

Determination of *in vitro* Cytotoxicity and *in vivo* Subacute Toxicity of a Phytomedicine Named "Phytomed"

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ABSTRACT

Background and Objective: Traditional medicine has a prominent place in the treatment of various diseases in Africa and Togo. The major handicap of traditional medicine is the insufficient data on the quality and toxicity of medicinal plants. This study was carried out to assess the safety of a phytomedicine which was named "Phytomed" for the study purpose. **Materials and Methods:** Before the toxicity tests, phytochemical screening was performed and the cardiac glycoside content was determined in the phytomedicine. The safety assessment involved, cytotoxicity (Brine shrimp lethality test) and acute and subacute toxicity (TG 423 and 407) tests. For the subchronic (28 days) toxicity test, the Wistar rat's body weight, or relative weight and haematological and biochemical parameters were measured and histological analysis was done. The cardiac activity of the formulation was then conducted on the *in situ* toad's heart. **Results:** The cytotoxicity test showed an LC₅₀ of 0.28 mg mL⁻¹. The 28 day repeated administration of Phytomed has induced a significant increase in platelet number at 0.43 and 0.85 mL kg⁻¹ and a significant decrease (p<0.05) in CPK at 0.85 mL kg⁻¹. The assessment of the phytomedicine effect on the toad *in situ* heart revealed a negative chronotropic effect. **Conclusion:** Phytomedicine did not induce toxicity. However, because of the significant increase in the number of platelets and the negative chronotropic effect observed, precautions should be taken during the use of this phytomedicine in the event of thromboembolic and cardiac disorders.

KEYWORDS

Phytomedicine, phytochemical screening, cardiac glycoside, cytotoxicity, *Artemia salina*, acute-subacute toxicity, cardiac activity

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INTRODUCTION

The use of plants in traditional medicine is a very old and widespread practice in the world, especially in developing countries¹. More than 80% of the African population use medicinal plants to treat themselves². Traditional medicine, therefore, has a prominent place in the treatment of various diseases in Africa and



Togo^{3,4}. However, despite its undeniable wealth, the major handicap of traditional medicine, both now and yesterday, has been and remains insufficient knowledge of doses and biochemical, pharmacological and toxicological properties of plants⁵. It is therefore, important to improve and rationalize the use of empirically administered extracts of African medicinal plants by demonstrating their pharmacological effects and safety. The increase in the use of traditional medicine, because it seems promising, without risk⁶. The risks of poisoning exist and many area cases of intoxication, in which plant-based products are implicated⁷.

Polyherbal formulations are often proposed in the simultaneous management of many diseases such as malaria, anaemia, diabetes, hypertension or erectile dysfunction. Because of their composition and the presence of many bioactive molecules, these formulations increase the risk of interaction and this does not always lead to the expected positive synergistic effect, but rather to intoxications⁸⁻¹⁰. The WHO is advocating the development of traditional medicine¹, which is widely used by our people in Africa because of its accessibility and acceptability¹¹. With this in mind, the collaboration between traditional therapists and scientists is crucial to ensuring the safety and effectiveness of plant-based health products.

This study was carried out as a part of this collaboration and is intended to assess the safety of a phytomedicine, used in the treatment of erectile dysfunction.

MATERIALS AND METHODS

Study area: The study was carried out in the laboratory of toxicology at the Department of Pharmaceutical Sciences, Faculty of Health Sciences, University of Lomé, Togo in March to November, 2019.

Plant material/formulation: Plant material was the phytomedicine provided by a traditional therapist. This wording has been named Phytomed in this study. It was marron-coloured, with a slightly alcoholic taste (37°) and conditioned in 500 mL vials. The composition of this formulation was not given by the traditional therapist. The traditional phytomedicine was used as provided by the traditional therapist without any changes.

