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Assessment of the *in vivo* acute toxicity of aqueous extracts of artavol® antimalaria herbal tea

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Acute toxicity testing plays a pivotal role in the initial safety assessment of products. It examines the effects of a single or multiple doses of a product administered to animals or taken by humans within a 24-hour period. This test holds particular importance in determining the toxic characteristics of products intended for use in humans, animals, and agriculture. Despite the availability of numerous herbal medicinal products in the Ugandan market, there is a notable absence of data regarding their safety profiles. This study focuses on the assessment of acute toxic effects of Artavol®, a herbal tea aimed at malaria prevention. The study follows the guidelines set forth by the Organisation for Economic Cooperation and Development (OECD). An analysis using Gas Chromatography-Mass Spectrophotometry (GC-MS) was carried out on Artavol® to identify its constituent compounds. The yield of the extract from Artavol® was determined to be 4.7%. The product was deemed safe within the limit dose of 5000 mg/kg. The GC-MS analysis of Artavol® revealed the presence of 40 compounds in the extract. Notably, the analysis identified artemisinin compounds (dihydroartemisinin and deoxyartemisinin) as well as coumarin compounds among others. This study reaffirms the previously reported safety of Artavol® up to the dosage of 5000 mg/kg. Furthermore, it confirms the existence of multiple compounds, previously reported coumarins, while also revealing the presence of compounds that were thought to be removed during processing. These latter compounds include dihydroartemisinin and deoxyartemisinin. To ensure the claimed complete deartemisation, further refinement in the processing is recommended.

Key words: Gas Chromatography-Mass Spectrophotometry (GC-MS), analysis, Acute toxicity, antimalaria tea, Artavol, *Artemesia annua.*

INTRODUCTION

Acute toxicity refers to the impact of single or multiple doses of a chemical substance administered to an animal or living system within a 24-hour timeframe. Traditionally, it is defined as the dose causing 50% mortality in test

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organisms, serving as an initial screening for drugs and chemicals to evaluate compound toxicity (Akhila et al., 2007; Lorke, 1983). For substances that cannot be administered all at once, multiple doses are divided and given within 24 hours, mirroring single-dose observation (OECD, 2022). The objective of acute toxicity testing is to identify the single large dose that would cause severe toxicity, including death, when extrapolated to humans or animals. Animals are monitored from intake to the anticipated completion of substance absorption, gauging intense effects post-absorption (Akhila et al., 2007; Erhirhie et al., 2018). In drug development, this applies to products potentially used as drugs. Traditional medicine, widespread in many regions, often assumes safety based on community use, but the World Health Organization (WHO) advises member countries to ensure the safety of their populations from traditional medicine products (World Health Organization, 2013). Despite growing regulation efforts, relaxed standards exist in many places (Carè et al., 2021; Ijaz and Boon, 2018; Kasilo and Trapsida, 2010; World Health Organization, 2013). The COVID-19 outbreak increased the use of herbal medicines, yet some products remain unregulated and possibly unsafe (Komolafe et al., 2021).

Though traditional remedies have historical use, varying preparation methods, solvent quality, and storage affect their safety (Mosihuzzaman and Choudhary, 2008; Ndhlala and Van Staden, 2012; Rousseaux and Schachter, 2003; Saad et al., 2006; Sahoo et al., 2010). Lack of standards and variations can expose users to contaminants like heavy metals and microbes (Mosihuzzaman and Choudhary, 2008). The Ugandan market features several antimalarial products, including Artavol®. Artavol®, a herbal tea for malaria prevention, is derived from Artemisia annua. It's reported that artemisinins are removed to prevent drug resistance. Flavonoids are suspected contributors to its antimalarial effects. Artavol® combines Artemisia annua, avocado seed extracts, and lemon grass extracts. This study employs OECD methods for acute toxicity and GC-MS analysis to validate Artavol®'s claimed safety and compound content.

MATERIALS AND METHODS

Study design and site

An experimental study was conducted over 3 weeks. The extractions were carried out in the Pharmaceutical Analysis Laboratory of Mbarara University of Science and Technology, the Animal experimentation was conducted in the Animal Research Facility of Mbarara University of Science and Technology and the GC-MS analysis was conducted at the Department of Government Analytical Laboratories (DGAL) in Wandegeya, Kampala.

