**	**Effect %/initial**	**Effect%/control**
Control	**0**	0.9556 ± 0.0348			
	1	2.956 ± 0.1617	< 0.0001***	209.33	-
	5	12.83 ± 0.7800	< 0.0001***	1 242.61	-
A1A	**0**	0.9611 ± 0.0309			
	1	1.033 ± 0.0235	0.0818	7.48	-201.85
	5	1.044 ± 0.0337	0.0877	8.62	-1,233.9
A1B	**0**	0.955 ± 0.0305			
	1	1.800 ± 0.1764	0.0002***	88.36	-120.97
	5	1.967 ± 0.1900	< 0.0001***	105.83	-1,136.78
A1C	**0**	0.9389 ± 0.0309			
	1	3.600 ± 0.1856	< 0.0001***	283.42	74.09
	5	9.222 ± 0.5286	< 0.0001***	882.21	-360.4
A2					
Group/Dilution	**Day**	**M ± SD**	**p/initial**	**Effect %/initial**	**Effect%/control**
A2A	**0**	0.877 ± 0.1014			
	1	1.056 ± 0.0294	0.1117	20.30	-189.03
	5	1.133 ± 0.0577	0.0437*	29.07	-1,213.5
A2B	**0**	0.9556 ± 0.0294			
	1	3.578 ± 0.1140	< 0.0001***	169.77	-18.89
	5	8.989 ± 0.5663	< 0.0001***	840.66	-401.95
A2C	**0**	0.956 ± 0.03056			
	1	3.156 ± 0.1260	< 0.0001**	230.26	20.93
	5	12.78 ± 0.8015	< 0.0001**	1,237.37	-5.24
A3					
Group/Dilution	**Day**	**M ± SD**	**p/initial**	**Effect %/initial**	**Effect%/control**
A3A	**0**	0.8778 ± 0.1014			
	1	1.056 ± 0.02940	0.1117	20.30	-189.03
	5	1.133 ± 0.05774	0.0437*	29.10	-1,213.51
A3B	**0**	0.9556 ± 0.02940			
	1	3.578 ± 0.1140	< 0.0001***	274.42	65.09
	5	8.989 ± 0.5663	< 0.0001***	840.66	-401.95
A3C	**0**	0.9556 ± 0.03056			
	1	3.156 ± 0.1260	< 0.0001***	230.26	21.27
	5	12.78 ± 0.8015	< 0.0001***	1,237.37	-5.24
A4					
Group/Dilution	**Day**	**M ± SD**	**p/initial**	**Effect %/initial**	**Effect%/control**
A4A	**0**	0.9611 ± 0.03203			
	1	1.089 ± 0.04547	0.0354*	13.30	-196.03
	5	1.144 ± 0.04747	0.0056**	19.03	-1,223.58
A4B	**0**	0.9556 ± 0.03056			
	1	1.589 ± 0.08407	< 0.0001***	66.28	-143.05
	5	1.667 ± 0.07071	< 0.0001***	74.44	1,168.17
A4C	**0**	0.9556 ± 0.03275			
	1	3.267 ± 0.1986	< 0.0001***	241.87	32.54
	5	5.878 ± 0.3643	< 0.0001***	515.11	-727.5

By diluting the solution 10 times (dilution B, 10^{-5}), substances A1, A4 and A6 stimulated the radicle elongation, as compared with the control sample (Figure 3). Moreover, it can be noted that, further diluting by 10x the tested solutions (from 10^{-4} to 10^{-5}), the increase in radicular elongation became highly significant statistically for the newly synthesized compounds.

As featured in Tables III – IV, at the highest dilution (C = 0.67 x 10^{-6}), against the control group, the registered effect was biphasic, stimulation of radicles growth in the first day evaluation followed by a more discrete growth according to the 5th day evaluation (Figure 4).

