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Short Communication

Evaluation of Betulin Mutagenicity by *Salmonella*/Microsome Test

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Abstract
Purpose: Betulin is a pentacyclic triterpene found in the outer barks of innumerous plants. This secondary metabolite is easily isolated from plants with the major interest in converting it to betulinic acid, which pharmacological properties were much more exploited than betulin. But, investments in the own betulin have been grown since no chemical step is necessary. In this study we focused the precursor betulin in order to evaluate its mutagenicity by *Salmonella*/microsome assay (Ames test).
***Methods*:** The Ames test was carried out using a commercial betulin exposed to *Salmonella typhimurium* strains TA98, TA100, TA102, and TA97a, in experiments with (+S9) and without (-S9) metabolic activation.
***Results*:** Betulin was unable to increase the number of revertants (+S9 and -S9 metabolic activation) showing the absence of any mutagenic effect by Ames test.
***Conclusion*:** This study allowed attribute safety to betulin being important for exploiting its pharmacological uses.

Introduction

Betulin, betulinol, betuline, or betulinic alcohol (3-lup-20(29)-ene-3β,28-diol)[1] is an abundant pentacyclic lupane-type triterpenoid ubiquitously occurring in many plants as described by Ferraz et al.,[2] besides being a compound easily isolable, which gives to betulin a role as a precursor biomolecule to betulinic acid.
Betulinic acid is produced by plants in small amounts[3,4] justifying the conversion from betulin to betulinic acid, which the pharmacological activities have been more exploited than betulin.[1,5] However, a new reasoning to avoid this expensive chemical steps of conversion (betulin to betulinic acid) is simply addressing the pharmacological focus to betulin.
It is known that the oil from birch bark of *Betulae pix* has been used for some skin diseases, the eczema and psoriasis;[6] betulin also exerts an anticonvulsant action in mice, showing ability in penetrating the blood-brain barrier due its lipophilic property;[7] betulin has antibacterial, antifungal, and antiviral properties,[1] anticancer and chemopreventive potential;[8] betulin from *Dipteryx alata* protects against the neuromuscular effects of *Bothrops jararacussu* snake venom either *in vitro*[9] as *in vivo*.[2]
Advantages of betulin is its good bioavailability when administered intraperitoneally (i.p.) or subcutaneously (s.c.) described in a preliminary pharmacokinetic analysis, besides it did have no subchronic toxicity in rats (injected i.p.) or dogs (injected s.c.).[10]
In this study we evaluated the mutagenicity of a commercial betulin towards four *Salmonella typhimurium* strains (TA98, TA97a, TA100, and TA102) by Ames *Salmonella*/microsome assay, since does not exist toxicological studies concerning the safety of this biomolecule.

Materials and Methods

Betulin

Commercial betulin was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used throughout this study.

Preliminary toxicity assay

Before the Ames test evaluation is mandatory to know the toxicity of betulin to *Salmonella* strains. Here, the toxicity of betulin was firstly submitted to TA98 and TA 100, both being histidine dependent as all other *Salmonella* tester strains, both contain a deletion mutation through the *uvrB-bio* genes;[11] a mutation (*rfa*) in all strains that leads to a defective lipopolysaccharide (LPS) layer;[11] and presence of plasmid pKM101.[12] The reversion event caused to TA98 and TA100 are frameshift and base-pair substitution, respectively. Betulin (100 mg) from Sigma Chemical Co. (St. Louis, MO, USA) were dissolved in dimethyl sulfoxide by which 10.0, 7.5, 5.0, and 2.5 mg/plate were initially assayed using TA98 and TA100 strains. Toxicity was apparent either as a reduction in the number of His+

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revertants or as an alteration in the auxotrophic background,[13] and were visualized in the two first concentration (10.0 and 7.5 mg/plate), which lead us to use betulin 5.0 mg/mL (the highest non-toxic dose) in a new set of assay, where 5.0, 3.75, 2.5, 1.25 and 0.63 mg/plate were selected for further Ames assay.