Animals: Five hundred and twenty-eight Brine shrimp (*Artemia salina*), 54 toads (*Bufo bufo*) and 27 Wistar rats (*Ratus norvegicus*) served as animal material for the experimentations. Wistar rats were provided by the Animal Physiology Department of the University of Lomé. Rats were acclimated at least one week before the debut of manipulations. They were fed standard rodent diets and had free access to water *ad libitum*. They were kept before and during the study at a temperature of 22±2°C with a relative humidity of 40% and the photoperiod was 12 hrs of light and 12 hrs of darkness. Animal care and handling were done as in previous study^{12,13}. Brine shrimp eggs were provided by the Toxicology Department of Pharmaceuticals Sciences of the University of Lomé. Toads were captured the night before the experimentation. Ethical approval was obtained from the Committee on Institutional Ethics in Education and Research under issue (Ref. CNCB-CEER 2801/2010).

Qualitative phytochemical screening: The test was performed to assess the presence of some phytochemical groups such as flavonoids, cardiac glycosides, tannins, reducing compounds, saponosides, sterols, triterpenes, coumarins and alkaloids^{14,15}.

Quantitative phytochemical screening (cardiac glycosides dosage): To 1 mL of the deflated extract was added 10 mL of Baljet reagent. After one hour of incubation, distilled water was added (20 mL). The absorbance of the mixture was read with a spectrophotometer at 495 nm. The cardiac glycoside content was determined from a range of calibrations with digoxin (10-100 dg mL⁻¹) and the results were expressed in mg of digoxin equivalent per gram of dry extract (mg EqD/g). The sample was tested in triplicate¹⁶.

Experimental design for toxicity studies

Cytotoxicity test: The test was conducted as described by Meyer *et al.*¹⁷ and Diallo *et al.*¹⁸. For this particular test, a volume of phytomed was evaporated to determine the mass contained in a given volume; thus, allowing the IC₅₀ determination. *Artemia salina* eggs (500 mg) were introduced into a bottle containing 500 mL of previously filtered seawater. The container was placed under automatic agitation for 48 hours. After hatching, live larvae were distributed in 11 test tubes at the rate of 16 larvae per tube in 1 mL of seawater. In the first 10 tubes, the Phytomed was added as a result of an order 2 dilution from a 6 mg mL⁻¹ stock solution. The last tube served as the control. The tubes were then incubated for 24 hrs. After this incubation period, the tubes were analyzed and the number of dead larvae was recorded. A larva was considered dead if it did not show movement for at least 30 sec of observation. The number of dead larvae in the control tube validated the test in case the mortality rate in the control tube was less than 15%. The test was repeated three times.

Acute toxicity test: The test was conducted as described by Dossou-Yovo *et al.*¹⁹. The Phytomed was sequentially administrated to three female Wistar rats over 48 hrs at 10 mL kg⁻¹ (maximum volume to be administered orally). They were observed over 14 days for possible signs of toxicity.

Subacute toxicity test: Repeated 28 days toxicity was carried out as described by Eliassou *et al.*²⁰. Volumes used for this test were defined based on the doses recommended to an adult (70 kg) by the traditional therapist. These doses were related to the weight of the animals. The animals were divided into four groups of six rats each. The control group (T) received distilled water. The second group received alcohol at 37° and the two other groups received respectively 0.43 mL kg⁻¹ (P1) and 0.85 mL kg⁻¹ (P2) of Phytomed. Preparation was administered orally for 28 days.

On the 29th day, after 12 hrs of fasting, the rats were anaesthetized by ether and blood was collected from the retro-orbital sinus in EDTA and dry-chemical tubes respectively. For biochemical analyses, urea, creatinine, transaminases (ALAT and ASAT), alkaline phosphatase (ALP) and creatine kinase (CPK) were measured. The ionogram was realized to determine the concentration of ions such as calcium, potassium, sodium and chloride. Concerning the haematological parameters, the number of red blood cells (RBC), hematocrit (HT), mean globular volume (MGV), haemoglobin (HB), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH) and platelet count (PLT) were determined.