Materials used in the Study

Artavol®, distilled water, saucepans for boiling water, stainless steel metallic steering paddle, stainless steel tank, tincture press, muslin cloth, jericans, funnels, minus 80-degree freezers, bench-top freeze drier, rotary evaporator, 70% ethanol, hexane, ethyl acetate, amber bottles for storing extracts, HPLC, glass observation chambers, plastic Rat cages, water bottles, porcelain/metallic plates, oral metallic cannula, gloves, halothane, anaesthetic chamber, 5ml syringes, water filtration system, animal feeds, bedding materials for the animals, and electronic balances were used in the study.

Animals used in the study and source

White albino Rats were used in the study. They were procured from the animal Research Facility of Mbarara University of Science and Technology after being bred specifically for Research. All Animals were females and were 4 months old although their dates of Birth varied within a one-week period. All Animals had weights between 130 to 150 g. The animals were kept in plastic cages lined with dry wood shavings, provided with pelleted foods in porcelain plates, and had access to water in 2 water bottles hanged through a wire mesh on top of the cages. They were kept in a natural environment of 12 hours of light and 12 hours of darkness.

Experimentation

The acute toxicity experiments were conducted in a clear glass observation chamber to allow for observations of reactions taking place following the administration of the product. The observations were also video recorded in order to allow for auditing to detect effects which could have occurred when the researcher or research assistants had moved from the experimental room. The bottom of the Glass observation chambers was either lined with wood shavings or kitchen tissue papers during experimentation. A total of 4 animals were used and treatment followed the Uganda National Guidelines for Use of Animals in Research and Teaching and the Guide for the care and use of Laboratory animals (Council, 2010: UNCST, 2021). This guideline was developed based on other International Guidelines for research Involving Animal Participants.

Product used

Artavol® was received in a metallic Tin (Figure 1), the product opening was covered in an aluminum foil covering, and inside were brown granules of Artavol®. The product description indicated that the product is composed of extracts of *Artemisia annua*, avocado seeds extracts, and lemon grass extracts. The product was dry and the granules were non-adherent to each other.

Preparation of extracts

Artavol® extraction

Extraction of Artavol® followed the methods used for the preparation of herbal tea. The product was weighed (1 teaspoonful), weight was recorded in order to help in the determination of the approximate yield from a dose which helped in determining the starting dose for the experiment. The 1 tea spoonful was extracted to determine how much a user would be taking from the recommended dose. For the larger extraction, Artavol® was weighed from their container tins (Figure 1) and dissolved in hot boiling water in a transparent graduated bucket, stirred for some time and allowed to stand, mimicking the process of tea making. On settling, the top clear fluid was decanted and later filtered off the other content and taken for freeze drying. This was repeated several times in order to obtain enough extracts for acute toxicity

Figure 1. A tin of Artavol® Used in the study.

study, sub-acute, teratogenicity and mutagenicity studies. The later studies have been conducted separately. The extracts were frozen first in the freezer and taken to the freeze dryer set at freezing temperature (-40°C) so as to reduce the duration of freeze drying. The final brown extract was crushed and stored in umber bottles in order to protect from sunlight.

GC-MS analysis of the artavol® extract

The researchers determined the characteristic chemical composition of the extracts which were being studied in this case using the GC-MS methods. The crude extracts of Artavol® were subjected to GC-MS analysis. Complete raw results of the analysis with chemical structures and chromatogram of each compound detected are presented in the supplemental data 1 for ARTAVOL® (GC-MS Analysis data for ARTAVOL)

Preparation of the sample solutions for GC-MS analysis

To prepare sample stock solution, 1 g of the neat sample was dissolved in Dichloromethane in a 10 ml volumetric flask to the mark. The test solution was then prepared by diluting 500 µl from the sample stock solution with 500 µl of distilled water to a final volume of 1000 µl (50% v/v solution) prior to injection. The vial was capped and the contents were mixed thoroughly before analysis. Care was taken to prevent the loss of volatile components by making all preparations in a closed system.

