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# *MACLEAYA CORDATA* EXTRACT AND SANGROVIT® GENOTOXICITY. ASSESSMENT *IN VIVO*

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**Background**: Sanguinarine (SG) has been reported to form DNA adducts *in vitro* and increase the levels of DNA single strand breaks in the blood and bone marrow of mice treated intraperitoneally with SG. Recently, we showed no genotoxic effects of orally administrated 120 mg/kg feed *Macleaya cordata* extract (a mixture of sanguinarine and chelerythrine) in pigs or rats in 90-day studies. The goal of this paper was to assess the possible genotoxicity of *M. cordata* extract when included as a dietary admixture to rodents at concentrations providing 600 mg/kg feed and 100, 7000 or 14000 mg/kg feed Sangrovit® (natural feed additive containing *M. cordata* extract and powdered *M. cordata*) in a 90-day pilot study.

**Methods and Results**: The rats consumed *ad libitum* either the standard diet or the diets containing 367 ppm of sanguinarine and chelerythrine in *M. cordata* extract, and 5, 330, or 660 ppm of total alkaloids in Sangrovit® for 90 days. The DNA adducts formation in liver was analyzed by $^{32}$P-postlabeling technique and DNA single strand breaks in lymphocytes were evaluated by Comet assay. The results showed that *M. cordata* extract and/or Sangrovit® induced no DNA damage to rat lymphocytes or hepatocytes after 90-days oral administration.

**Conclusions**: Data from the studies described in this paper and the fact that Sangrovit® given to the rats in our experiments were higher than the recommended dose (50 to 100 mg/kg feed), argue strongly in favour of the use of Sangrovit® in live stock.

## INTRODUCTION

*Macleaya cordata* (Willd.) R.Br. (Papaveraceae) is used in traditional Chinese medicine for its anti-inflammatory and antimicrobial activities[1]. *M. cordata* contains isoquinoline alkaloids including sanguinarine (SG), chelerythrine (CH), dihydrosanguinarine (DHSG), dihydrochelerythrine (DHCH), protopine, homochelidonine, α-allocryptopine, angoline, bocconine, bocconoline, chelitunine, chelirubine, coptisine, cryptopine, dehydrocheilanthifoline, ethoxychelerythrine, ethoxysanguinarine, macarpine, oxysanguinarine, and protopine-*N*-oxide[2]. Its alkaloid extract (*M. cordata* extract) called sanguiritrin contains mainly SG and CH and a minor quantity of protopine and α-allocryptopine[3]. Both alkaloids SG and CH are considered to be responsible for the plethora of pharmacological activities of *M. cordata*. *M. cordata* extract and powdered *M. cordata* aerial parts are active components of Sangrovit®, a natural feed additive for rearing pigs, broilers, and dairy cattle[4]. Sangrovit® is standardized as 1.5 w/w % SG. The Achilles heel of safe uses of SG and/or CH containing preparations, oral hygiene products and Sangrovit®, is SG reported genotoxicity. We reported that SG forms DNA adducts *in vitro*[5]. Ansari et al.[6] found increased levels of DNA single strand breaks in the blood and bone marrow of mice treated intraperitoneally with SG. Recently we showed no genotoxic effects of orally administrated 120 mg/kg feed *M. cordata* extract (sanguiritrin) in pigs or rats in 90-day studies[7, 8].

The goal of this paper was to assess the possible genotoxicity of *M. cordata* extract when administrated as a dietary admixture to rat at concentrations providing 600 mg/kg feed and 100, 7000 or 14000 mg/kg feed Sangrovit® in a 90-day pilot study.

## MATERIAL AND METHODS

*Chemicals and reagents*. Sangrovit® and *M. cordata* extract (sanguiritrin) were obtained from Phytobiotics Futterzusatzstoffe GmbH, Eltville, Germany. For determination of alkaloid content, Sangrovit® was extracted with acidified (1% HCl) methanol and analyzed by HPLC. Its alkaloid composition was (g/kg): sanguinarine (13.51 ± 0.25); chelerythrine (6.90 ± 0.09); α-allocryptopine (20.26 ± 1.96); protopine (4.30 ± 0.54); homochelidonine (1.63