The rats were then sacrificed by ether. The heart, kidneys, testes, spleen and liver were collected, weighed and the weight obtained was used to determine the relative weight of each organ expressed, by the formula:

$$PR = \frac{\text{Organ weight}}{\text{Weight rat}} \times 100$$

During the period of experimentation, rats were observed macroscopically to detect possible adverse effects. Thus, mortality, behavioural and physiological parameters (aggressiveness, appetite and diarrhoea, mobility) were assessed daily during the 28 days of the experimentation.

Effect of Phytomed on *in situ* toad heart: The cardiac activity test was conducted following the method described by Aboudoulatif *et al.*²¹. The toad was decerebrated, demodulated and then put on dorsal decubitus. The heart was clouded by cutting successively, the skin, the musculoskeletal wall and the pericardium. The heart apex has been clustered to an end wire linked to the force transducer. The recording of heart rate and amplitude was done with LabChart 8 application software. The heart was exposed to a range of 10 µL to 1 mL of the formulation.

Statistical analysis: The results obtained were analyzed using the GraphPad Prism 6.02 software and expressed as mean with the Standard Error on the Mean (MESM). Analysis of Variance (ANOVA) was used to compare different groups. Differences between the two groups were determined by Tukey's test. The significance threshold was set at $p < 0.05$.

RESULTS

Qualitative phytochemical screening: The qualitative phytochemical screening of Phytomed (Table 1) revealed the presence of tannins, flavonoids, reducing sugars, saponins, alkaloids, triterpenes and cardiac glycosides.

Quantitative phytochemical screening (cardiac glycoside dosage): The optical densities obtained from the digoxin range allowed us to plot the standard curve (Fig. 1) from which the cardiac glycosides value contained in the Phytomed was determined. The value obtained was 9.67 ± 0.43 mg digoxin/mL of Phytomed.

Toxicity tests

Cytotoxicity: The number of dead larvae in each tube allowed us to plot a logarithmic trend curve (Fig. 2) expressing the number of dead larvae as a function of solution concentration. From this trend curve, the LC_{50} was determined to be 0.28 mg mL^{-1} .

Acute toxicity: No observable toxicity signs were observed and no behavioral changes were observed during the 14 days of observation. No deaths were recorded.

Table 1 : Phytochemical screening of a phytomedicine named "Phytomed"

Chemical groups	Results
Tannins	+
Flavonoids	+
reducing sugars	+
cardiac glycosides	+
Saponins	+
Alkaloids	+
Triterpenes	+
Sterols	-
Anthracenes	-
Coumarins	-

+: Présence and -: Absence

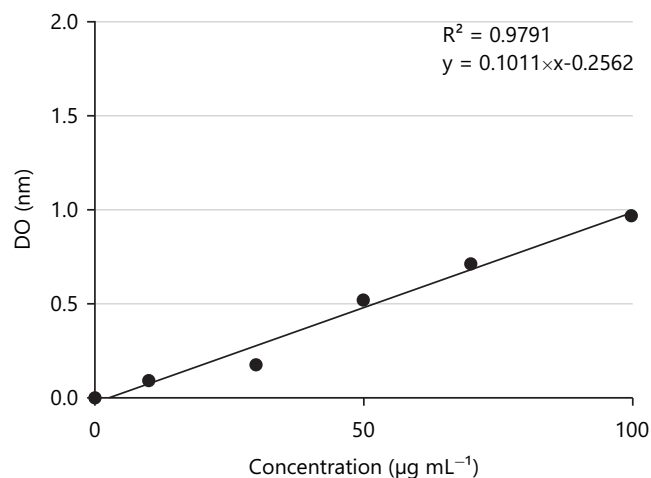


Fig. 1: Standard curve of the digoxin range

Cardiac glycoside content was determined from a range of calibration with digoxin ($10\text{-}100 \text{ dg mL}^{-1}$) and the results were expressed in mg of digoxin equivalent per gram of dry extract (mg EqD/g). The sample was tested in triplicate

