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# Toxicology of a Peruvian botanical remedy to support healthy liver function

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**Abstract**

**Background:** The purpose of these studies was to determine the safety of a botanical treatment for supporting healthy liver function developed in Peru. The formulation, A4$^+$, contains extracts of *Curcuma longa* L. rhizome (A4R), *Cordia lutea* Lam. flower (A4F) and *Annona muricata* L. leaf (A4L). The tests were used to support an application for a non-traditional Natural Health Product Licence from the Natural Health Product Directorate of Health Canada and future clinical trials.

**Methods:** Besides reviewing the scientific and clinical information from Peru on the ingredients and conducting an initial Ames test for mutagenicity, we analysed A4$^+$ for its chemical profile and tested genotoxicity (micronucleus test) and general toxicity (28-day repeated dose).

**Results:** A4$^+$ and extracts from the three plants provided distinctive chemical fingerprints. A4L contained acetogenins, requiring a second chromatographic method to produce a specific fingerprint. The Ames test proved positive at the highest concentration (5,000 µg/mL) but A4$^+$ showed no evidence of genotoxicity in the more specific mouse micronucleus test. The 28-day repeated dose (general toxicity) study in rats showed no toxicity at 2,000 mg/kg.

**Conclusions:** We conclude that under the conditions of these studies, A4$^+$ shows no evidence of toxicity at the levels indicated. A no-observed adverse effect level (NOAEL) of 2,000 mg/kg was assigned.

**Keywords:** *Annona muricata*, *Cordia lutea*, *Curcuma longa*, herbal medicine, liver disease, toxicity testing

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## Introduction

A4$^+$ is a herbal product formulated to support healthy liver function. It contains extracts of three herbs: *Cordia lutea* Lam. flower, *Curcuma longa* L. rhizome and *Annona muricata* L. leaf, combined in the w/w ratio of 80:10:10. The herbal components originate from the Amazon rainforest and the Coastal plains of Peru. Individually, these plants are used as traditional remedies. All have known beneficial effects on liver health and monograph support using *Cu. longa* and *A. muricata* to treat patients with liver disorders [1, 2]. *Co. lutea* is used traditionally for hepatitis [3].

This unique combination of herbs and their relative proportions was developed by Dr. José Cabanillas, a Peruvian physician with extensive experience of traditional medicine in the Amazon basin in consultation with traditional Peruvian Elder Healers. Interviews with five of these Healers on the traditional use of *Cu. longa* rhizome, *Co. lutea* flower and *A. muricata* leaf indicated that these plants have been used for generations to treat liver conditions and maintain liver health, with no recorded side effects [4].

*Cu. longa* L., commonly known as turmeric, is a plant of the ginger family (Zingiberaceae) [5]. It has been used for medicinal purposes for centuries as an anti-cancerous, anti-inflammatory and hepatoprotective drug [5, 6]. Its hepatoprotective property is attributable to its antioxidant activity and ability to reduce production of pro-inflammatory cytokines [5]. Its main active constituent, curcumin, has also been shown to increase apoptosis of damaged hepatocytes [6].

*A. muricata* L., commonly known as graviola, is a member of the Annonaceae family. All parts of the plant have been extensively used in traditional medicines. In particular, the leaf has been used for its anti-diabetic, anti-rheumatic and hepatoprotective properties [7].

*Co. lutea* Lam., commonly known as Overal, belongs to a genus of shrubby plants in the Boraginaceae family [3, 8]. Many plants in this genus have been used for medicinal purposes for inflammation, malaria, cough and lung disease [8]. *Co. lutea* has traditionally been used for kidney and prostate inflammation in addition to liver complications [3].

The purpose of these studies was to determine A4$^+$ toxicity to support an application for a product license application to the Natural Health Product Directorate of Health Canada and future human clinical studies. The studies reported here are a 28-day repeated dose (general toxicity) study in rats, a bacterial reverse mutation assay (Ames test) and a micronucleus test for genotoxicity in mice.

# Materials and methods

## Analytical methods

A4$^+$ was extracted from the flower of *Co. lutea* (A4F), rhizome of *Cu. longa* (A4R) and leaf of *A. muricata* (A4L) in a ratio (w:w) of 80:10:10, respectively, using a hydroalcoholic menstruum, composed of 70 % ethyl alcohol and 30 % water. The overall yield gave 12.96 g of A4$^+$ extract per litre. The A4$^+$ product label discloses each capsule to contain 77.8 mg of *Co. lutea*, 9.7 mg *A. muricata* and 9.7 mg of *Cu. longa*. Microcrystalline cellulose was used as bulker/excipient.

The *Cu. longa* and *A. muricata* were sourced from the Peruvian amazon. The *Co. lutea* was derived from the Coastal plains of Peru. Vouchers are deposited at the "Herbarium Amazonense (Flora Peruana)" at the Universidad Nacional de la Amazonia Peruana, Iquitos Peru (Supplemental Data, Section A).

Single ingredient extracts (crystalline dry material) along with a mixture (crystalline dry material) were provided by Sabell Corporation, Calgary, Canada. Samples were dissolved as 10 mg/mL in 80 % methanol (A4R, A4F) or 100 % methanol (A4L) and clarified by centrifugation. Samples (20 µL) were analysed using high-performance liquid chromatography (HPLC) coupled to diode array absorbance (DAD) and positive mode electrospray mass spectrometric (LC/MS) detection. A common elution procedure using a solvent gradient provided a well-separated characteristic fingerprint for each of the three plant materials. Details of the method are found in Supplemental Data, Section B.