± 0.13); dihydrosanguinarine (0.25 ± 0.01) and traces of oxysanguinarine, oxychelerythrine and dihydrochelerythrine. The content of alkaloids in *M. cordata* extract was (g/kg): SG (528.95 ± 6.56), and CH (82.05 ± 0.95). SG was isolated from the alkaloid extract of *M. cordata* using column chromatography on alumina, 98.1% HPLC purity, mp 279-282 °C (277-280 °C, ref.[9]) was obtained. IR, UV, MS and NMR spectra were consistent with the structures of the above alkaloids. NADPH, calf thymus DNA and nuclease P1 were obtained from Sigma Chemical Co. (St. Louis, MO). β-Naphthoflavone (β-NF) and bicinchoninic acid were from Aldrich Chemical Co., (Milwaukee, WI). All other chemicals were of analytical purity or better. Enzymes and chemicals for the $^{32}$P-postlabeling assay were obtained commercially from previously described sources[10].

*Experimental diet*. 100, 7000, 14000 mg Sangrovit®, (containing 5, 330 or 660 mg of isoquinoline alkaloids respectively 2, 140, and 290 mg of SG and CH) or 600 mg *M. cordata* extract (containing 367 mg of SG and CH) were blended with powdered commercial diet (800 g), microcrystalline cellulose (190 g) and magnesium stearate (10 g).

*Control diet*. The powdered commercial diet (800 g) was blended with microcrystalline cellulose (190 g) and magnesium stearate (10 g).

The diets were prepared monthly and analyzed by HPLC periodically to confirm concentration, homogeneity and stability of alkaloids in the diet. The pellets were stored in paper bags and kept dry.

*Animals*. The study was approved by the Ethics Committee, Ministry of Education, Czech Republic and conducted in compliance with the Experimental Animals Protection Act No. 167/1993 L.C. Male Wistar rats (n=30; 240 ± 10 g bw) were purchased from BioTest Ltd., Konarovice, Czech Republic. The rats were acclimatized one week before the experiment, kept in plastic cages containing dust-free sawdust, two animals per cage. On the day of treatment, the animals were 10 weeks old with a mean body weight ± standard deviation of 312 ± 12 g (n = 30). They were randomized to five groups (6/group); control - Group 1 (316 ± 9 g bw), Group 2, 100 (314 ± 8 g bw), Group 3, 7000 (307 ± 14 g bw) and Group 4, 14000 mg Sangrovit®/kg feed (309 ± 17 g) or Group 5, 600 mg *M. cordata* extract/kg feed (313 ± 11 g bw).

During the acclimatization period and during testing, the conditions in the animal room were as follows: temperature (23 ± 2 °C; checked daily); relative humidity (30 to 70 %); light/dark cycle 12 hrs/12 hrs. The health of the animals was checked daily and body weights were monitored twice a week and prior to sacrifice.

*Administration of Sangrovit® and* M. cordata *extract.* The animals consumed *ad libitum* either the standard diet or the diets containing 5, 330, or 660 ppm of total alkaloids in Sangrovit®, and 367 ppm of SG and CH in *M. cordata* extract, for 90 days. The total amount of administered Sangrovit® or *M. cordata* extract depended on the individual quantity of consumed feed, which was 38 ± 2 g on average. The daily alkaloid doses for 5, 330 and 660 ppm, and 367 ppm (Groups 2, 3, 4, and 5), respectively, fell from 0.6 (Group 2), 40.8 (Group 3), 81.2 (Group 4), and 61.1 mg/kg/day (Group 5) at the beginning to 0.3, 23.7, 47.9, and 29.8 mg/kg/day at the end of the experiment, respectively, i.e. averaged doses over 90 days were 0.5 (Group 2), 32.3 (Group 3), 64.6 (Group 4), and 45.4 mg/kg/day (Group 5).

*Liver collection and preparation.* The animals were deprived of feed 12 hrs before terminal *i.m.* anaesthesia by fentanyl (4 μg/100 g bw), medetomidin (20 μg/100 g bw) and diazepam (0.5 mg/100 g bw). After opening the abdominal cavity, macroscopic examination of the main organs (GI tract, heart, kidneys, liver, lungs, and other organs) was performed. The $Na_2$EDTA-blood was used for lymphocyte isolation for genotoxicity assay (0.5 ml). Livers of animals from all groups were removed, washed in cold phosphate-buffered saline and stored at −80 °C prior to analysis.

*DNA isolation*. Liver samples (1–2 g) were powdered under liquid nitrogen in a dismembrator (Braun, Melsingen, Germany) and DNA isolated from 0.5–1 g of tissue by the phenol/chloroform extraction method. DNA was dissolved in sterile water and the solution stored at −80 °C. The DNA content was quantified spectrophotometrically.