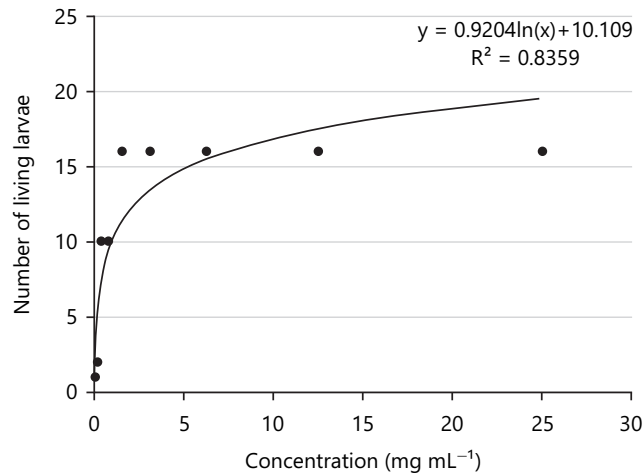


Fig. 2: *In vitro* cytotoxicity of phytomedicine named "Phytomed" on *Artemia salina* larvae

Sixteen live larvae were brought into contact with the series of phytomedicine at progressive concentrations. After 24 hours of incubation. The mean percentage mortality was plotted against the concentrations. The concentration (LC_{50}), at which 50% of the larvae were killed, was determined from the graph and n (the number of replies) = 5

Table 2: Effects of phytomedicine named "Phytomed" on rats body weight

Week	Control	Alcohol 37°	«Phytomed»	
			0.43 mL kg ⁻¹	0.85 mL kg ⁻¹
0	124.25±4.55	146.25±6.86	143.50±5.97	143.00±6.44
1	127.75±4.66	149.00±4.60	151.00±7.42	153.25±5.96
2	147.75±4.32	155.75±7.18	160.25±10.05	159.00±5.97
3	162.25±3.35	159.00±9.74	168.50±9.06	169.00±9.65

Each value represents the Mean±Standard Error on the Mean (SEM) and $n = 6$

Table 3: Effects of phytomedicine named "Phytomed" on organs relative weight (%)

Organ	Control	Alcohol 37°	«Phytomed»	
			0.43 mL kg ⁻¹	0.85 mL kg ⁻¹
Spleen	0.60±0.13	0.62±0.15	0.54±0.05	0.25±0.02
Heart	0.45±0.03	0.43±0.02	0.41±0.01	0.38±0.03
Kidney	0.63±0.02	0.58±0.01	0.56±0.02	0.59±0.03
Liver	3.56±0.17	3.40±0.15	3.18±0.13	3.14±0.13
Testicles	1.41±0.11	0.86±0.28**	1.22±0.05	1.28±0.03

Each value represents the Mean±Standard Error on the Mean (SEM), $n = 6$ and ** $p < 0.01$ (alcohol 37° vs control)

Subacute toxicity

Clinical observation: The 28 days oral administration of Phytomed did not cause any change in the behavior of the treated group rats. No death or adverse effects were observed during the experimentation.

Effect of Phytomed on animal's body weight: Phytomed did not induce any significant changes in the mean weight of rats during the 28 days of administration (Table 2).

Effect of Phytomed on organs relative weight: The relative weight of rat organs after 28 days of the experiment showed a significant decrease in the relative weight of testes in the batch receiving alcohol at 37° (Table 3).

Effect of Phytomed on hematological parameters: Hematological parameters showed a significant increase concerning the number of platelets in the 37° alcoholic group ($p < 0.0001$) and in treated groups ($p < 0.05$) (Table 4).

Effect of Phytomed on biochemical parameters: For biochemical parameters, CPK significantly decreased ($p < 0.05$) in the group which received alcohol at 37° and Phytomed at 0.85 mL kg⁻¹ compared to the control. There was also a significant decrease in alkaline phosphatase in the alcoholic batch ($p < 0.0001$) (Table 5).