## Bacterial reverse mutation assay: Ames test

This test was conducted under contract by the Marine Biotechnology Research Centre (MBRC, Analytical Division, 265, 2nd Street East, Rimouski, QC, Canada G5L 9H3). MBRC conducted in vitro bacterial reverse mutation Ames assay tests to determine the mutagenic properties of *Cu. longa* rhizome, *Co. lutea* flower, *A. muricata* leaf and A4$^+$. The samples were in the form of extracts dissolved in 100 % dimethylsulfoxide (DMSO) at a concentration of approximately 125 mg/mL. The standard Ames assay is commonly used for initial screening to detect point mutations induced by test compounds in *Salmonella typhimurium* bacteria in an in vitro system [9, 10]. Bacterial cultures were exposed to test compounds in the presence and absence of an exogenous metabolic activation enzyme system. After incubation, positive bacterial revertant colonies were counted and compared with the number of spontaneous positive bacterial revertant colonies generated in vehicle control wells and in comparison to known mutagenic compounds. Details of the method are found in Supplemental Data, Section C.

## Toxicology studies

These Good Laboratory Practice (GLP) studies were conducted in accordance with the Organisation for Economic Co-operation and Development (OECD) GLPs [11] and internal institutional Standard Operating Procedures with the exception of test item characterization and dose concentration analysis, which was not conducted in compliance with GLP as A4$^+$ contains extracts of herbs for which no analytical standard is currently available. All animal procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Liquid extract of A4$^+$ was lyophilized to produce a powder, which for dosing was reconstituted as slurry in Nanopure water containing 0.2 % v/v of simethicone USP to prevent foaming. Details of the method are found in Supplemental Data, Section D.

## Twenty-eight-day repeated dose study

The design was a repeated dose oral toxicity study conducted under GLP according to the OECD 407 (28-day repeated dose oral toxicity) guidelines [12]. Four groups of five per sex per group of Sprague Dawley rats (Charles River Laboratories, Saint-Constant, Canada) weighing 275–315 g (males) and 188–211 g (females) at the initiation of treatment were randomly allocated to rack and position and acclimated for 6 days. The study involved administration of three dosage levels (large multiples of the human dose) of A4$^+$ (125, 500 and 2,000 mg/kg) plus vehicle control to male and female rats daily for 28 days, followed by euthanasia and necropsy. The study design is shown in Table 1.

Rats were dosed with 6 mL/kg of vehicle control or A4$^+$ via oral gavage once daily for 28 days. Animals were observed twice daily and body weights recorded on study day 1 and weekly thereafter and

**Table 1:** Twenty-eight-day repeated dose toxicity study design with dosing details.

| Treatment group | Treatment | No. of animals per group | | Dosage level, mg/kg BW | Dosage concentration, mg/mL | Dosage volume, mL/kg BW |
|---|---|---|---|---|---|---|
| | | Male | Female | | | |
| 1 | Vehicle control | 5 | 5 | 0 | 0 | 6 |
| 2 | Low dose A4$^+$ | 5 | 5 | 125 | 20.81 | 6 |
| 3 | Mid-dose A4$^+$ | 5 | 5 | 500 | 83.25 | 6 |
| 4 | High dose A4$^+$ | 5 | 5 | 2,000 | 333 | 6 |

on study day 29 prior to necropsy. Food consumption was measured weekly. Standard functional observational batteries (FOB) were conducted on all animals during the last week of the study. Details of the FOB are included in Supplemental Data, Section E. Urinalysis was conducted prior to necropsy.

All rats were euthanized by exsanguination under isoflurane anaesthesia on study day 29 following final blood collections and underwent a necropsy under the supervision of a board-certified veterinary pathologist. Blood was collected prior to euthanasia via venipuncture of the abdominal vena cava and assayed for clinical chemistry (Table 2, Hitachi 912 Automatic Analyser, Hitachi, Mississauga, Canada), haematology (Abbott Cell-Dyn® 3700 CS, Abbott, Mississauga, Canada) and coagulation (Coagamate® XM, Fisher Scientific, Edmonton, Canada). At necropsy, all major organs were examined for gross abnormalities, weighed and samples fixed in 10 % neutral buffered formalin. The brain-to-body-weight ratio was calculated using the last total body weight measured. Histological examination was conducted by a board-certified veterinary pathologist on tissues from the high-dose and vehicle control groups only. For details on the clinical chemistry and haematology parameters measured, tissues collected at necropsy and organ weights, see Supplemental data, Section F.

