

NON-CLINICAL TOXICITY AND PHYTOTOXICITY OF TWO HERBAL EXTRACTS

ROBERT ANCUCEANU¹, CRISTINA ELENA ZBÂRCEA^{2*}, MARIA COJOCARU-TOMA³,
TATIANA CALALB³, ADRIANA IULIANA ANGHEL¹, SIMONA NEGREȘ², MIHAELA DINU¹,
MARILENA VIORICA HOVANET¹

¹University of Medicine and Pharmacy "Carol Davila" Bucharest, Faculty of Pharmacy, Department of Pharmaceutical Botany and Cell Biology, 6 Traian Vuia Street, 020956, Bucharest, Romania

²University of Medicine and Pharmacy "Carol Davila" Bucharest, Faculty of Pharmacy, Department of Pharmacology, 6 Traian Vuia Street, 020956, Bucharest, Romania

³State University of Medicine and Pharmacy "Nicolae Testemițanu", Faculty of Pharmacy, Department of Pharmacognosy and Pharmaceutical Botany, 165 Stefan cel Mare și Sfint Street, Chisinau, Republic of Moldova

*corresponding author: cristinazbar@yahoo.com

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Abstract

Two herbal extracts, one of *Panax notoginseng* (Burkill) F. H. Chen and the other from oat bran were tested for their toxicity in NMRI mice, *Artemia franciscana* Kellogg and for their phytotoxicity on *Triticum aestivum* L. No lethality was recorded and no changes in the parameters followed up were seen for any of the two extracts tested on mice. Based on the experimental data the two extracts may be classified in the Globally Harmonized System (GHS) category 5. In the *Artemia* test, the *Panax notoginseng* extract was virtually non-toxic (0% lethality); the oat bran extract had slight toxicity, but for all practical purposes it may also be considered non-toxic. In the *Triticum* test a concentration-dependent inhibitory effect on root elongation ($p < 0.001$), was seen at concentrations of 0.5% and higher ($p < 0.001\%$ versus control) in the case of *Panax notoginseng* and at concentrations of 0.25% for the oat bran extract ($p < 0.012$); the results are in agreement with those seen on *Artemia franciscana*.

Rezumat

Două extracte vegetale, unul de *Panax notoginseng* (Burkill) F.H.Chen și celălalt de tărațe de ovăz, au fost testate în privința toxicității pe șoareci NMRI, *Artemia franciscana* Kellogg și în privința fitotoxicității pe *Triticum aestivum* L. Nu s-a înregistrat letalitate și nicio modificare la parametrii urmăriți pentru niciunul din cele două extracte testate la șoarece. Pe baza datelor experimentale cele două extracte pot fi clasificate în categoria 5 a Sistemului Armonizat Global (GHS). În testul *Artemia*, extractul de *Panax notoginseng* a fost practic non-toxic (letalitate 0%); extractul de tărațe de ovăz a avut o toxicitate ușor superioară, dar poate fi considerat practic non-toxic. În testul *Triticum* s-a observat un efect inhibitor dependent de concentrație asupra elongației radiculare ($p < 0,001$), observat la concentrații de 0,5% și mai mari ($p < 0,001\%$ versus martor) în cazul extractului de *Panax notoginseng* și la concentrații de 0,25% și mai mari pentru extractul de tărațe de ovăz ($p < 0,012$); rezultatele sunt în acord cu cele observate pe *Artemia franciscana*.

Keywords: collagen, minocycline, spongioid delivery systems, freeze-drying

Introduction

Panax notoginseng (Burkill) F.H.Chen is one of the several *Panax* species whose dried roots are used under the name "ginseng". It is cultivated in China, mainly in the Southern Provinces Yunnan and Guangxi [20]. The species have been used for a wide variety of indications, from circulatory disorders to alleviation of pain and inflammation [7]; it is often cited as having immunomodulatory properties [15]. Because a number of ginsenosides interact with glutamate receptors inhibiting or activating them [12, 19], we have speculated that a *P. notoginseng* extract might have protective effects against neuropathic pain and initiated a non-clinical

experiment to test this hypothesis in a rodent model (the results will be reported elsewhere).