*$^{32}$P-Postlabeling analysis*. The nuclease P1 enrichment version[11] and the 1-butanol extraction-mediated enrichment procedure[12] of the $^{32}$P-postlabeling assay[13] were performed by digesting DNA samples (12.5 μg) with micrococcal nuclease (750 mU) and spleen phosphodiesterase (12.5 mU) in digestion buffer (20 mM sodium succinate, 8 mM $CaCl_2$, pH 6.0) for 3 h at 37 °C in a total volume of 12.5 μl. Here, 2.5 μl of the digests were removed and diluted 1:1500 to determine the amount of normal nucleotides. In the nuclease P1 version, digests (10 μl) were enriched for adducts by incubation with 5 μg (5 U) of nuclease P1 in 3 μl of a buffer containing 0.8 M sodium acetate, pH 5.0, 2 mM $ZnCl_2$ for 30 min at 37 °C. The reaction was stopped by adding 3 μl of 427 mM Tris base. The extraction with 1-butanol to enrich adducts was carried out as described earlier[12]. Four microlitres of labeling mix consisting of 400 mM bicine pH 9.5, 300 mM dithiothreitol, 200 mM $MgCl_2$, 10 mM spermidine, 100 μCi [γ-$^{32}$P]-ATP (15 pmol), 0.5 μl of 90 μM ATP and 10 U T4 polynucleotide kinase were added. After incubation for 30 min at room temperature, 20 μl were applied to a polyethylenimine (PEI)-coated cellulose thin-layer chromatography (TLC) plate (Macherey-Nagel, Düren, Germany) and separated as described[5]. For determination of the amount of normal nucleotides 5 μl of the 1:1500 dilution of digests were mixed with 2.5 μl of Tris buffer (10 mM, pH 9.0) and 2.5 μl of labeling mix (see above), and incubated for 30 min at room temperature. The labeling mixture was diluted by mixing 4 μl with 750 μl of 10 mM Tris buffer, pH 9.0. This solution (5 μl) was applied to a PEI-cellulose TLC plate and run in 0.28 M $(NH_4)_2SO_4$, 50 mM $NaH_2PO_4$, pH 6.5. Two parallel analysis of each DNA sample isolated from rat livers, by both versions of the $^{32}$P-postlabeling assay, were carried out.

Adducts and normal nucleotides were detected and quantified by storage phosphor imaging on a Packard