**Statistical analyses:** All statistical analyses were performed separately for each sex using SAS Release 9.2 for Windows XP. Statistical procedures were selected on the distribution of the data and the validity of the assumptions. Statistical significance was declared when $p \le 0.05$. Body weights were analysed using repeated measures analysis of variance (ANOVA). The model included treatment group, study days (7, 14, 21, 28 and 29) and treatment group by study day interaction as fixed effects and the body weights from study day 1 (pre-dose) as covariate. Additionally, body weights on study day 1 (pre-dose) were compared between treatment groups by ANOVA. Average daily food consumption was analysed using repeated measures ANOVA. The model included treatment group and time period (study days 1–7, 7–14, 14–21, 21–28). Average daily food consumption (g/day) for each individual during each time period was calculated by dividing the total individual food consumption (in grams) by the number of days within the time period. Clinical chemistry, haematology and coagulation parameters from study day 29 were compared between groups using an ANOVA model. The exceptions to this were total bilirubin, basophils and absolute basophils, which were analysed using Cochran–Mantel–Haenszel's row mean score statistic due to low variability in the data. Categorical urinalysis and FOB parameters were compared between groups (1–4) using Fisher's exact test, while ordinal urinalysis and FOB parameters were compared between groups (1–4) using Cochran–Mantel–Haenszel's row mean score statistic. Specific gravity (from urinalysis) and average foot splay, temperature and max forelimb and hind limb grip strengths (from FOBs) were compared between groups using one-way ANOVA. The maximum forelimb grip strength was normalized using natural log transformation. Organ weights were analysed as absolute weights and as a proportion of total body weight using a one-way ANOVA model to compare treatment groups. For statistical analysis of body weight, food consumption, clinical chemistry, haematology, and coagulation parameters, micronucleus parameters, continuous urinalysis and FOB parameters, and organ weights, if the model revealed statistical significance ($p \le 0.05$), Tukey–Kramer adjusted comparisons were used to determine if pairwise differences existed.

## Genotoxicity: micronucleus study

A GLP mammalian erythrocyte micronucleus study on Sabell A4$^+$ was conducted in Balb/c mice according to the OECD 474 guideline [13]. A4$^+$ was formulated as described earlier. Cyclophosphamide monohydrate (Sigma Aldrich, Oakville, Canada) 4 mg/mL in sterile water was used as a positive control.

**Table 2:** Clinical chemistry parameters analysed.

| Parameter analysed | Method of analysis | Reagent manufacturer |
|---|---|---|
| Alanine aminotransferase | IFCC without pyridoxal (UV test) | Roche |
| Albumin | Bromocresol green | Roche |
| Albumin/globulin ratio | Calculated (ALB/GLOB) | N/A |
| Alkaline phosphatase | ALP-IFCC liquid | Roche |
| Aspartate aminotransferase | IFCC without pyridoxal | Roche |
| Calcium | *o*-Cresolphthalein complexone | Roche |
| Chloride | Ion-selective electrode with dilution | Roche |
| Cholesterol | CHOD-PAP (enzymatic colorimetric) | Roche |
| Creatine kinase | CK-liquid IFCC | Roche |
| Creatinine | Jaffe (kinetic) rate blanked and compensated | Roche |
| Globulin | Calculated (total protein – albumin) | N/A |
| Glucose | GOD-PAP | Roche |
| Sorbitol dehydrogenase | Enzymatic – NADH to NAD$^+$ | Catachem |
| Phosphorus | Molybdate | Roche |
| Potassium | Ion-selective electrode with dilution | Roche |
| Sodium | Ion-selective electrode with dilution | Roche |
| Total bilirubin | Jendrassik | Roche |
| Total protein | Biuret | Roche |
| Triglycerides | GPO-PAP (enzymatic colorimetric) | Roche |
| Urea nitrogen | Urea kinetic (UV) | Roche |

Six groups of five per sex per group of Balb/C mice (Charles River Laboratories, Saint-Constant, Canada), weighing 18.2–23.1 g (males) and 16.9–19.9 g (females) were group housed in standard shoebox cages. Mice were acclimated to rack and position for 7 days prior to the start of the study.

This study involved the administration of three consecutive daily 6 µL/g body weight doses of A4$^+$ (125, 500 and 2,000 mg/kg) plus vehicle controls via oral gavage as well as a positive control substance, cyclophosphamide, and its vehicle controls (water) via intraperitoneal injection, to male and female mice, as per Table 3. Body weights were taken on all animals on study day 1 (pre-dose) and used to calculate dose. Euthanasia was via $CO_2$ asphyxiation approximately 24 h post-final dosing. At necropsy, both femurs were collected from each animal and a smear of bone marrow was stained with Wright's Giemsa. Bone marrow slides were scored blindly. The ratio of polychromatic erythrocytes (PCE) to total erythrocytes was determined by examining 200 erythrocytes per animal. The number of micronucleated PCEs (MNPCE) in 2,000 PCE per animal was then determined.

**Statistical analyses:** The statistical analyses were performed using SAS Release 9.2 for Windows XP. Statistical procedures were selected based on the distribution of the data and the validity of the assumptions. Statistical significance was declared when $p \le 0.05$. The number of PCEs in 200 erythrocytes per animal and the number of MNPCEs in 2,000 PCE per animal were compared between sexes and groups by ANOVA. MNPCE was normalized using a natural log transformation of the value +1. The ratio of the number of PCE per 200 erythrocytes and the ratio of the number of MNPCE per 2,000 PCE were also calculated for each animal and summarized by sex and group.