Gas chromatography conditions

The following chromatographic conditions were used following system suitability tests and method validation (US-FDA, 2020). The volatiles in the sample were analyzed by an Agilent GC-MS operated using a GC-MS Mass Hunter qualitative analysis software with helium as the carrier gas at 1.103 ml/min flow rate, pulsed splitless, injection volume of 5.0 µl. DB-5MS UI capillary column with the dimensions 30 m by ID 250 µm with a film thickness of 0.25 µm was used for separation. The oven temperature was programmed from 70 to 280ºC, with the initial temperature of 70 ºC maintained for 2 min, followed by a gradient of 150ºC at 25ºC/min for 1 min, then 200ºC at 3ºC/min for 1 min and 280ºC at 8ºC/min for 10 min. Total run time of 41.9 min. The mass selector was maintained at an ion source temperature of 280ºC, and electron impact (EI) mass spectra were obtained at the acceleration energy of 70eV. Fragment ions were analyzed in the full scan mode over 30 to 600 m/z mass range. The filament delay time was set at 0 min.

Dissolution of extracts prior to administration to the rats

The Artavol® extract was dissolved in distilled water at concentration of 250 mg/ml before administration to the animals since it is water soluble. This helped a lot in ensuring that the volume that was reached and administered at highest dose of 5000 mg/kg remained small for easy administration to the animals.

Treatment of experimental animals

The OECD test guidelines 425, the Up-and-down methods for toxicity testing using the limit test at 5000 mg/kg was used in this study (OECD, 2022). The starting dose for each animal was automatically calculated by the AOT425 Startpack Software on entering an assumed LD_{50} value which was done basing on previous report of no toxicity at 5000 mg/kg (Ogwang et al., 2011). At the start of the experiment and subsequent doses were determined by the software after entry of the results of the preceding experiment as indicated in the AOT software output in the results section. This was done till a stop signal was indicated by the software when the maximum limit dose of 5000 mg/kg was reached and no toxicity was entered in the software Table 1 and a total of 4 animals were used. Each of the four animals used were dosed at different times 48 hours following the previous one if no signs of toxicity or death were recorded. The route of administration was oral and each animal was dosed once in cases where the calculated doses did not exceed 2 mls / 100 g body weight or twice, 30 min apart when the calculated dose could not be administered once. Volume of administration was greatly influenced by the solubility properties of the product. During observations, the animals were placed in a glass observation cages immediately following treatment and a video recording was started to keep track of what would be happening to the animal in each chamber of the observation cage where each animal was placed. The animals were observed for signs of toxicity such as; failure to eat or drink, rough hair coat, unusual posture, vocalization, urination, diarrhea, stereotypical behaviors, lethargy, coma, etc. All rats used in the experiments were fed on NOVITA mice pencils and provided with filtered water in plastic water bottles which the animals could access freely. All animals used at the end of the 14 days were kept for further breeding after giving a further wash-out period of 4 more weeks as there was no reason to euthanize them at the end of the experiment because no minor or serious sign of toxicity was observed. At this period, they were housed in groups in plastic cages with wood shavings as beddings which were changed after every 4 days.

Ethical consideration

This study followed National guidelines for use of animal in research and Teaching (UNCST, 2021) and was approved by the Mbarara University of Science and Technology Research Ethics

Table 1. the GC-MS analysis detected 40 different compounds in the Artavol® extract.