Oat brans contain important amounts of ferrulic acid, which was shown in non-clinical studies to have beneficial effects in the control of neuropathic pain [28, 31, 32]. On the other hand, oat brans, like other cereal brans are generally rich in sinapic acid, which was shown to have anxiolytic effects mediated by GABA_A receptors [29]. In addition, it has been proven that sinapic acid inhibits TNF α expression [30], an effect which, in theory, could also contribute to a potentially useful effect of this substance in alleviating neuropathic pain. Ferrulic acid (more abundant in oat brans) and sinapic acid (less abundant) are present in their free forms in the

herbal product, but in relatively small amounts; amounts 10 - 30 times higher may be released from the cell walls by acid or alkaline hydrolysis. We therefore developed a method intended to extract first the free hydroxycinnamic acids, followed by an alkaline hydrolysis and extraction of the hydroxycinnamic acids thus release. Alkaline hydrolysis was preferred because previous studies showed that it is superior in its efficacy to acidic hydrolysis [14]. This extract was also assessed in a neuropathic pain model and the results will be reported elsewhere.

Although both extracts are derived from plants widely and long used for therapeutic or nutritional purposes, they have different compositions than their natural sources, which may impact their safety. Therefore, we were interested in assessing their potential toxicity using a rodent model, a brine shrimp model and a phytotoxicity model.

Materials and Methods

Extracts

We intended to use roots of *Panax notoginseng* (Burkill) F. H. Chen to prepare an appropriate extract in the laboratory, but we could not find in Romania a provider (importer) of whole or powdered roots of the species. We have identified on Amazon.de a product marketed as "Notoginseng infusion", with a root image on the package (manufacturer Green Nature Ltd., Hong Kong) and including the mention "GMP certified", and we assumed that it contained a powdered root. When opening the package, we found cubicles containing, according to the manufacturer, "Panax notoginseng 95% extract" (with no additional information on the nature or mode of preparation) and 5% sugar. Because we could not procure pure root powder from other sources, we have used this commercial extract for our researches. We have examined microscopically the extract to identify potentially relevant elements confirming the identity of the herbal source or to detect the potential presence of non-declared ingredients (as such cases have been previously identified and reported by us) [2]. It seems that the extract was obtained by the manufacturer by extraction with water (or a more diluted solvent), as it was 100% soluble in water.

Oat bran was purchased from Sanovita SRL, through an herbal store. We investigated the presence of impurities or foreign matter using a light microscope, on powder clarified with acidified 80% chloral hydrate. Oat bran (150.0 g) have been pulverized and passed through a 425 micron sieve, extracted with ethanol 70% (v/v) three times, at ambient temperature, for 4 hours each time. The extractive solutions were filtered through qualitative filter paper and the pooled filtrated solutions were

concentrated with a rotary evaporator at 60°C and lyophilized (-58°C). The extraction yield for this fraction (containing free hydroxycinnamic acids) was 2.87%. The herbal product exhausted with ethanol 70% was thereafter subjected to successive extraction (repeated three times) with a 1M NaOH in ethanol 70% solution, four hours for each extraction operation. The extractive solutions were filtered by qualitative filter paper and the filtrated solutions were pooled and neutralized with hydrochloric acid to pH~7. The solutions thus neutralized were concentrated using a rotary evaporator (60°C) and then subjected to cryodesiccation (-58°C). Because the obtained lyophilized had a high content in salt (resulted from the neutralization of NaOH with HCl), it was treated with a mixture of ethanol:acetone 2:1, concentrated by rotary evaporation and again subjected to lyophilisation. The yield for this second fraction was 2.43% and it was mixed with the first one, the yield for the global extract thus obtained being of 5.31%. The extract was light-weight, spongy, yellowish and with a sweetish taste. Its chemical analysis will be reported elsewhere, together with the pharmacological evaluation.