# Results

## Chemical analysis of A4R (rhizome of *Cu. longa* L.)

Analysis of A4R provided adequate separation of components with strong DAD signals at 254 nm and distinct mass spectrometry signals (Figure 1). At 254 nm at least

**Table 3:** Micronucleus study design with dosing details.

| Treatment group | Treatment | No. of animals per group | | Dosage level, µg/g BW | Dosage concentration, mg/mL | Dosage volume, µL/g BW |
|---|---|---|---|---|---|---|
| | | Male | Female | | | |
| 1 | Reference item 1 (vehicle control) | 5 | 5 | 0 | 0 | 6 |
| 2 | Low dose A4$^+$ | 5 | 5 | 125 | 20.81 | 6 |
| 3 | Mid-dose A4$^+$ | 5 | 5 | 500 | 83.25 | 6 |
| 4 | High dose A4$^+$ | 5 | 5 | 2,000 | 333 | 6 |
| 5 | Water (reference item 3) | 5 | 5 | 0 | 0 | 10 |
| 6 | Cyclophosphamide (positive control, reference item 2) | 5 | 5 | 40 | 4.0 | 10 |

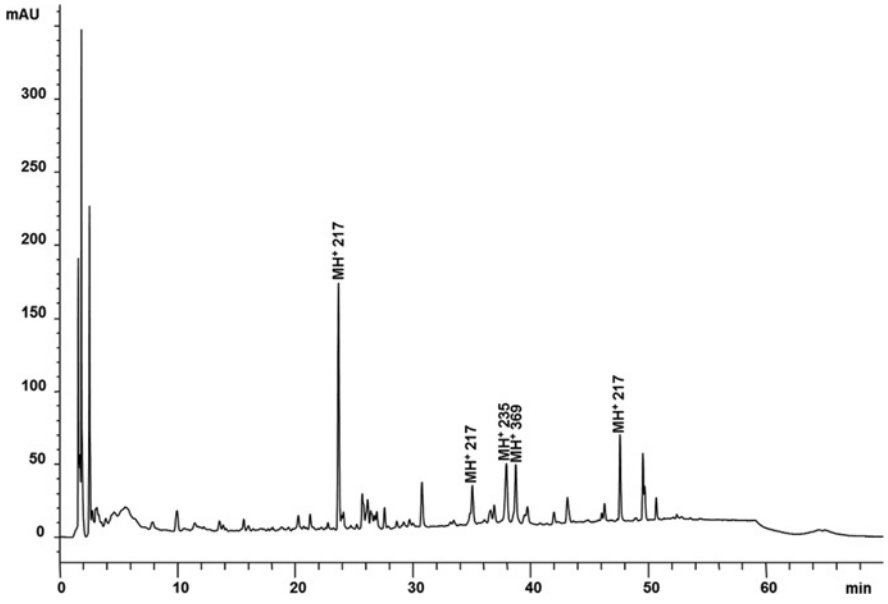


**Figure 1:** Representative chromatogram of A4$^+$ rhizome extract separated using the A4R method with detection at 254 nm.
MH$^+$ 217 is tentatively identified as tumerone, furanodiene of curzerene or their structural isomers, MH$^+$ 235 is tentatively identified as curcumenol and MH$^+$ 369 is likely curcumin and co-chromatographs with authentic curcumin.

eight major peaks were determined. Several signals characteristic of compounds common to the genus *Curcuma* were observed. The mass spectrometric signal (protonated molecular ion, $MH^+$) = 217 seen at least three distinct retention times is likely due to the presence of tumerone, furanodiene and curzerene ($C_{15}H_{20}O$) or structural isomers. The mass spectrometric signal $MH^+$ = 235 seen at least one distinct retention time is likely due to the presence of curcumenol ($C_{15}H_{22}O_2$). The mass spectrometric signal $MH^+$ = 369 seen at least one distinct retention time was characteristic of curcumin ($C_{21}H_{20}O_6$).

## Chemical analysis of A4F (flower of *Co. lutea* Lam.)

Analysis of A4F provided adequate separation of components with strong DAD signals at 254 nm and distinct mass spectrometry signals (Figure 2). At 254 nm at least two major peaks and eight moderate peaks were determined. The mass spectrometric signals for the two major peaks were identical ($MH^+$ 611). $MH^+$ signals were also seen at 625, 595, 584, 479 and 303. The spectrometric signal for $MH^+$ 611 with fragments at 495 and 303 suggested the loss of rhamnose and glucose and leaving quercetin as the aglycone. This pattern is characteristic of rutin. Other flavone glycoside possibilities exist ($C_{30}H_{26}O_{14}$, $C_{31}H_{30}O_{13}$ and $C_{28}H_{34}O_{15}$), but many are excluded due to the characteristic fragmentation pattern. There are at least 102 known flavonol glycosides with these chemical formulae [14]. The spectrometric signal for $MH^+$ 625 with fragments at 479 and 317 suggests the loss of rhamnose and glucose and leaving a flavone (MW 316, possibly methylquercetin) as the aglycone. This pattern, characteristic of a flavone (MW 316) rutinoside ($C_{28}H_{32}O_{16}$) may be a methylated rutin. The spectrometric signal for $MH^+$ 595 with fragments at 449 and 287 suggests the loss of rhamnose and glucose, leaving a flavone (MW 286) as the aglycone. This pattern is characteristic of a flavone (possibly kaempferol or luteolin, MW 286) rutinoside ($C_{27}H_{30}O_{15}$) [14]. There are at least 111 known flavonol glycosides with this molecular weight. The spectrometric signal for $MH^+$ 479 with a fragment at 317 suggests the loss of glucose and leaving a flavone (MW 316, possibly methylquercetin) as the aglycone. This pattern is characteristic of a flavone glucoside ($C_{22}H_{22}O_{12}$). There are at least 73 known flavonol glycosides with this molecular weight [14]. The spectrometric signal for $MH^+$ 303 is characteristic of a flavone such as quercetin ($C_{15}H_{10}O_7$), hesperetin ($C_{16}H_{14}O_6$) or diffutidin ($C_{17}H_{18}O_5$). There are at least 73 known flavones with these chemical formulae [14].