Effect of Phytomed on *in situ* toad's heart: The results of the formulation effect on the *in situ* toad's heart were shown in Table 6. A significant decrease in frequency was observed from 40 µL of the formulation.

Table 4: Effects of phytomedicine named "Phytomed" on hematological parameters

Parameter	Control	Alcohol 37°	«Phytomed»	
			0.43 mL kg ⁻¹	0.85 mL kg ⁻¹
WBC (10 ⁹ µL ⁻¹)	6.83±0.77	6.52±0.44	6.55±0.83	8.43±0.80
RBC (10 ¹² L ⁻¹)	7.52±0.38	7.07±0.52	7.54±0.10	6.38±0.20
HB (g dL ⁻¹)	13.42±0.70	12.97±1.09	14.00±0.26	12.53±0.28
HT (%)	40.48±0.79	40.13±0.66	37.23±0.44	38.65±1.00
MGV(fl)	54.35±1.99	58.38±4.51	49.43±0.47	59.93±1.31
MCH (Pg)	17.83±0.23	18.50±1.42	18.55±0.29	19.45±0.82
MCHC (g dL ⁻¹)	33.07±1.31	32.18±2.40	37.50±0.29	32.48±1.11
PLT (10 ⁹ L ⁻¹)	501.50±26.35	667.33±67.36****	603.75±88.14*	601.75±76.71*

Each value represents the Mean±Standard Error on the Mean (SEM), n = 6, * $p < 0.05$, **** $p < 0.0001$ (alcohol 37° or «Phytomed» vs Control). RBC: Red blood cells, WBC: White blood cells, HB: Haemoglobin, HT: hematocrit, MGCV: Mean globular volume, MCH: Mean corpuscular haemoglobin, MCHC: Mean corpuscular haemoglobin concentration and PLT: Platelet count

Table 5: Effect of phytomedicine named "Phytomed" on biochemical parameters

Parameters	Control	Alcohol 37°	«Phytomed»	
			0.43 mL kg ⁻¹	0.85 mL kg ⁻¹
ASAT (UI L ⁻¹)	156.40±18.99	118.50±9.86	142.50 ±7.31	142.25±14.62
ALAT (UI L ⁻¹)	68.83±9.36	68.67±12.99	67.50±8.49	83.50±5.56
ALP (UI L ⁻¹)	484.60±76.81	187.17±36.02****	526.50±39.78	493.75±26.62
Creatinine (mg dL ⁻¹)	0.43±0.05	0.43±0.08	0.53±0.03	0.45±0.05
Urea (g L ⁻¹)	0.33±0.04	0.42±0.03	0.25±0.05	0.21±0.01
Glycemia (mg dL ⁻¹)	70.67±5.34	61.33±3.95	96.50±8.45	91.50±5.42
CPK	764.60±94.73	626.83±116.66*	718.25±85.35	608.00±101.76*
Ca ²⁺ (mg dL ⁻¹)	103.80±5.01	110.50±17.08	101.75±2.29	103.50±1.55
Na ⁺ (mmol L ⁻¹)	146.00±0.98	146.00±0.92	145.65±0.99	147.25±3.14
K ⁺ (mmol L ⁻¹)	6.03±0.53	5.69±0.37	5.12±0.05	5.00±0.14
Cl ⁻ (mmol L ⁻¹)	105.05±0.94	103.78±0.44	103.50±0.90	105.08±2.17

Each value represents the Mean±Standard Error on the Mean (SEM), n = 6, * $p < 0.05$, **** $p < 0.0001$ (Alcohol 37° or «Phytomed» vs control). ASAT: Aspartate aminotransferase, ALAT: Alanine aminotransferase, ALP: Alkaline phosphatase, CPK: Creatine phosphokinase, Ca²⁺: Calcium, Na⁺: Sodium, K⁺: Potassium and Cl⁻: Chloride