Mouse toxicity

According to the OECD guidelines for acute oral toxicity testing after a single dose administration *per os*, we have used an alternative method, the fixed dose procedure, using the limit test [1]. The experiment was conducted on 15 male mice, strain NMRI, divided in three groups of 5, with a mean (standard deviation) weight of 22.35 g (1.57), sourced from the biobase of the University of Medicine and Pharmacy "Carol Davila", Bucharest. The mice had *ad libitum* access to food and water. The temperature and relative humidity were maintained between 21 - 24°C and 45 - 60% and assessed with a thermometer and hygrometer, respectively. All procedures were performed in compliance with the rules on bioethics in the research on experimental animals for scientific purposes, according to Law no. 43/2014 on the protection of animals used for scientific purposes and Directive 2010/63/UE of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, including approval of the local ethics committee. The experiment took place for 14 days, in the Laboratory of Experimental Pharmacology of the Faculty of Pharmacy, Bucharest, Romania.

The extracts were administered in fasting conditions, for four hours before administration. Food was added two hours post-administration and the rodents had access to water along the whole experiment. The animals were randomly selected, marked for individual identification and kept in their cages. The limit test is used mainly when there

is available information showing that the test substance or extract is nontoxic. The limit dose was 2000 mg/kg bw for each test group, administered as an aqueous suspension 20% (w/w), initially to one animal and followed by later administration to four additional animals in each group, because no death was recorded in the first. The control group (C) received 0.1 mL/10 g distilled water (vehicle used to prepare suspensions). After administration, the mice were observed individually during the first 60 minutes, then periodically during the first four hours and daily for 14 days. The occurrence of the following effects was observed: tremor, hyper-salivation, diarrhoea, lethargy, sleep and coma. The body weight was determined at baseline and during days 2, 4, 7, 10, and 14 days from the beginning of the experiment.

Artemia franciscana nauplii toxicity

The brine shrimp lethality test was based on adaptations of the B. M. Meyer *et al.* (1982) [16] and T. W. Sam (1993) methods [24], as reported by other sources [5, 26]. Artificial marine water was prepared by dissolving a commercial salt mixture (CoralMarine, Grotech) in distilled water, at a concentration of 33.4 g/L. *Artemia franciscana* (Kellogg, 1906) cysts, from Grate Salt Lake (USA), were purchased from Ocean Star International (USA), repackaged by S.K. Trading (Thailand). The process of hatching was started about 48-50 hours before the initiation of the lethality test, by introducing about 2 grams of cysts in 500 mL artificial marine water, at 25°C, under continuous aeration. The hatching started after about 40 hours and the recently hatched organisms (nauplii) were used within a 10 hours interval from hatching. The nauplii were separated from cyst residues and concentrated to an appropriate density for experimental use, with the help of a light source placed towards the lower part of a large crystallizing dish [5] in which they were placed. Afterwards they were transferred with a micropipette in a small 20 mL crystallizing dish, from which they were collected for testing.

For the *Panax notoginseng* extract the following concentrations were tested: 1860, 930, 465, 232 and 116 µg/mL; for the oat bran extract, solutions of 2012, 1006, 503, 252 and 126 µg/mL were used. In both cases the concentrations were chosen to be two-fold (log2) dilutions varying roughly between 2000 and 100 µg/mL, based on the authors' previous experience with the effects of herbal extracts in this test. All extracts were dissolved in marine artificial water and subsequent dilutions were carried out with the same solvent. The test included a negative control group (artificial marine water) and five concentrations of the test solutions, each with three replicates of 10 - 12 nauplii. The test was carried out in a 24 (6 x 4) multiwell test plate.

In each plate 2 mL of the relevant solution was placed and subsequently 10 - 12 larvae were transferred from the crystallizing dish (after concentration in a free well of the plate) in each well. The plate was covered with the lid and incubated at 25°C, in the dark. After 24 hours the dead nauplii were counted; larvae with no internal or external movements for at least 10 seconds were considered dead.