SN	RT	Compounds found in aqueous extract of Artavol®	SI
1	8.2240	1H-2-Benzopyran-1-one, 3,4-dihydro-	86.4
2	8.3177	5-Ethoxy-3,4-dihydro-2H-pyrrole-2-carboxylic acid, ethyl ester	68
3	8.4381	Dimethyl methylphosphonate	71.6
4	8.4636	7-Hydroxythujone	70.6
5	8.6191	2-Diethylamino-N-methyl-2-phenyl-acetamide	70.4
6	8.9622	Coumarin	88.5
7	9.3609	Borane, diethyl(decyloxy)-	72.4
8	9.7211	2,4-Di-tert-butylphenol	70
9	9.7217	Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters	71.6
10	9.8189	.alpha.-Methyl-.alpha.-[4-methyl-3- pentenyl]oxiranemethanol	73.8
11	9.9600	2-Hydroxy-1-(1'-pyrrolidiyl)-1-buten-3-one	81.2
12	10.0375	Methyl orotate	74.6
13	10.3432	.alpha.-Methyl-.alpha.-[4-methyl-3- pentenyl]oxiranemethanol	71.2
14	10.3579	Propanamide, N-methyl-2-(methylamino)-	89.4
15	10.4448	1-Ethyl-1H-pyrazole-3,4-diamine	72.2
16	10.8094	Bicyclosesquiphellandrene	70.4
17	10.8911	Ethanone, 1-(4-hydroxyphenyl)-2-phenyl-	69.8
18	11.4814	(1S,4S,4aS)-1-Isopropyl-4,7-dimethyl-1,2,3,4,4a,5- hexahydronaphthalene	81.2
19	11.9874	(1S,4S,4aS)-1-Isopropyl-4,7-dimethyl-1,2,3,4,4a,5- hexahydronaphthalene	80.1
20	12.5728	4,4,5,8-Tetramethylchroman-2-ol	72.8
21	12.7914	(3-Fluorophenyl) carbamic acid, 2-isopropyl-5- methylphenyl ester	70.8
22	12.7916	3-Methyl-4-isopropylphenol	74.0
23	13.3477	3-(4-Isopropylphenyl)-2-methylpropionaldehyde	74.9
24	14.2716	2,4-Dimethyl 1,4-pentadiene	70.1
25	14.2879	4-Amino-5-formamidomethyl-2-methylpyrimidine	72.3
26	14.3834	(3E,10Z)-Oxacyclotrideca-3,10-diene-2,7-dione	71.6
27	15.3835	Pyrimidine, 4,6-dimethoxy-5-acetyl-	76.8
28	15.3938	2,5-Dihydroxy-4-methoxyacetophenone	76.3
29	16.8920	2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester	71.2
30	20.0266	Dibutyl phthalate	85.6
31	20.3171	6-Hydroxy-7-methoxycoumarin	71.8
32	21.1256	2-Amino-5,6-dimethylbenzimidazole	70.8
33	22.4453	Deoxyartemisinin	77.1
34	22.4587	Deoxyartemisinin	77.7
35	24.5838	Cyclohexane, 1-methyl-4-(1-methylethenyl)-, cis-	71.1
36	25.5222	Butyl citrate	71.6
37	25.9251	Cedrol	76.6
38	26.2716	Dihydroartemisinin, 3-desoxy-	62.4
39	27.6435	Ether, 2-chloro-1-methylethyl isopropyl	71.9
40	32.4941	1,4-Benzenedicarboxylic acid, bis (2-ethylhexyl) ester	70.3

Committee with approval number MUREC 1/7 and Uganda National Council for Science and Technology with Registration number HS540ES. The experimental procedure was conducted following the OECD guidelines for acute oral toxicity testing (OECD, 2022)

from 1 tea spoonful of the Artavol® product used in the tea or porridge. The 0.2g was used here to determine how much Artavol® extract one takes on adding 1 tea spoonful to their tea or porridge as directed in the product insert.

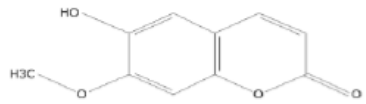
RESULTS

Extraction yield

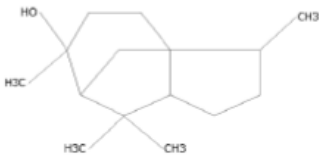
A yield of 4.7%, Artavol, were obtained (0.2g) which is

GC-MS analysis results of the two products tested

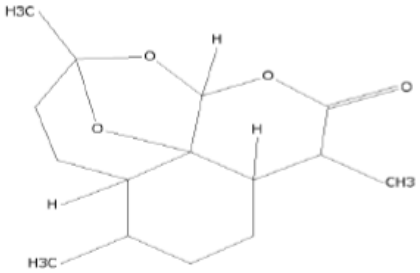
The Gas Chromatography-Mass spectrophotometric



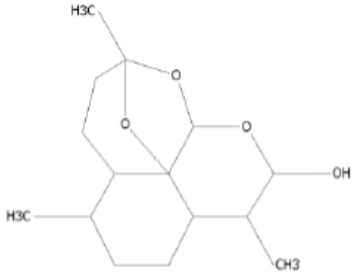
6-Hydroxy-7-methoxycoumarin



Cedrol



Deoxyartemisinin



Dihydroartemisinin, 3-desoxy-

Figure 2. Chemical structure of selected compounds found in the Artavol® extract.

analysis of the Artavol® extract conducted indicated the presence of multiple compounds in the products. Artavol® had 40 compounds Table 1, with some selected compounds presented in-text (Figure 2 selected compounds detected in Artavol®). Although claims from the product insert in artavol® indicated that the product is devoid of artemisinin compounds, the GC-MS analysis conducted in this study has indicated that the products contains artemisinin and other compounds earlier reported present in the product.