In vitro mutagenicity assay

Mutagenic activity was tested by the *Salmonella*/microsome assay, using the *S. typhimurium* tester strains TA98, TA100, TA102, and TA97a,[14] kindly provided by B.N. Ames (Berkeley, CA, USA), with and without metabolization by the preincubation method.[15] This assay was made as described by Yoshida et al.,[16] as following: the strains from frozen cultures were grown overnight for 12–14 h in Oxoid Nutrient Broth No. 2. The S9 fraction, prepared from livers of Sprague-Dawley rats treated with the polychlorinated biphenyl mixture Aroclor 1254 (500 mg/kg), was purchased from Molecular Toxicology Inc. (Boone, NC, USA) and freshly prepared before each test. The metabolic activation system consisted of 4% of S9 fraction, 1% of 0.4M $MgCl_2$, 1% of 1.65M KCl, 0.5% of 1M D-glucose-6-phosphate disodium and 4% of 0.1M nicotinamide adenine dinucleotide phosphate (NADP), 50% of 0.2M phosphate buffer and 39.5% sterile distilled water.[14] Defined the betulin concentrations by preliminary toxicity tests, in all subsequent assays were used the upper limit of the dose range tested was either the highest non-toxic dose or the lowest toxic dose. The concentrations varied from 0.63 to 5.0 mg/plate for betulin. The various concentrations of betulin to be tested were added to 0.5 mL of 0.2M sodium phosphate buffer (pH 7.4), or to 0.5 mL de 4% S9 mixture, with 0.1 mL of bacterial culture and then incubated at 37°C for 20 min. Next, 2 mL of top agar (0.6% agar, histidine and biotin 0.5 mM each, and 0.5% NaCl) was added and the mixture was poured on to a plate containing minimal glucose agar (1.5% Bacto-Difco agar and 2% glucose in Vogel-Bonner medium). The plates were incubated at 37°C for 48 h and the His(+) revertant colonies were counted manually. All experiments were carried out in triplicate. The standard mutagens used as positive controls in experiments without S9 mix were 4-nitro-*O*-phenylenediamine (10 μg/plate) for TA98 and TA97a, sodium azide (1.25 μg/plate) for TA100 and mitomycin (0.5 μg/plate) for TA102. 2-anthramine (1.25 μg/plate) was used with TA98, TA97a and TA100 and 2-aminofluorene (1.25 μg/plate) with TA102 in the experiments with metabolic activation. DMSO served as the negative (solvent) control (50 μL/plate). Figure 1 shows representatives His(+) revertant colonies (A) and positive control (B).

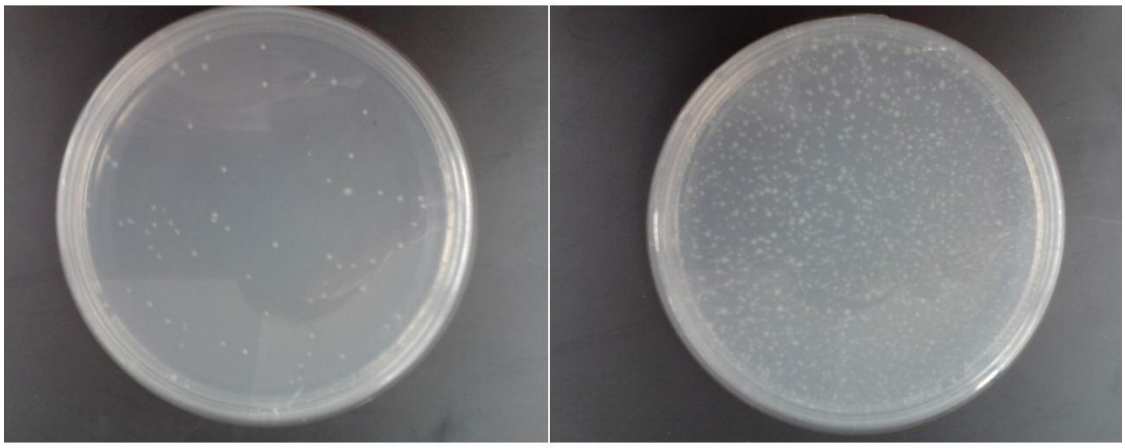

Figure 1. Photography of bacterial colonies grown in representative His(+) revertants (A) and positive control (B).

The mutagenic index (MI) was calculated for each concentration tested, this being the average number of revertants per plate with the test compound divided by the average number of revertants per plate with the negative (solvent) control. A sample was considered mutagenic when a dose-response relationship was detected and a two-fold increase in the number of mutants (MI $\geq$ 2) was observed with at least one concentration.[17]

Statistical analysis

The results of the mutagenicity tests were analyzed with the Salanal statistical software package (U.S. Environmental Protection Agency, Monitoring Systems Laboratory, Las Vegas, NV, version 1.0, from Research Triangle Institute, RTP, North Carolina, USA), adopting the Bernstein et al. model.[18] The data (revertants/plate) were assessed by analysis of variance (ANOVA), followed by linear regression.