Table IV

Evolution of radicle elongation for the compounds: Control, A5, A6, A7

A5					
Group/Dilution	**Day**	**M ± SD**	**p/initial**	**Effect %/initial**	**Effect %/control**
Control	**0**	0.9556 ± 0.0348			
	1	2.956 ± 0.1617	< 0.0001***	209.33	-
	5	12.83 ± 0.7800	< 0.0001***	1,242.61	-
A5A	**0**	0.9611 ± 0.03977			
	1	1.744 ± 0.2231	0.0032**	81.50	-127.8
	5	1.900 ± 0.2297	0.0010***	97.69	1,144.92
A5B	**0**	0.9333 ± 0.02357			
	1	3.967 ± 0.1878	< 0.0001***	325.15	115.82
	5	9.844 ± 0.9097	< 0.0001***	955.10	-286.9
A5C	**0**	0.9331 ± 0.03436			
	1	3.997 ± 0.1818	< 0.0001***	328.35	119.02
	5	12.73 ± 0.4291	< 0.0001***	1,264.26	21.65
A6					
Group/Dilution	**Day**	**M ± SD**	**p/initial**	**Effect %/initial**	**Effect %/control**
A6A	**0**	0.9500 ± 0.04330			
	1	1.344 ± 0.07658	0.0004***	41.47	-167.86
	5	1.489 ± 0.07896	0.0002***	56.73	-1,185.88
A6B	**0**	0.9556 ± 0.01002			
	1	1.700 ± 0.1213	< 0.0001***	77.89	-131.44
	5	1.833 ± 0.07071	< 0.0001***	918.16	324.45
A6C	**0**	0.9611 ± 0.03706			
	1	3.433 ± 0.1190	< 0.0001***	257.19	47.86
	5	10.26 ± 0.7280	< 0.0001***	967.52	-275.09
A7					
Group/Dilution	**Day**	**M ± SD**	**p/initial**	**Effect %/initial**	**Effect%/control**
A7A	**0**	0.9000 ± 0.1044			
	1	1.156 ± 0.04120	0.0369*	28.44	-180.89
	5	1.300 ± 0.06236	0.0046**	44.44	-1,198.17
A7B	**0**	0.9556 ± 0.03859			
	1	1.433 ± 0.06667	< 0.0001***	49.95	-159.38
	5	1.522 ± 0.06620	< 0.0001***	59.27	-1,183.34
A7C	**0**	0.9500 ± 0.03727			
	1	3.956 ± 0.08184	< 0.0001***	316.42	107.09
	5	7.578 ± 0.4403	< 0.0001***	697.68	-544.93

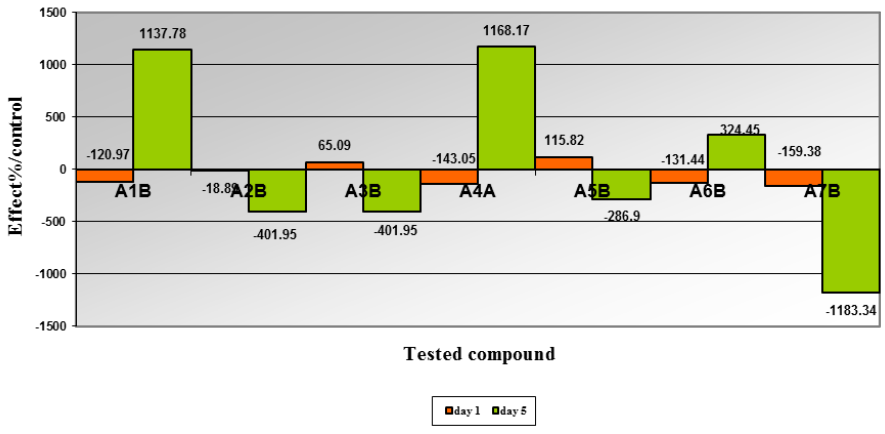

Figure 3.

Modifications in radicle elongation induced by compounds A1 – A7, compared to control group (days 1, 5), dilution B

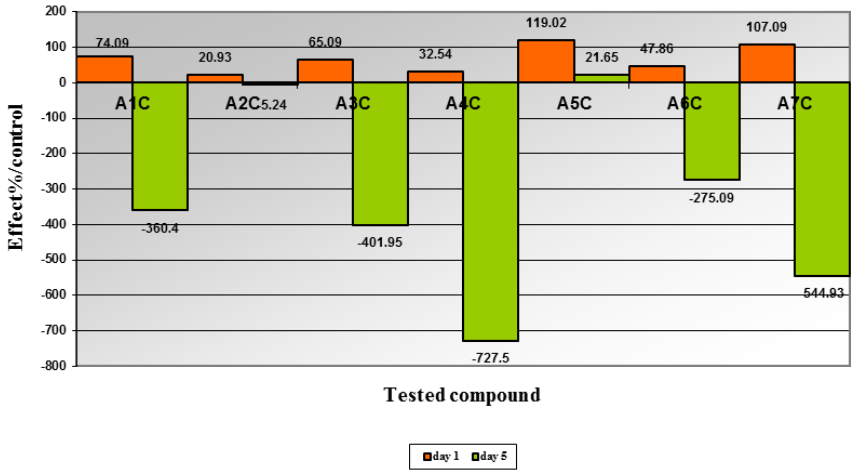

Figure 4.
Modifications in radicle elongation induced by compounds A1 – A7, compared to control group (days 1, 5), dilution C

In vivo toxicity

Body weights of the animals were statistically significant higher against the baseline (Figure 5), as was the case for the animals in the control group.

Immediate systemic effects (recorded during the first hour after the administration) for the tested compounds were as follows:

- A3: intense vasodilation accompanied by hyperaemia in tail, muscle tone disorders, with odd stance of no limb support;
- A4: hyper-reflectivity to acoustic stimuli, eyelid ptosis, obvious vasodilation in tail, affected muscle tone;
- A5: mild vasodilation, sluggish movement, contortion-like posture;
- A6: vasodilation in tail, odd stance of no limb support, hyper reflectivity (3 animals from the group);
- A7: intense vasodilation in tail and paws, accompanied by hyperaemia, motor hyperactivity and aggressive behaviour registered in day 2 and 3 of the study.