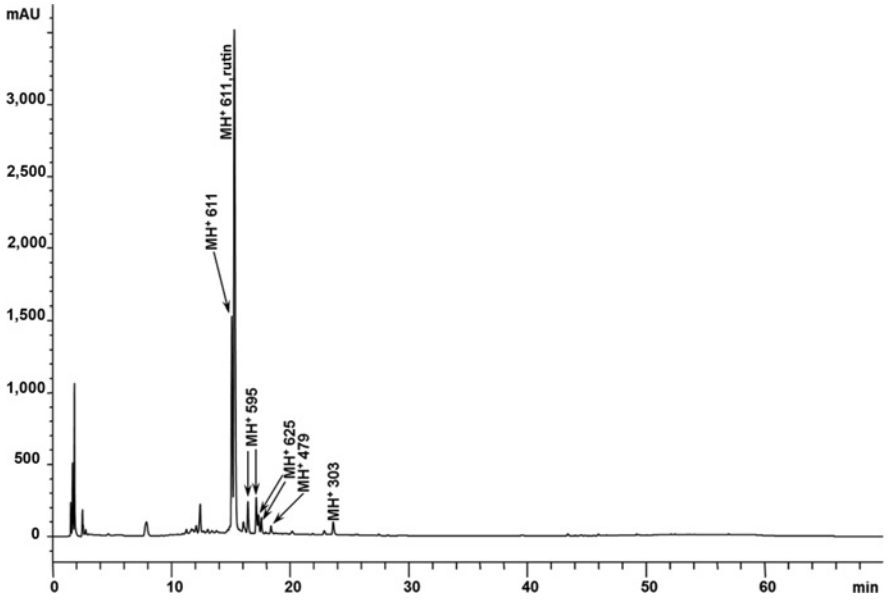


**Figure 2:** Representative chromatogram of $A4^+$ flower extract separated using the A4F method with detection at 254 nm.
$MH^+$ 611 with fragments 495 and 303 are tentatively identified as rutin or related structural isomers. Peak indicated as rutin co-chromatographs with authentic standard. $MH^+$ 595 with fragments 449 and 287 are tentatively identified as flavonol rutinosides of kaempferol (MW 286) or related substance. $MH^+$ 625 with fragments 479 and 317 is likely a flavonol rutinoside of a flavonol with MW 316 or related substance. $MH^+$ 479 is tentatively identified as a flavonol glucoside of a flavonol with MW 316 or related substance. $MH^+$ 303 is likely quercetin and co-chromatographs with authentic standard.

## Chemical analysis of A4L (leaf of *A. muricata* L.)

Analysis of A4L provided adequate separation of components with strong DAD signals at 254 nm and distinct mass spectrometry signals (Figure 3). At 254 nm, at least four major peaks and three moderate peaks were determined. The mass spectrometric signals for the five larger peaks are all characteristic of flavonol glycosides. The $MH^+$ signals for these peaks include 611, 595 and 465. The peaks with $MH^+$ signals at 611 and 595 (described above) are also chromatographically identical with those seen in A4F. The spectrometric signal for $MH^+$ 465 with a fragment at 303 suggests the loss of glucose, leaving a flavone (MW 303, possibly quercetin) as the aglycone. This pattern is characteristic of a flavone glucoside (possibly quercetin glucoside $C_{21}H_{20}O_{12}$). There are at least 68 known flavonol glycosides with this molecular weight [14].

Mass spectrometric signals for three groups of acetogenins were also seen in the *A. muricata* leaf extract (Figure 4). The mass spectra matched masses previously reported for a number of acetogenins [15–20]. A chromatographic method designed to optimally separate the acetogenins was developed and with this method it was possible to chromatographically isolate three molecular weight groups of acetogenins. There were at least 39 chromatographically distinguishable acetogenins in this material. There are no commercially available standards for these acetogenins but comparative estimates between preparations and plant structures are possible using UV absorbance and mass spectrometric signals. Three additional compounds associated with molecular weight 380 are also present (see Supplemental Data, Figure 1). The identities of these compounds remain undetermined.

## Chemical analysis of $A4^+$ (crystalline material)

The crystalline material consisting of a mixture of A4R, A4F and A4L was examined. The $A4^+$ crystalline material provided evidence for all compounds described in the three components that make up the formula. The most prevalent signals came from the A4L and A4F components with the A4R material providing minor signals (see Supplemental Data, Figures 2 and 3).

## Bacterial reverse mutation assay: Ames test

Based on the interpretation criteria for the mutagenic response under the experimental conditions of the