Results and Discussion

Our experience with *Dipteryx alata* Vogel,[19] one of many betulin-plants,[2] showed an unexplained facilitatory effect on mouse nerve-muscle synapse,[9] besides it possesses antiophidian properties against the neuromuscular and myotoxicity, two known toxic effects of *Bothrops jararacussu*,[20] and *Crotalus durissus terrificus*[21] venoms

(protection of betulin against the toxic effects of *Bothrops jararacussu* venom > *Crotalus durissus terrificus* venom).
Aiming to confirm the antiophidian efficacy of betulin against *Bothrops jararacussu* venom, we further assayed it in *in vivo* experimental model, using rat external popliteal/sciatic nerve-tibialis anterior muscle (EPSTA) preparation.[2] Intraperitoneally (i.p.) injections of betulin were compared to intravenously (i.v.) commercial bothropic antivenom (CBA) injections, in venom-pretreated animals. No statistically difference was observed between betulin and CBA, showing a promising complementary therapeutical use for betulin, either in veterinary as in human area.
However, chemicals can induce damage in germ line causing fertility problems and leading to mutations in future generations, besides they also are capable of inducing cancer. Gene mutations can occur with only a single base changes (base-pair substitution mutants), or one or a few bases inserted or deleted (frameshift mutants), and are readily measured in bacteria and other cell systems.[22] Thus, the evaluation of betulin mutagenicity is an inevitable step in the safety assessment.
Here, the Ames *Salmonella*/mutagenicity assay, a short-term bacterial assay, was chosen for testing betulin, since it is able in identifying substances that can produce genetic damage that leads to gene mutations. After preliminary test, five concentrations of betulin were submitted to Ames test: 0.63, 1.25, 2.5, 3.75, and 5.0 mg/plate.
Table 1 shows betulin at various concentrations exposed to *S. typhimurium* TA98, TA97a, TA100 and TA102 tester strains, without metabolic activation.

Table 1. Revertants/plate, standard deviation and mutagenicity index (in brackets) for the strains TA98, TA100, TA102 and TA97a of *S. typhimurium* after treatment with various doses of phytochemical Betulin without metabolic activation (−S9)

Treatments mg/plate		Number of revertants (M ± SD)/plate and MI			
		TA 98	TA 100	TA 102	TA 97a
		- S9	- S9	- S9	- S9
Betulin	**0.0**[a]	19 ± 2	120 ± 15	341 ± 5	178 ± 12
	0.63	19 ± 4 (1.0)	105 ± 2 (0.9)	338 ± 27 (1.1)	221 ± 27 (1.2)
	1.25	21 ± 5 (1.1)	89 ± 1 (0.7)	367 ± 28 (1.1)	185 ± 33 (1.0)
	2.50	19 ± 3 (1.0)	96 ± 10 (0.8)	299 ± 18 (0.9)	173 ± 16 (0.9)
	3.75	20 ± 5 (1.1)	99 ± 22 (0.8)	314 ± 67 (0.9)	197 ± 12 (1.1)
	5.00	18 ± 3 (1.0)	112 ± 10 (0.9)	350 ± 54 (1.0)	161 ± 52 (0.9)
	Control +	1044 ± 56[b]	1161 ± 292[c]	968 ± 77[d]	1175 ± 43[b]

M ± SD = mean and standard deviation; MI = mutagenicity index; [a]Negative control: dimethylsulfoxide (DMSO - 50 µL/ plate); Control+ = Positive control - [b]4 -nitro-*o*-phenylenediamine (NOPD – 10.0 µg/ plate – TA98, TA97a); [c]sodium azide (1.25 µg/ plate – TA100); [d]mitomycin (0.5 µg/ plate – TA102), in the absence of S9 (−S9).