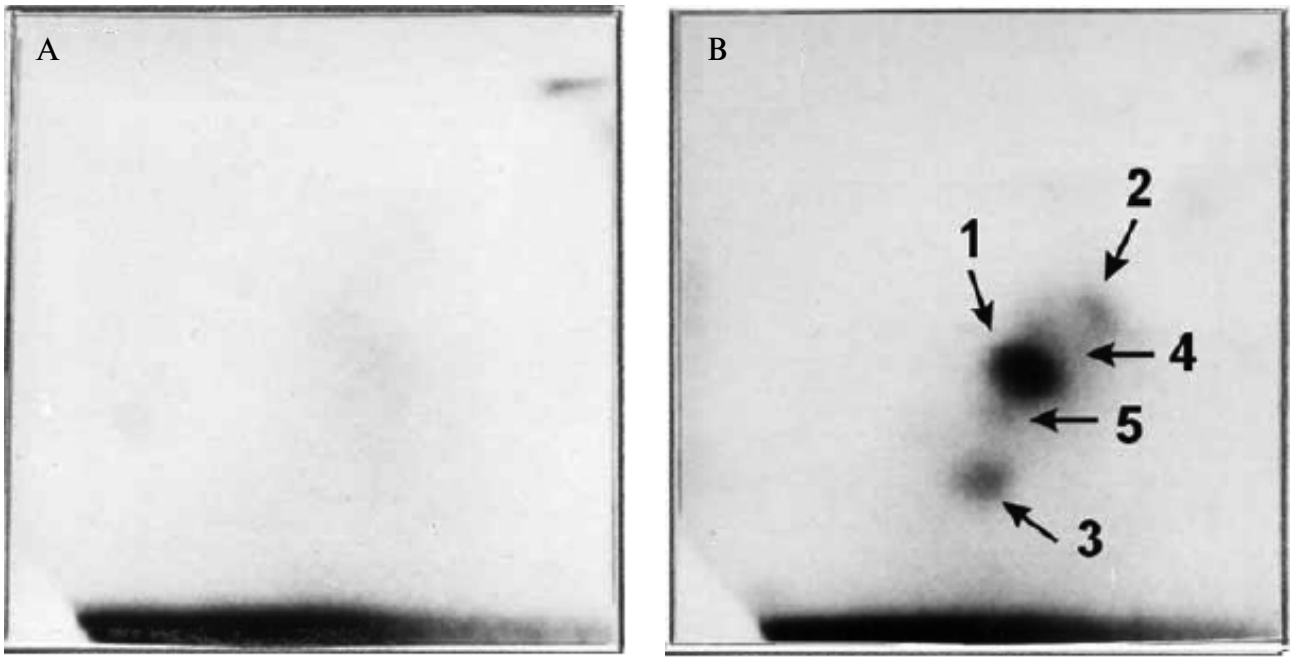


**Fig. 1.** Analysis of DNA adduct formation by Sangrovit® *in vivo* (Group 4; A) and in DNA from *in vitro* incubation of calf thymus DNA with sanguinarine activated with hepatic microsomes of rats induced with β-NF (B) (spots 1 – 5 indicate the DNA adducts formed by sanguinarine *in vitro*). The nuclease P1-enrichment version of $^{32}$P-postlabeling assay was used for analysis. Chromatographic conditions: D1, 1 M sodium phosphate, pH 6.8; D3, 3.5 M lithium formate, 8.5 M urea, pH 4.0; D4, 0.8 M LiCl, 0.5 M Tris-HCl, 8.5 M urea, pH 9.0; D5, 1.7 M $NaH_2PO_4$, pH 6.0. Origins, in the bottom left-hand corner, were cut off before exposure; D3 direction from bottom to top and D4 direction on four directional TLC on PEI-cellulose from left to right.

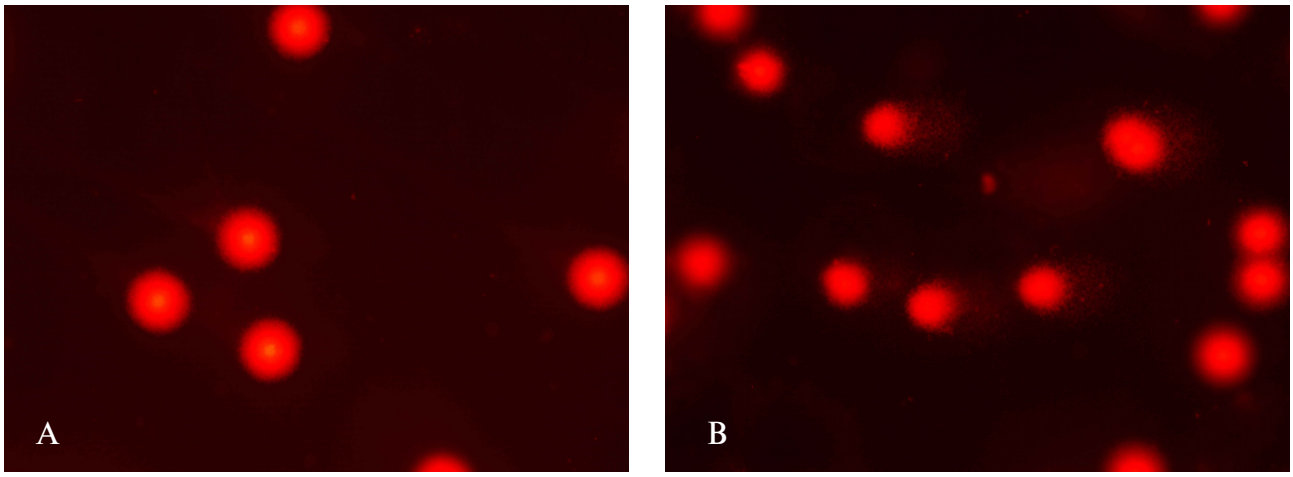


**Fig. 2.** Analysis of DNA single strand breaks formation by Sangrovit® *in vivo* (Group 4; A) and in DNA of lymphocytes incubated with sanguinarine *in vitro* (positive control; B).

Instant Imager. Count rates of adducted fractions were determined from triplicate maps after subtraction of count rates from adjacent blank areas. Excess [γ-$^{32}$P]ATP after the postlabeling reaction was confirmed. Adduct levels were calculated in units of relative adduct labeling (RAL) which is the ratio of counts per minute (cpm) of adducted nucleotides to cpm of total nucleotides in the assay.