Table 6: Effect of phytomedicine named "Phytomed" on the *in situ* toad's heart

	Amplitud (g)	Frequency (bpm)
Control	1.19±0.11	67.21±6.10
«Phytomed» 10 µL	1.24±0.12	62.72±5.36
«Phytomed» 20 µL	1.28±0.13	56.67±4.83
«Phytomed» 40 µL	1.32±0.13	53.66±4.22*
«Phytomed» 80 µL	1.31±0.12	50.38±3.77**
«Phytomed» 160 µL	1.27±0.14	47.37±1.88****
«Phytomed» 320 µL	1.19±0.18	48.48±1.71***
«Phytomed» 640 µL	1.01±0.15	49.60±1.44**
«Phytomed» 1 mL	0.99±0.13	50.54±2.76**

Each value represents the Mean±Standard Error on the Mean (SEM), n = 6, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ («Phytomed» vs control) bpm: Beat per minute

DISCUSSION

In this study, the toxicological assessment of phytomedicine was carried out. This toxicological assessment was preceded by phytochemical screening. This process characterizes the large chemical groups present in a plant to determine the phytochemical composition of the different plant products. These chemical groups are often responsible for plants' pharmacological and/or toxicological properties. The screening revealed the presence of certain chemical groups such as flavonoids, tannins, alkaloids, cardiac glycosides, triterpenes and saponins. Various properties are attributed to these chemical groups. Thus, saponins would stimulate sexual activity²². Tannins and flavonoids are often cited for hemostatic properties²³. Flavonoids are also known for their antioxidant properties²⁴. However, these phytochemical groups, despite their pharmacological virtues, may present toxicity risks, especially in the event of misuse and/or overdose. Thus, chemical groups such as cardiac glycosides, which are beneficial at low doses in the management of heart failure, may be fatal at high doses^{25,26}. Similarly, alkaloids are incriminated in cases of intoxication, especially based on their ability to induce tumors concerning pyrrolizid alkaloids.

The toxicological study of Phytomed focused on the assessment of cytotoxicity and both sides on the assessment of its acute and subacute toxicity.

Brine shrimp's lethality test is a simple and reproducible cytotoxicity test that allows determining the toxicity of plant extracts or bioactive molecules²⁷. This classification of the toxic effect is made by determining the lethal concentration capable of killing 50% of the larvae (CL₅₀). The LC₅₀ of the formulation is 0.28 mg mL⁻¹, a value greater than 0.1 mg mL⁻¹. Phytomed is therefore not cytotoxic according to the Mousseux scale²⁸.

At the maximum volume administered in a single dose, no toxic effects that could result in the death of treated rats or affect their behavior were observed. But this result would not mean that repeated use of the extract would not cause organ damage.

The 28 days repeated dose administration explored the effect of substances on various parameters. During the 28 days of administration of the formulation, no deaths or clinical abnormalities were observed.

Changes in rat weight did not show any significant difference between the control and treated groups. The formulation had no deleterious effect that could influence the weight of male Wistar rats at 0.43 and 0.85 mL kg⁻¹.

Reducing body weight may be a sensitive and simple toxicity index and a 5% reduction in body weight is considered an empirical predictor of pathological discoveries²⁹. The variation in body weight of the treated groups versus control group rats did not show any significant difference after 28 days of the experiment. The relative weights of the organs provide information on possible hypertrophy and atrophy of these organs³⁰. The relative weight of organs such as the heart, spleen, testis, liver and kidney did not show any significant difference between formulation and control groups. However, there was a significant decrease in the relative weight of testes in the alcoholic group compared to the control group. This would therefore indicate testicular atrophy. Previous studies have demonstrated the harmful effects of alcohol on testes^{31,32}. These adverse effects would result in a decrease in the number of testicular cells, atrophy of the seed tubes and a decrease in the relative weight of testes among others³³. However, the relative weight of the organs did not show any significant difference between the treated groups and the control, meaning that the alcohol in the formulation did not affect the testis. Thus, plant extracts in the formulation could mitigate the effect of alcohol.