Phytotoxicity test

The Constantinescu bioassay (*Triticum* test), as described in other publications, [4, 6] was used. Each extract was tested in a different experiment. For the *Panax notoginseng* extract 6 (1.0%, 0.5%, 0.10%, 0.05%, 0.01%, 0.001%) and for the oat bran extract 5 serial dilutions (1.0%, 0.5%, 0.25%, 0.12%, 0.06%) were prepared. The concentrations were chosen based on our previous experience with the effects of herbal extracts in this test; because we assumed that the oat bran extract was less toxic than *Panax notoginseng*, we used slightly higher concentrations at the lower end of the concentration range for the former. A negative control group consisting of distilled water was used in each case. The comparison of root length was performed 72 hours from baseline and the genetic material was stained with acetic orcein. Microscopic examinations were performed on a Nikon Labophot 2 light microscope.

Statistical analysis

All analyses were carried out in R 3.2.0, using the 'car' [9], 'fBasics' [23] and 'drc' [22] packages. Animal weights were compared for all groups with (classical) one-way ANOVA; wheat rootlet lengths were compared with one-way Welch ANOVA for the *Panax notoginseng* extract and with Welch ANOVA performed on ranks for the oat bran extract. For sensitivity analysis we also applied one-way ANOVA with White-corrected covariance matrices ('car' R package) for wheat lengths and the results were shown to be robust. The assumption of homoscedasticity was assessed using the Levene test ('car' R package) and by the visual examination of scale-location plots. The assumption of normality was assessed by the visual inspection of quantile-quantile plots and histograms, and formally by the Shapiro-Wilk test ('fBasics' package). No outliers were excluded from analysis.

The dose-response curve for the *Artemia franciscana* lethality test was non-linearly modelled with a two-parameter log-logistic function (the lower limit being set at 0 and the upper limit at 1), using the 'drc' R package. The model was selected from several ones based on the log likelihood value, Akaike's information criterion (AIC) and the p - value from a lack-of-fit test, all of which were consistent.

Results and Discussion

Mouse toxicity

No lethality was recorded and no changes in the parameters followed up were seen for any of the extracts tested. The evolution of body weight of the mice in the three lots during the experiment is shown graphically in Figure 1. The changes in body weight expressed as a percentage of the initial values are shown in Figure 2 and the changes expressed as percentage of the control group, in Figure 3. Such lack of toxicity is often a feature of many herbal extracts, as also reported for an extract of *Euonymus europaeus* L. fruit (*Celastraceae*) [25] or for a *Punica granatum* L. peel extract [11]. Due to lack of toxicity we used only male mice, as using female mice would have been a waste of resources.

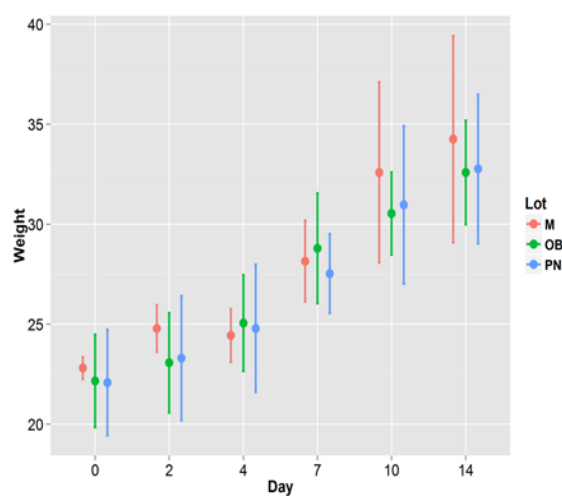


Figure 1.

Evolution of body weight of the animals during the experiment (M = control group; OB = oat bran extract; PN = *Panax notoginseng* extract)

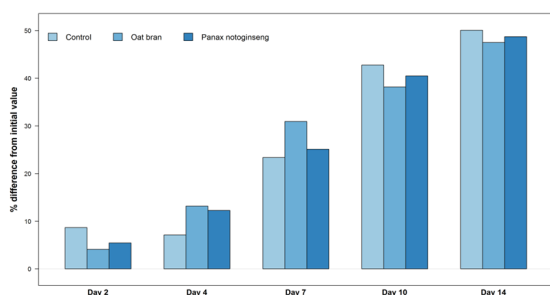


Figure 2.