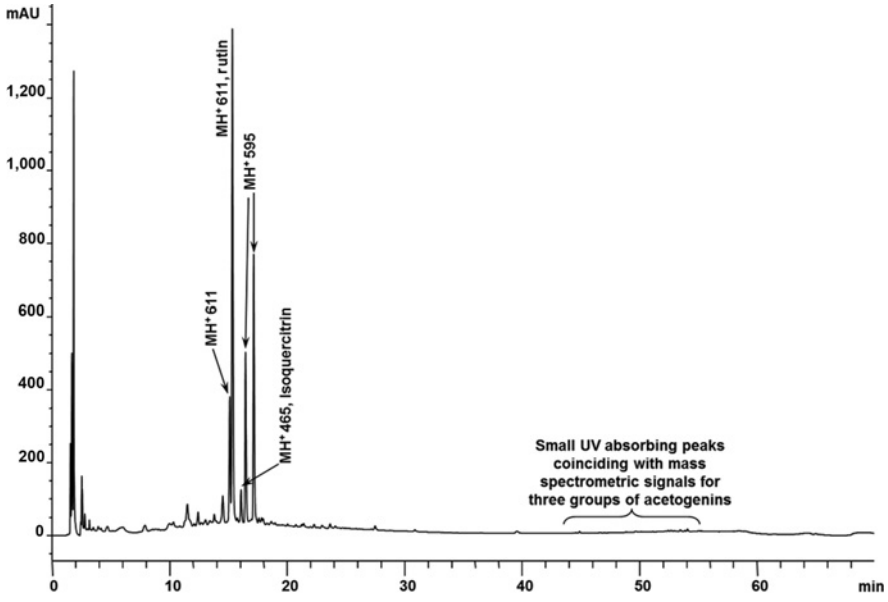


**Figure 3:** Representative chromatogram of $A4^+$ leaf extract separated using the A4L method with detection at 254 nm.
$MH^+$ 611 with fragments 495 and 303 are tentatively identified as rutin or related structural isomers. Peak indicated as rutin co-chromatographs with authentic standard. $MH^+$ 595 with fragments 449 and 287 are likely flavonol rutinosides of kaempferol (MW 286) or related substance. $MH^+$ 465 with fragment at 303 is likely a flavonol glucoside of quercetin (MW 302) and co-chromatographs with authentic isoquercitrin.

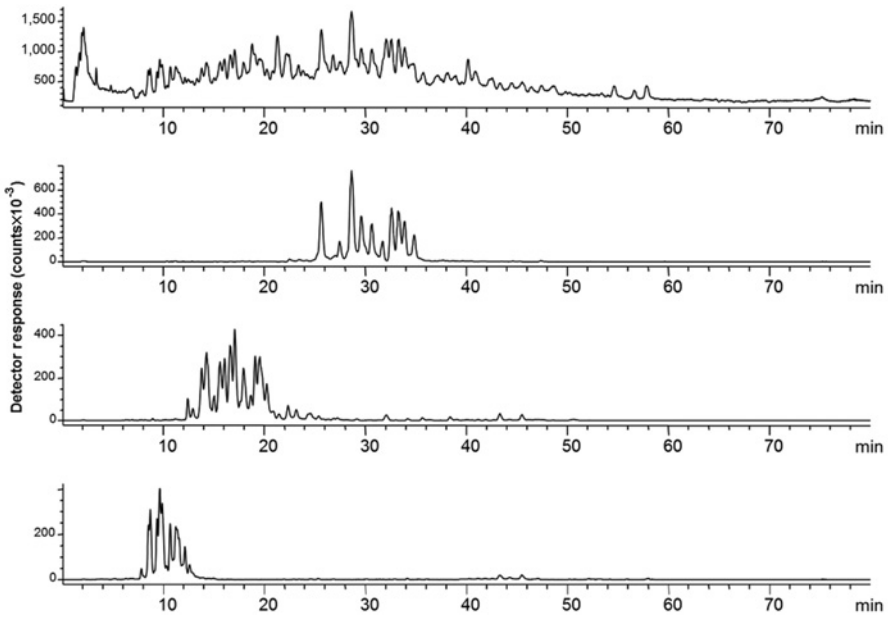


**Figure 4:** Representative chromatograms of ion extractions for acetogenin-related molecular weights ($MH^+$ 597, 616, 629) from $A4^+$ leaf extract separated using the A4L3 method with positive mode electrospray mass spectrometric detection.
Upper panel provides trace using SCAN mode. Second panel provides trace of extracted ion $MH^+$ 597 indicative of a group of acetogenins with MW 596 ($C_{35}H_{64}O_7$). At least 10 chromatographically distinct peaks are observed. Third panel provides trace of extracted ion $MH^+$ 613 indicative of a group of acetogenins with MW 612 ($C_{35}H_{64}O_8$). At least 14 chromatographically distinct peaks are observed. Fourth panel provides trace of extracted ion $MH^+$ 629 indicative of a group of acetogenins with MW 628 ($C_{35}H_{64}O_9$). At least 11 chromatographically distinct peaks are observed.

study, all three extracts were deemed mutagenic. *Cu. longa* rhizome was mutagenic in the presence of metabolic enzymatic S9 fraction with TA100 bacteria at the highest dose of 5,015 µg/mL (ratio 6.0). *Co. lutea* flower was mutagenic in the presence and absence of metabolic enzymatic S9 fraction with TA98 bacteria at high doses of 5,014 (absence ratio 6.67; presence ratio 4.07) and 1,003 µg/mL (absence ratio 4.18; presence ratio 2.19), as well as in the presence of the metabolic enzymatic S9 fraction with TA100 bacteria at a high dose of 5,014 µg/mL (Ratio 2.59). *A. muricata* leaf was mutagenic in the presence of the metabolic enzymatic S9 fraction with both TA98 and TA100 bacteria at doses of 5,017 µg/mL (TA98 ratio 1.57; TA100 ratio, 7.92), 1,003 (TA98 ratio 5.41; TA100 ratio 13.98) and 201 mg/mL (TA98 ratio 2.09).

This assay was performed in accordance with the manufacturer's recommendations and was based upon the OECD guideline document no. 471 [21].