Acute toxicity results

The acute toxicity testing indicated that both extracts are not toxic up to a limit dose of 5000mg/kg. The Result showing dose progressions and suggested starting dose based on the AOT425 software for Artavol® granule and Flavonoid isolated from *Artemisia annua* acute toxicity test are presented in the Figure 3.

DISCUSSION

The findings from the acute toxicity study on Artavol® indicated that the product is safe up to a limit dose of 5000 mg/kg and thus the LD_{50} is greater than 5000 mg/kg (Figure 3). This thus puts Artavol® as a product in the GHS of toxicity rating of chemicals at the category of 5 (Kwon and Yu, 2005; Morris-Schaffer and McCoy, 2020). Considering the dosage of Artavol® at 1 spoon per day for 1 week and 1 spoon every week, the levels of exposure would be much smaller than used in the acute toxicity test even after taking for 1 week on a daily basis. Further, the yield of flavonoids which would be expected from the one spoon of Artavol® although not estimated would be very small. It was also noted that, Artavol® product swells when dissolved in hot water and this, makes it impossible to take a dose of more than 50 mg at once as estimated from the extract used in this study. During the study period, animals were observed for various signs of toxicity including; urination, restlessness,

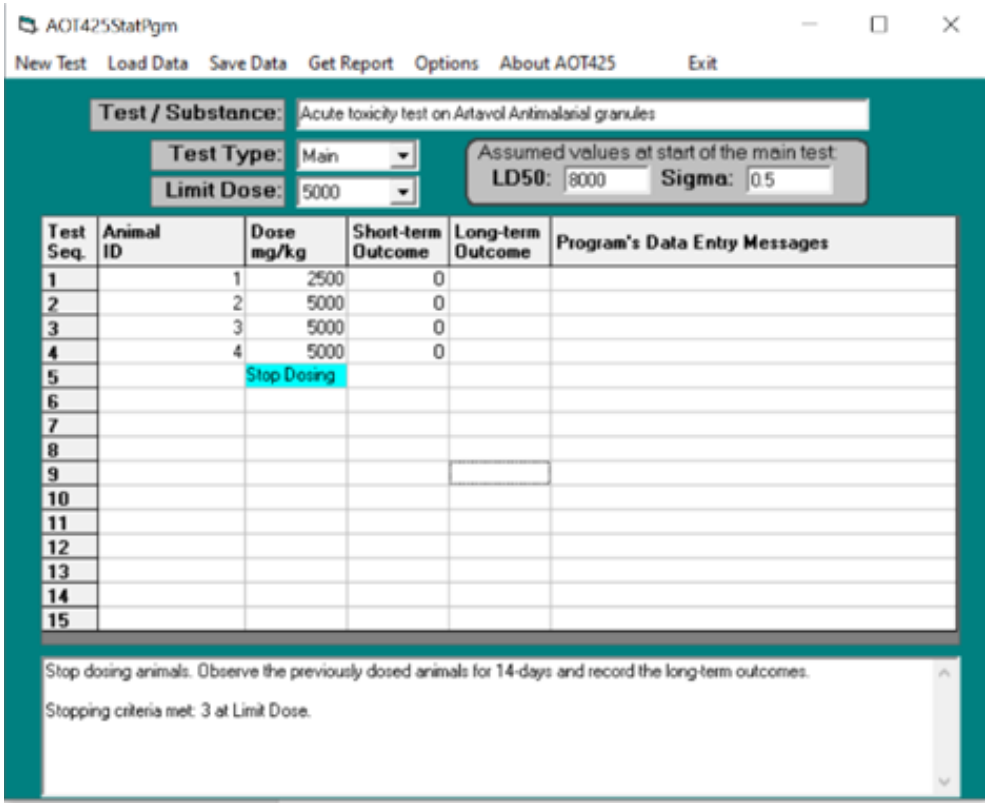


Figure 3. AOT425 Statpgm output for the acute toxicity test on Artavol®.