*Preparation of microsomes and assays*. Hepatic microsomes of male Wistar rats pre-treated with β-NF were prepared as described previously[14]. Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with serum albumin as a standard[15]. The concentration of cytochrome P450 (CYP) was estimated according to Omura and Sato[16] based on the complex of reduced cytochrome P450 with carbon monoxide. Specific contents of cytochromes P450 was 1.30 ± 0.43 nmol/mg protein for hepatic microsomes of rats pre-treated with β-NF (10 rats/group), respectively.

The results are presented as means ± standard deviations (n = 10).

*Incubations of sanguinarine with DNA and hepatic microsomes* in vitro. The incubation mixtures contained in a final volume of 0.75 ml: 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, microsomes containing 1 nmol cytochrome P450, 1–100 μM SG as chloride salts dissolved in water and 1 mg of calf thymus DNA (4 mM). The reaction was initiated by addition of NADPH. Incubations were carried out at 37 °C for 60 min. Control incubations were performed either without NADPH or with the whole activating system and SG but without DNA, or with the activating system and DNA but without SG. After incubation (37 °C, 60 min), the mixtures were extracted twice with ethyl acetate (2 x 2 ml). DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described earlier[14]. The content of DNA was determined spectrophotometrically. $^{32}$P-postlabeling analyses of DNA were performed using both enrichment versions (nuclease P1 enrichment and extraction with 1-butanol) as described above.

*Detection of DNA single strand breaks:* The DNA breaks were measured in peripheral lymphocytes using an alkaline version of Comet assay[17]. Lymphocytes were isolated on Histopaque 1077 gradients. DNA damage was analyzed after staining by ethidium bromide on an Olympus IX 70 fluorescence microscope. One hundred cells per slide were analyzed and divided into four classes of DNA damage standard scale. Total DNA damage was calculated: (number of cells in class 1 × 1 + number of cells in class 2 × 2 + number of cells in class 3 × 3 + number of cells in class 4 × 4)/100. The maximal damage was given a value of 400. As positive control (DNA single strand breaks) the lymphocytes isolated from control rats were incubated with SG (5 μM, 60 min, 37 °C, in dark) and the Comet assay was carried out.

## RESULTS AND DISCUSSION

*M. cordata* extract and Sangrovit® genotoxic potential was studied in rats exposed to 600 ppm *M. cordata* extract or 100, 7 000 and 14 000 ppm Sangrovit® in feed in 90-days experiment. In order to evaluate their possible genotoxicity, two experimental approaches were utilized; analyses of DNA adduct formation in livers and DNA single strand breaks in lymphocytes of exposed animals.

DNA adducts were analyzed employing the $^{32}$P-postlabeling technique, namely the nuclease P1 version and extraction with 1-butanol, as these methods are found most appropriate for detection of DNA adducts formed by SG and CH *in vitro*[5]. DNA isolated from incubation of SG activated by hepatic microsomes treated with β-NF *in vitro*[5] was also used for analysis of DNA adducts in the present study as a positive control (Fig. 1). In contrast to experiments *in vitro*, using both versions of the $^{32}$P-postlabeling assay, no DNA adducts were detectable in the hepatic DNA of either control rats or those treated with Sangrovit® (Fig. 1) or *M. cordata* extract (results not shown).

Comparable results were obtained by Comet assay detecting the single strand breaks of DNA. No significant differences were found between lymphocyte DNA of control and Sangrovit® or *M. cordata* treated animals (Fig. 2). DNA single strand breaks were detected *in vitro* after incubation of isolated lymphocytes with SG (positive control). However, using the alkaline Comet assay, Ansari et al.[6] found that intraperitoneal administration of isoquinoline alkaloid SG isolated from argemone oil in mice caused DNA damage in blood and bone marrow cells. Utilizing the same method employed by these authors, our *in vivo* experiment showed that neither Sangrovit® nor *M. cordata* extract in the feed induced any DNA damage to lymphocytes or hepatocytes, which is in disagreement with Ansari et al.[6]. Moreover, no covalent DNA modification by Sangrovit® or *M. cordata* extract (DNA adducts) in liver DNA *in vivo* was found in this study. These results and the fact that Sangrovit® (appetiser feed additive) given to the rats in our experiments were higher than the recommended dose (50 to 100 mg/kg feed), argue strongly in favour of the use of Sangrovit® in live stock.

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