## Toxicology: 28-day repeated dose

There were no statistically significant intergroup differences in body weights over the course of the study. There were no intergroup differences in food consumption for either sex over the course of the study, nor were there any group by time period interactions. For both sexes, however, there were expected significant differences between time periods.

There were no significant intergroup differences in FOB measurements among the animals of either sex, with the exception of tail pinch response, where the males of the control group had more abnormal responses than those of the low-dose group. There were no significant intergroup differences in urinalysis parameters among the animals of either sex. There were no significant intergroup differences in clinical pathology parameters among the animals of either sex. For haematology results, the high-dose females had significantly higher mean segmented neutrophil counts than the rest (0.21 vs. 0.11, 0.12 and 0.12) and higher mean absolute segmented neutrophil counts than the two lower dose groups but not the controls (1.55 vs. 0.73 and $0.84 \times 10^9$/L). The high-dose females had significantly lower mean lymphocyte counts than the controls or low-dose females (0.76 vs. 0.86 and 0.84). Also, the mid-dose males had significantly lower lymphocyte counts than the low-dose males (0.79 vs. $0.88 \times 10^9$/L). There were no significant intergroup differences for coagulation parameters among the animals of either sex. The only significantly different organ weight

was in the males, where the controls had a significantly greater mean lung to body weight ratio than the mid-dose males (0.00370 vs. 0.00321).

Regarding mortality, no animal deaths occurred during the study. During necropsy, no gross abnormalities were observed in any group. On histological examination, some minor abnormalities were observed in all groups but there was no consistent pattern of lesions found by the pathologist in any organ or group that could be related to $A4^+$.

### Repeated dose micronucleus study in mice

Study dose verification for target and measured dosage concentration are shown in Supplemental Data, Table 4. No mortalities occurred during the study. For body weights, the females had a weight range of 16.1–19.1 g, with a mean of 17.7 g. The males had a weight range of 16.2–22.2 g, with a mean of 19.6 g. All weights were within ± 20 % of the mean body weight. There were no significant group differences for body weights.

Summary statistics for PCE, MNPCEs and the ratios of PCE per 200 erythrocytes and MNPCE per 2,000 PCE by sex and group (1, 4 and 5 only) are presented in Table 4. The number of PCE PER 200 erythrocytes differed significantly between groups for females ($p = 0.0005$) and males ($p < 0.0001$). Statistical analysis of the bone marrow scores revealed that the positive control compound cyclophosphamide reduced PCE scores similarly in both sexes, an indicator of bone marrow toxicity. No changes in PCE scores were associated with high-dose $A4^+$ test item.

MNPCE scores, the primary indicator of genotoxicity in this test, were significantly higher in the positive control animals of both sexes than in either high-dose $A4^+$-treated animals or negative controls. The $A4^+$-treated animals of both sexes had similar low MNPCE scores to the control animals with no significant differences between groups.

It was concluded that under the repeated dose conditions of the experiment, $A4^+$ was negative for the production of elevated micronucleus counts and did not exhibit bone marrow toxicity. A no-observed adverse effect level (NOAEL) of 2,000 mg/kg was assigned for genotoxicity and bone marrow toxicity based on the results of this study.

## Discussion

All three extracts provided excellent and distinctive chemical fingerprints. There was some overlap in the chemical constituents of A4F and A4L. The extract of *Cu. longa* rhizome contained signals characteristic of the genus *Curcuma*. Mass and UV absorbance signals likely associated with curcumin, curcumenol, tumerone, furanodiene and curzerene were determined. The extract of *Co. lutea* flower contained signals characteristic of flavonol glycosides, most likely rutin, a rutin isomer, kaempferol or luteolin rutinoside, quercetin, a flavonol (MW 316) rutinoside and a flavonol (MW 316) glucoside. A number of minor components remained unidentified.

**Table 4:** Summary statistics for the number of PCE per 200 erythrocytes, ratio of PCE to total erythrocytes, the number of MNPCE per 2,000 PCE and the ratio of MNPCE to total PCE by sex and group.

| Group | Variable | Females | | Males | |
|---|---|---|---|---|---|
| | | Mean | Std. dev. | Mean | Std. dev. |
| 1 (vehicle control) (n = 5) | PCE/200 RBCs | 101.400 | 11.371 | 127.800 | 13.255 |
| | PCE to total RBC ratio | 0.507 | 0.057 | 0.639 | 0.066 |
| | MNPCE/2,000 PCEs | 4.400 | 2.302 | 2.600 | 0.548 |
| | MNPCE to PCE_RATIO | 0.002 | 0.001 | 0.002 | 0.001 |
| 4 (high) (n = 5) | PCE/200 RBCs | 102.400 | 15.043 | 102.000 | 11.979 |
| | PCE_TOT_RBC_RATIO | 0.512 | 0.075 | 0.510 | 0.060 |
| | MNPCE/2,000 PCEs | 5.000 | 3.000 | 3.600 | 2.408 |
| | MNPCE_PCE_RATIO | 0.003 | 0.002 | 0.002 | 0.001 |
| 5-Cyclophosphamide (positive control) (n = 8) | PCE/200 RBCs | 72.000 | 10.994 | 74.875 | 12.124 |
| | PCE_TOT_RBC_RATIO | 0.360 | 0.055 | 0.374 | 0.061 |
| | MNPCE/2,000 PCEs | 19.625 | 4.373 | 18.875 | 2.416 |
| | MNPCE_PCE_RATIO | 0.010 | 0.002 | 0.010 | 0.001 |

The extract of *A. muricata* leaf contained signals characteristic of flavonol glycosides, including rutin, a rutin isomer and kaempferol or luteolin rutinoside and a quercetin glucoside. Further work with pure authentic standards will allow confirmation of identities and quantification.