It is important to remark the DNA (deoxyribonucleic acid) sequences of the target mutations of *Salmonella* tester strains used in this study. The *hisD3052* mutation carried by TA98 is a -1 frameshift mutation which affects the reading frame of a nearby repetitive -C-G-C-G-C-G-C-G- sequence,[23] which reversion of the mutation back to the wild-type state by 2-nitrofluorene and various aromatic nitroso derivatives of amine carcinogens. In this study we used 4 -nitro-*o*-phenylenediamine as a positive control. Note in TA98 column that brackets expressing the MI are all < than 2.0, showing no mutagenicity in all tested concentrations.
The *hisG46* marker in TA100 strain results from the substitution of a leucine (GAG/CTC) by a proline (GGG/CCC),[24] that is reverted to the wild-type state by mutagens that cause base-pair substitution mutations primarily at one of the GC pairs, as that showed by sodium azide (positive control). In this study, no mutagenic activity was seen in any betulin concentrations when submitted to TA100.
TA102 strain contains AT base pairs at the *hisG428* mutant site, which mutation is carried on the multi-copy plasmid pAQ1 aiming to amplify the number of target sites, which in turn confers tetracycline resistance, a convenient marker to detect the presence of plasmid. In this strain, the *uvrB* gene was retained (differently of other *Salmonella* strains) making the bacterium DNA repair proficient, and enhancing the ability of this strain to detect DNA cross-linking agents, as those caused by bleomycin and mitomycin C (used here as positive control).[25] The *hisG428* mutation is an ochre mutation (TAA) in the *hisG* gene, which the reversion involves transitions and transversions events. In our study none concentrations of betulin caused mutagenicity when submitted to TA102.
The *hisD6610* mutation carried by TA97 is +1 frameshift mutation (cytosine) resulting in a run of 6 cytosines (-C-C-C-C-C-C-), which reversion of the mutation back to the wild-type state occurs by the same compounds as seen by TA98,[26] as 4 -nitro-*o*-phenylenediamine used here as positive control. Betulin in all concentrations used in this study caused no mutagenicity in TA97a strains.
Table 2 shows betulin at various concentrations exposed to *S. typhimurium* TA98, TA100, TA102, and TA97a tester strains, with metabolic activation (+S9).

Table 2. Revertants/plate, standard deviation and mutagenicity index (in brackets) for the strains TA98, TA100, TA102 and TA97a of *S. typhimurium* after treatment with various doses of phytochemical Betulin with (+S9) metabolic activation

Treatments (mg/plate)		Number of revertants (M ± SD)/plate and MI			
		TA 98	TA 100	TA 102	TA 97a
		+ S9	+ S9	+ S9	+ S9
Betulin	**0.0**[a]	24 ± 9	107 ± 20	205 ± 11	84 ± 16
	0.63	27 ± 4 (1.1)	117 ± 6 (1.1)	219 ± 19 (1.1)	102 ± 24 (1.2)
	1.25	27 ± 7 (1.1)	97 ± 5 (0.9)	199 ± 21 (1.0)	84 ± 6 (1.0)
	2.50	29 ± 3 (1.2)	105 ± 15 (1.0)	223 ± 38 (1.1)	80 ± 15 (0.9)
	3.75	27 ± 1 (1.1)	95 ± 17 (0.9)	240 ± 27 (1.2)	71 ± 16 (0.8)
	5.00	37 ± 5 (1.5)	94 ± 17 (0.9)	183 ± 21 (0.9)	74 ± 13 (0.9)
	Control +	1551 ± 81[e]	2627 ± 297[e]	1043 ± 39[f]	1252 ± 30[e]

M ± SD = mean and standard deviation; MI = mutagenicity index; [a]Negative control: dimethylsulfoxide (DMSO - 50 µL/ plate); Control+ = Positive control - [e]2-anthramine (1.25 µg/ plate – TA 97a, TA98, TA100); [f]2-aminofluorene (10.0 µg/ plate – TA102), in the presence of S9.

As some carcinogenic chemicals are biologically inactive unless they are metabolized to active forms by cytochrome P450 enzymes, an exogenous mammalian organ activation system needs to be added to the petri plate together with the chemical and the bacteria.[11] Here, we purchased a commercial metabolic activation system that consists of a 9000xg supernatant fraction of a rat liver homogenate (S9 microsomal fraction), that in presence of nicotinamide adenine dinucleotide (NADH) and cofactors for nicotinamide adenine dinucleotide phosphate (NADPH) (S9 mix), enzymes are delivered to the test system. Positive controls used in this metabolic activation step were 2-anthramine (TA 97a, TA98, TA100) and 2-aminofluorene (TA102), with visible grown of colonies in the histidine absence, showing the mutation ability of these mutagens. Even in the presence of metabolic activation all concentrations of betulin exposed to *S. typhimurium* strains were unable to cause any mutation.

The lack of mutagenicity for betulin, a triterpenoid with many pharmacological properties,[1,2,6-9] that has a good bioavailability but no subchronic toxicity (rats and dogs),[10] is very promising. Some positive correlation can be taken with the facilitatory nature of betulin on mouse nerve-muscle synapse[9] with its anticonvulsant action.[7] At the same mode, phenobarbital, a known anticonvulsant, also acts increasing the amplitude of contractile response in mouse phrenic nerve-diphragm preparation,[27] probably involving the glutamatergic regulation, since the role of glutamate as an acetylcholine co-transmitter in motoneurons was already established.[28,29]

Conclusion

In conclusion, betulin is a safety bioactive molecule (for intraperitoneal and subcutaneous administrations, limited by its solubility), with absence of mutagenicity by *Salmonella*/microsome assay.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors report no conflicts of interest in this work.

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