Analysis of haematological parameters allows a predictive projection of the action of the extract on human blood cells³⁴. The results revealed a significant increase in the number of platelets in the alcoholic group ($p < 0.0001$) and in the treated batches ($p < 0.05$) compared to the control batch. This increase in platelet number would not be related to the presence of alcohol in the formulation, since excessive alcohol consumption would induce thrombocytopenia and a decrease in platelet activity^{35,36}. The formulation, therefore, would not cause toxicity in blood cells, especially since other haematological parameters did not reveal significant differences. The liver is one of the most important organs of the organism according to its role in the metabolism of substances, especially those absorbed orally. Hepatic toxicity assessment is based on very important data, transaminase assay (ALAT, ASAT), alkaline phosphatase (PAL) and glucose^{37,38}. Increased transaminase rates are often associated with liver cell injury or lysis because they are essentially cytosolic enzymes. Alkaline phosphatase is a marker of biliary activity³⁹. No significant differences were observed for transaminases, so the Phytomed would not have a toxic effect on the liver. This is consistent with the organ relative weight results which did not reveal any significant difference as well. The significant decrease in ALP observed in the alcoholic batch would be due to alcohol, which would cause a decrease in the ALP rate⁴⁰.

In synergy with the liver, the kidneys contribute to the good functioning of the organism. They are mainly involved in the excretion process and are therefore exposed to the effect of xenobiotics. Serum electrolytes, on the one hand, creatinemia and uremia on the other, allow the renal function to be explored^{41,42}. No significant differences were noted concerning the ions explored in this study. Similarly, no significant difference between treated and control groups was revealed regarding uremia and creatinemia. So, the Phytomed would not have induced nephrotoxicity. Other biochemical parameters, such as CPK, explore the integrity of heart tissue. Thus, an increase in its rate could result from heart injury⁴³. A significant decrease in treated batches was observed after the experiment. This decrease could indicate a cardio-protection effect of Phytomed which could thus be due to the presence of flavonoids^{25,44}.

The study of the effect of Phytomed on the *in situ* mechanical activity of the toad's heart revealed, above all, a negative chronotropic effect from 40 μL to 1 mL. However, the decrease in heart rate which reflects this negative chronotropic effect remains significant in this volume interval. As regards the amplitude of cardiac contractions was not significantly modified. The observed chronotropic effect could be due to the cardiac glycosides contained in the Phytomed. These cardiac glycosides would cause a decrease in heart rate by reducing sympathetic activity and, to a lesser extent, by increasing parasympathetic activity at the sinus level. The effect of the Phytomed on the *in situ* toad's heart, however, did not lead to cardiac arrest, so it would be relatively non-toxic to the heart despite the induced changes.

Due to the significant increase in the number of platelets and the negative chronotropic effect observed, precautions should be taken during the use of this traditional formulation in the event of thrombosis disorders. Future work should be carried out to determine the toxic action mechanisms leading to the increase in the number of platelets, the negative chronotropic effect and these effects in case of chronic use.

CONCLUSION

In this study, the toxicological assessment of a traditional formulation called Phytomed was carried out. Before this toxicological study, a phytochemical screening revealed the presence of certain phytochemical groups, including alkaloids, cardiac glycosides, flavonoids and tannins. Phytomed is not cytotoxic and did not induce toxicity following a daily administration at 0.43 mL kg^{-1} and 0.85 mL kg^{-1} for 28 days. Its effect on *in situ* toad's heart revealed a negative chronotropic effect but no cardiac toxicity as there was no cardiac arrest.

SIGNIFICANCE STATEMENT

This study reveals the safety of the formulation provided by a traditional therapist for short-term use but without evaluating its therapeutic efficacy. Despite its safety, this medical preparation should be taken with caution as further studies are needed to determine the mechanism of action that causes the increase in platelets, the negative chronotropic effect and its effects with chronic use.

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