Evolution of body weight of the animals as a percentage of the initial body weight

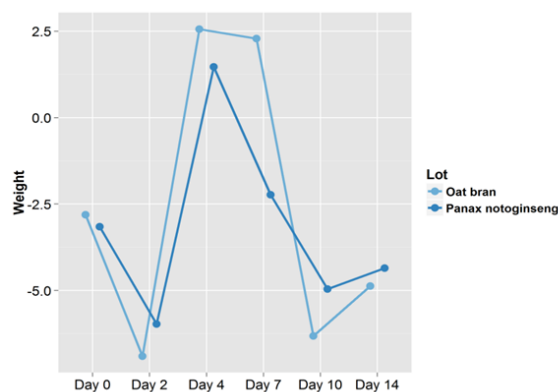


Figure 3.

Evolution of mean body weight of the test animals as a percentage of the control group mean body

The body weight of the animals has grown statistically significant or marginally significant ($p < 0.03$ for the control and oat bran group; $p = 0.0625$ for the *Panax notoginseng* group) compared with the initial values in each lot; such a growth is physiological, as the animals have been fed appropriately during the experiment. For the test groups, the body weight variations were small and not unidirectional, indicating no negative effect on body weight in comparison with the control group (differences were not more than 7% from the control group).

Based on these results, according to the OECD guideline 420 for assessing oral toxicity following single dose administration, the two extracts may be classified in the Globally Harmonized System (GHS) category 5.

Artemia franciscana lethality test

For the *Panax notoginseng* extract, at none of the concentrations tested (116-1860 $\mu\text{g/mL}$) was recorded any lethality on nauplii. In other words, the LC_{50} (and even LC_{10}) is higher than 1860 $\mu\text{g/mL}$. In the scientific literature, extracts with an LC_{50} of 30-100 $\mu\text{g/mL}$ have been classified as "mildly toxic" [17]; in comparison, the *Panax notoginseng* extract may be considered as virtually non-toxic, a characterization in agreement with the results from the mouse toxicity experiment.

For the oat bran extract, rather surprisingly a slight toxicity was detected, although for all practical purposes it may be considered non-toxic. An LC_{10} of 1571.12 $\mu\text{g/mL}$ and an LC_{50} of 9214.43 $\mu\text{g/mL}$ were calculated (Figure 4). For comparison, LC_{50} for alcoholic extracts (methanolic and ethanolic) of *Morella serata* (Lam.) Killick (*Myricaceae*) have been estimated to less than 1 $\mu\text{g/mL}$ [3], whereas for extracts of various parts of *Euphorbia hirta* L. (*Euphorbiaceae*), LC_{50} values varying between 82.7 and 1589 $\mu\text{g/mL}$ were reported [21].

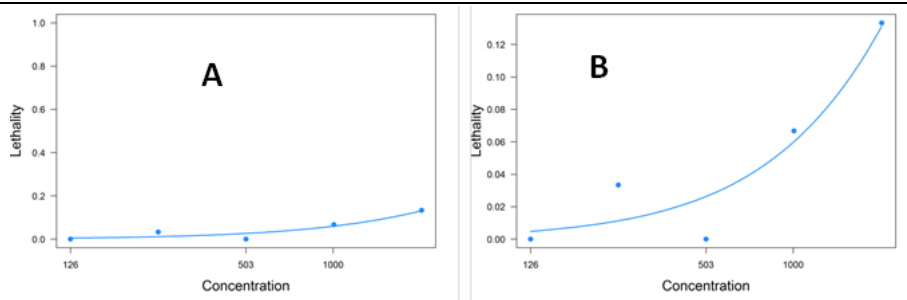


Figure 4.

Artemia franciscana lethality concentration-response curve for the oat bran extract. The x scale is logarithmic.