stereotypical behavior, groaning, writhing, tearing, pilo-erection, failing to eat or drink, drowsiness, sleep and death in 24 hours and over a period of 14 days. As indicated in the results section for acute toxicity study, no death was recorded either immediately, in 24 hours or in 14 days. A study by (Udoh et al., 2016) indicated that the $LD5_0$ of Dihydroartemisinin was found to be 547.70 mg/kg although this was not the case or even close in this study for which dihydroartemisinin was detected in the product. This indicates that the levels of dihydroartemisinin in the product must be very low. This confirms the reported no toxicity at 5000 mg/kg for Artavol® as in (Ogwang et al., 2011).

According to the study by Ogwang et al. (2012) it was documented that *Artemisia annua* tea had a protective effects of 50% in the population of workers in a flower farm who used it over a period of 9 months and it also concluded that the observed effects could be due to the flavonoid content (Ogwang et al., 2012). The findings above which has indicated that the LD_{50} is greater than 5000 mg/kg for Artavol®, does not guarantee their safety at doses above these levels and whether their toxicity profiles would remain the same at higher doses. The current findings which found both product having a LD_{50} greater than 5000 mg/kg thus confirms the earlier results reported (Ogwang et al., 2012). The claim that the product was devoid of artemisinin compounds as in Ogwang et al. (Ogwang et al., 2012) is not true as dihydroartemisinin-3-desoxy and deoxyartemisinin with confirmed chemical structures (Figure 1 Artavol® select) were found in the product from the Gas-Chromatography Mass spectrophotometric analysis conducted and a list of other compounds detected in Artavol® (Table 1). The antimalarial effect of the product reported could most likely be due to the presence of these artemisinin compounds in the product. Compounds found in Lemon grass which are reported to possess antimalarial properties such as cedral were also found in the product, a further confirmation that lemon grass was used in the product (Chukwuocha et al., 2016; Dapper et al., 2008; Ekpenyong et al., 2015; Mukarram et al., 2021; Shah et al., 2011). Reports on the antimalarial activities of extracts of Avocado seed extracts which is a component of Artavol have also been made (Adesina et al., 2016; Leite et al., 2009).

Further deartemisinisation is thus required to get rid of the found artemisinin products. Our estimates from the behaviors of Artavol® once it was dissolved in hot water and the yield indicated that it would be difficult for a person to dissolve an amount which would give a yield of the total extract to a level of 1500mg/kg at once. This means that, there is no possibility of a person taking a dose which can cause toxicity requiring emergency treatment. A previous case report (Ruperti-Repilado et

al., 2019) had reported a case of cholestatic jaundice in an Ethiopian returnee patient who had been on *Artemisia annua* herbal tea. During the 14-day observation period of the animals treated in this study, efforts were made by continually checking for symptoms of jaundice in rats treated with the highest dose of 5000mg/kg but no jaundice was observed. It is however, difficult to predict if this could have been contributed by differences in the end product metabolites in rats as compared to the humans. This aspect would require further investigations and analysis during the repeat dose studies. The absence of observed toxicity from Artavol® doses used means that the OECD Method may not be the best for studying herbal products with toxicity profiles above 5000 mg/kg. From the above, other methods such as Lorke (1983) method may be better for studying such herbal products for the purpose of determining dose levels at which toxic effects would begin. A review study (Heghes et al., 2022) noted that the toxicity of natural coumarins in rats was 290-680mg/kg and in comparison to the composition of the Artavol® for which no toxicity was noted, this is an indication that the concentration of the coumarins in the products is very low.

Conclusions

The study findings clearly indicate that Artavol® has not demonstrated any acute toxic effects up to a dosage limit of 5000 mg/kg body weight. The GC-MS analysis has shown that Artemisins have not been completely removed from Artavol®. Furthermore, it has provided evidence that Artavol® contains dihydroartemisinin-3-desoxy and deoxyartemisinin, suggesting incomplete deartemisation. These compounds could contribute to the antimalarial effects of the product. In conclusion, further investigation is needed into higher doses exceeding 5000 mg/kg body weight. Such investigation should employ the Lorke's method, as the OECD 425 guidelines do not permit studies exceeding the 5000 mg/kg limit.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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