There were at least 39 chromatographically distinguishable acetogenins in the *A. muricata* leaf extract. Acetogenins are of interest both for their putative neurotoxicological effects at high doses and their potentially therapeutic properties, particularly against cancer [22]. A large number of minor peaks present in the complex *Annona* extract remain unidentified.

All of the three constituents of $A4^+$ were mutagenic in the Ames test at the highest dose of 5,015 µg/mL (ratio 6.0). The mutagenicity of both *Co. lutea* flower and *A. muricata* leaf in vitro is likely due to the presence of quercetin and other flavonoids that are known to give a positive Ames test [23–25], despite a lack of genotoxicity in vivo [24–26]. The anti-mutagenic activity reported for *Curcuma* previously [27, 28] may be due to differences in concentrations examined.

When unidentified potentially genotoxic compounds are present in a compound, an in vivo genotoxicity test is indicated [25, 29]. Therefore, we conducted a sensitive high repeated dose in vivo micronucleus test which was negative as discussed later.

The repeated dose micronucleus study in mice assay produced negative control counts of MNPCE consistent with the results reported by Krishna and Hayashi [30]. The negative control assay range for MNPCE/1,000 PCE was 0.4–3.8 for males and 0.6–3.6 for females, similar to that reported by Shelby et al. [31]. In this study, the means were in the middle of those ranges. Furthermore, the means from the high-dose $A4^+$ treatment group for both sexes were similar and not statistically different from controls, which is the most important indicator that under the conditions of the study, $A4^+$ did not show genotoxicity. Similarly, the positive control ranges for MNPCE/1,000 PCE reported by Krishna and Hayashi [30] were 7.7–42.7 for the males and 8.0–44.7 for the females. In this study, the means for both sexes were within these ranges. For both males and females, the positive control (Group 5) means were significantly greater than the means for the treatment vehicle negative controls (Group 1) and for the $A4^+$ high-dose treatment group (Group 4), indicating that elevated numbers of MNPCE were detected where they were expected to occur, but not with the highest dose of $A4^+$. Therefore, no evidence of genotoxicity of $A4^+$ was uncovered at the highest dose administered.

A secondary endpoint of the micronucleus test is the number of PCE per 200 erythrocytes. Reduced PCE numbers in a treatment group compared with controls may indicate bone marrow toxicity. In both sexes, PCE levels were significantly lower in the positive control group than in the negative control group. There was no significant effect of $A4^+$.

During the 28-day toxicity study, $A4^+$ administration at any of the doses did not significantly affect body weight, food consumption, urinalysis, clinical pathology parameters, blood coagulation, organ weights or macroscopic findings at the highest daily dose. Liver toxicity related to herbal supplements appears to be increasing, which is in part attributed to the presence of contaminants [32]. Therefore, it is important for any new herbal preparation to be thoroughly tested for toxicity as well as to authenticate the product by chemical analysis. In this study, we found no hepatic toxicity following standard toxicity tests.

For FOB measurements, the differences in tail pinch response were deemed not biologically significant given that the abnormal responses occurred only in the control group and no other intergroup differences were observed. It was concluded that none of the doses of $A4^+$ affected the neurobehavioral parameters measured in the FOB from a toxicological viewpoint. Small, statistically significant differences in haematology cell counts were seen, but these were deemed of low biological significance since all of the counts were close to the normal range [33]. It was concluded that $A4^+$ did not exhibit toxicity, including neurotoxicity, under the conditions of this study. A NOAEL of 2,000 mg/kg was assigned.

## Conclusions

In the 28-day repeated dose test, $A4^+$ did not exhibit toxicity under the conditions of this study. A repeated dose micronucleus study in mice was negative for genotoxic effects and did not exhibit bone marrow toxicity. With the results of this study, a NOAEL of 2,000 mg/kg may be assigned for 28-day repeated dose toxicity, genotoxicity and bone marrow toxicity.

## List of abbreviations

| | |
|---|---|
| $A4^+$ | a herbal natural health product to support healthy liver function |
| A4R | extract of *Curcuma longa* rhizome |
| A4F | extract of *Cordia lutea* flower |

| | |
|---|---|
| A4L | extract of *Annona muricata* leaf |
| ANOVA | analysis of variance |
| DAD | diode array absorbance |
| DMSO | dimethylsulfoxide |
| FOB | functional observational batteries |
| GLP | Good Laboratory Practice |
| HPLC | high-performance liquid chromatography |
| LC/MS | positive mode electrospray mass spectrometry |
| MNPCE | micronucleated PCEs |
| NOAEL | no-observed adverse effect level |
| OECD | Organisation for Economic Co-operation and Development |
| PCE | polychromatic erythrocytes |
| PT | prothrombin time |
| PTT | partial thromboplastin time |

**Author contributions:** HS directed the toxicology studies and drafted the manuscript. DS conducted the analytical chemistry. FG coordinated the study and manuscript preparation. SA provided medical and herbal expertise. AC assisted with background research and coordination of the project. JC invented the A4$^+$ formulation and provided the ingredients for testing. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

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