Further pharmacodynamic researches on baseline glycaemia will be performed with a dose 10x smaller than the one used to determine acute toxicity.

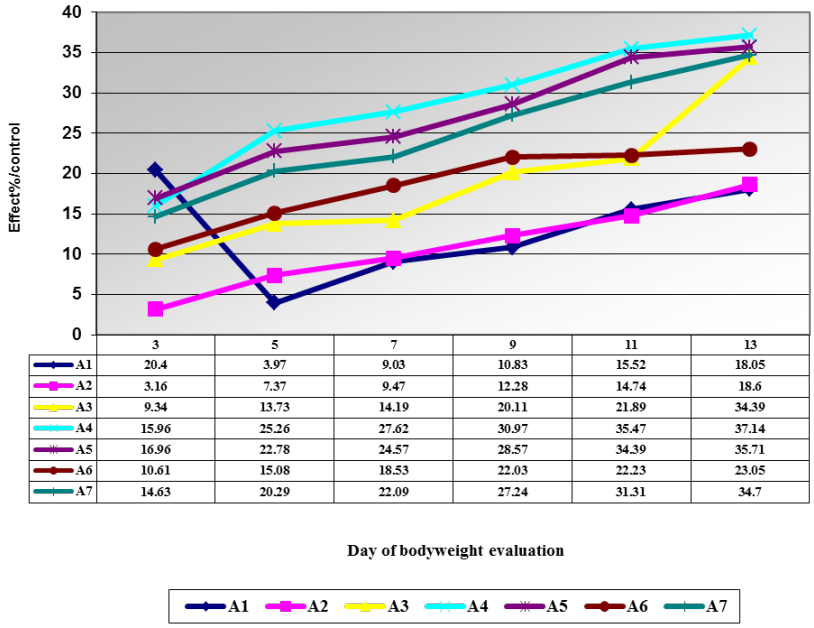

Figure 5.
Alterations of body weight in animals treated with compounds A1 – A7, against baseline

QSAR equation, a multilinear regression model, has proven a very high discriminative capability for the prediction of biological activity in correlation to specific mechanisms of cellular/acute toxicity

levels for the tested compounds (the molecular descriptors that this QSAR model has employed were constitutive and/or conformational).

Nevertheless, remaining within the present predictive model, the research team suspects that a lesser number of substances (14) used as „training set" may lead to differences between calculated data and the ones obtained experimentally.

In our opinion information obtained by *in vitro* evaluation on plant cells has provided the basis for consecutive determination of *in vivo* acute toxicity in laboratory animals. For example, increasing by 10x the solution's concentration (from 10^{-6} to 10^{-5}) has not inhibited the radicular elongation against the baseline, which remained highly significant statistically as described in Tables III – IV. This aspect suggests that, in probing towards the lethal doses 50%, one can select the doses observing the relation:

Probing dose 2 (Dp_2) = Dp_1 mg/kg bw x 10,

where Dp_1 represents the start probing dose.

The sustained radicle growth when doses increased 10 times suggests that toxicity of the tested compounds in plant cell is low, a parameter that is correlating with the safety index of authorized pharmaceutical products. According to regulations in force, the safety index determined in laboratory animals (LD_{50}/ED_{50} ratio) for medicines must be greater than 10 (no risk of toxic effects upon 10x increase in dose).

In vivo researches demonstrated that neither the outer aspect of the animals nor their motor behaviour suffered alteration and that at 1,000 mg/kg bw no toxicity was recorded. Based on these observations the tested compounds were placed in the class of low toxicity, according to Hodge-Sterner scale [11].

Nevertheless, the correlation refers to rodent laboratory animals and extrapolation of toxicological conclusions to humans is difficult given the differences in the enzymatic equipment of the species.

Table V

Classification of chemical substances by LD_{50}: Hodge and Sterner Scale [11]

Degree of toxicity	Term of assessing the toxicity	LD_{50} (per os) mg/kg bw
1	Extremely toxic	$LD_{50} < 1$
2	Highly toxic	$1 < LD_{50} \leq 50$
3	Moderate toxic	$50 < LD_{50} \leq 500$
4	Low toxic	$500 < LD_{50} \leq 5000$
5	Practically non-toxic	$5000 < LD_{50} \leq 15000$
6	Relatively without toxicity	$15000 \leq LD_{50}$

Conclusions

The authors concluded that, by using alternative methods of screening for acute toxicity (QSAR, *in vitro* toxicity), the number of laboratory animals necessary for determining this parameter was reduced to minimum. Moreover, starting from the data yielded by the present research, it was possible to establish the doses which the authors have used in further pharmacodynamic research already underway.

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