A. The curve on the full y scale. B. Zoomed-in representation, with the y axis limited to 12% lethality.

Triticum phytotoxicity test

The variation of the wheat main rootlets under the influence of the *Panax notoginseng* extract after 72 hours of treatment is shown in Figure 5A and under the influence of the oat bran extract after the same treatment duration, in Figure 5B. There was a concentration-dependent inhibitory effect on root length ($p < 0.001$), seen only at concentrations of 0.5% and higher ($p < 0.001\%$ versus Control) in the case of *Panax notoginseng* and at concentrations of 0.25% for the oat bran extract ($p < 0.012$). The

results are in agreement with those seen on *Artemia franciscana*, where the oat bran extract was slightly more toxic than that of *Panax notoginseng*. In the wheat rootlets treated with both extracts at the higher (inhibitory) concentrations, relatively rare dividing cells were seen (in division phases with normal features); most cells were in interphase, some with hypertrophied nucleoli. At the lower (non-inhibitory) concentrations the cells were similar to those of the control group, with cells in normal division phases (a few with discrete tropokineses).

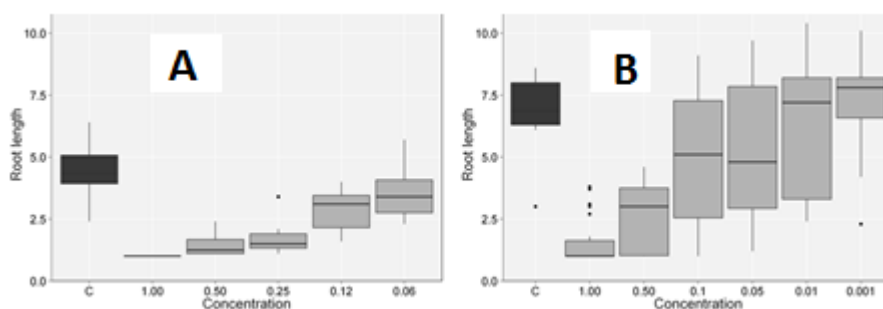


Figure 5.

Variation of the wheat root length as a function of concentration, measured at 72 h from baseline.

A. Oat bran extract. B. *Panax notoginseng* extract.

It is not clear why a product derived from cereal brans should have a toxicity slightly higher than that of an extract derived from a saponin-containing plant. It may be related to the nature of the extracts, as the one from *Panax notoginseng* is fully water soluble and thus might have low amounts of saponins. On the other hand, ginsenosides, the main components of *Panax notoginseng* have a rather atypical saponin structure (four ring dammarane type) and has shown good tolerability in chronic toxicity experiments in rats at doses up to 375 mg/kg bw/day [13]. Because the reagents used to prepare the cereal bran extract were of analytical grade (and not food grade), it is also possible that reagent or solvent impurities may have contributed to the slightly higher toxicity of this extract.

It is to be assumed that herbal extracts of innocuous herbals sources such as cereal bran or ginseng are

likely to be harmless in their effects on animal or plant organisms, but because toxicity is a property heavily dependent on concentration and extraction leads to a selective concentration in certain compounds, such an assumption needs experimental verification. Our results confirmed the harmless character of the two extracts, although the one derived from cereal bran had slight toxicity in high doses. The oat bran extract was intended to be enriched in phenolics, especially sinapic and ferulic acid, both natural substances known for their low toxicity [10, 18]. A wheat bran extract has been evaluated in a subchronic toxicity study and triggered no adverse effect at doses up to 4354 mg/kg bw/day [8]. No such study was identified for an oat bran extract, but it is to be expected that its safety profile is similar, as indicated by our data. The toxicological literature on *Panax notoginseng*

(Burkill) F. H. Chen is very limited, but the scarce data indicate that extracts obtained from the typical herbal product are more toxic than indicated by our findings, toxic effects being reported in non-clinical experiments on the kidney, liver and other organs [28]. The high hydrosolubility of the extract and the sweet taste of the commercial extract assessed by us tend to suggest a rather atypical composition, which might explain the low level or absence of toxicity in our tests.

Conclusions

Mouse and brine shrimp toxicity, as well as wheat phytotoxicity experiment have shown that the two extracts tested are likely to be harmless in human subjects (Globally Harmonized System category 5). The cereal bran extract was slightly more toxic than the *Panax notoginseng* one in the more sensitive assays on *Artemia franciscana* and *Triticum*.

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