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Research Article

Toxicological Studies of Hydroethanolic Leaf Extract of *Xylopia aethiopica* (Dunal) A. Rich. (Annonaceae) on Wistar Rats

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Abstract

Introduction: The impact of aromatic plants used in cooking on health is indisputable.

This study aims to evaluate the *in vitro* cytotoxicity of *Artemia salina*, acute and the 28 days subchronic toxicity of *X. aethiopica* dried fruit used like spice in Togo by oral administration of the extract to female Wistar rats.

Results: The *A. salina* assay showed that the hydroalcoholic extract of *X. aethiopica* presented any toxicity. The LC₅₀ values of *X. aethiopica* on brine shrimp were 0.64 ± 0.13 mg/mL. The acute toxicity study revealed neither behavioral disturbances nor death in rats. The lethal dose (DL₅₀) of this extract is greater than 5000 mg/kg body weight. The results of subchronic toxicity showed no significant change in body and organs weight gain in rats at test doses throughout the treatment period. No changes in haematological parameters were observed apart from a significant increase in platelet number at doses of 500 and 1000 mg/kg. Biochemical parameters such as Urea, Creatinine, Triglycerides, Total cholesterol, Serum glutamic pyruvic transaminase (SGPT), C-reactive protein (CRP) and glycemia were not significantly changed. Serum glutamic oxaloacetic transaminase (SGOT) and Alkaline phosphatase (ALP) activities increased at doses of 500/1000 mg/kg and 1000 mg/kg respectively. Creatine Phosphokinase (CPK) activity decrease at the dose of 500 and 1000 mg/kg. Blood electrolytes showed no significant change. The histological sections showed no organ damage.

Conclusions: Aqueous extract of *X. aethiopica* did not lead to any adverse effects in rats after acute and subchronic treatment at 500 and 1000 mg/kg doses.

Keywords: *Xylopia aethiopica*, Cytotoxicity, Toxicity, Wistar rats.

INTRODUCTION

Apart from serving as a source of food, plants invariably remain a major source of medicine in most parts of the world¹. This is the case of spices produced and used throughout the world and known for their organoleptic qualities and their therapeutic virtues. *Xylopia aethiopica* is a common plant in West Africa, with wide applications in trado-medical management of several diseases². In Togo, it is commonly used as spice in the preparation sauce. Traditionally, it has been utilized for inducing placental discharge postpartum, management of rheumatism, asthma, headache, bronchitis, neuralgia and colic pain³. Studies have revealed that the fruit has anti-malaria, antibacterial and antifungal properties². The use of this dried fruit as spice may be abused by its users, as it has been indicated in folklore management of several disorders. It is therefore important, to investigate the toxic potential of *X. aethiopica*.

This study aims to evaluate the *in vitro* cytotoxicity of *X. aethiopica* leaves on *Artemia salina*, the acute toxicity and its

28 days subchronic toxicity by oral administration of the extract on female Wistar rats.

I. MATERIALS AND METHODS

Collection and extraction of plant material

Xylopia aethiopica leaves were collected at the Nukafou market located in the second district of the city of Lomé (TOGO) after identification by the Botany and Ecology Department of the University of Lomé. A voucher specimen was registered in the herbarium under the number TOG015571.

Leaves were cleaned and dried under climatization (20°C). Then 1000 g of dried and pulverized leaves were soaked in 15 L of ethanol-water (50-50) for 72 hours with intermittent agitation. After double filtration with cotton and Whatman paper, the filtrate was evaporated using a rotating evaporator "Rotavapor IKA RV 10 (Germany)". The yield of this extraction was calculated according to the formula: R = (weight of extract / fruit dry weight) x 100.

Animals

12-week-old female Wistar rats (150-200 g) were used for acute and sub-chronic experimentations on the one hand. They were provided by the animal facility of the Department of Animal Physiology and were acclimated at least one week prior the beginning of the manipulations. The animals were fed with rodent standard diets and water *ad libitum*. Animal's care and handling were conformed to accepted guidelines⁴⁻⁵. Ethical approval was obtained from the institutional Ethical Committee for Teaching and Research under the number (ref no. CNCB- CEER 2801/2010). *Artemia salina* eggs were used for the study of cytotoxicity on the other hand.

Phytochemical screening

*Qualitative

The screening was performed to assess the presence of some chemical groups such as alkaloids, saponins, total phenols, hydrolyzable tannins, condensed tannins, sterols, terpenes, anthracenes, cardiotonic heterosides, coumarins and flavonoids. Saponins, total phenols, hydrolyzable tannins, condensed tannins, sterols, terpenes, anthracenes, cardiotonic heterosides, coumarins and flavonoids were checked⁶⁻⁷. For alkaloids, Bouchardat, Dragendorff, and Mayer reagents were used to determine their presence.

*Quantitative

The content of cardiotonic glycosides was determined from a calibration range with digoxin (10-100 µg / mL) and the results were expressed as mg digoxin equivalent per gram of dry extract (mg EqD / g). Three trials were performed for the sample⁸.

Assessment of larval toxicity of the substance

Bioactivity of the extract on larvae was monitored by the brine shrimp lethality test⁹. Different concentrations of extract were prepared. Using a cone micropipette, a colony of 16 live larvae was brought into contact with a series of solutions in progressive concentrations (from 25 mg/mL to 0.049 mg/mL) of plant drug extracts. These media and controls were allowed to stir and live larvae were counted 24 hours after incubation. The mean percentage mortality was plotted against the logarithm of concentrations. The concentration (LC50), at which 50% of the larvae were killed, was determined from the graph⁹⁻¹⁰⁻¹¹.

Acute toxicity test

The limit dose of 5000 mg/kg was carried on three male Wistar rats according to the Organization for Economic Cooperation and Development (OECD) guidelines¹². The animals were observed afterwards at approximately 30 min, 1 h, 2 h, and 4 h on day 1 and once daily for 14 days for mortality, bodyweight, visual observations (changes in eyes and mucous membranes, skin and fur), and behavioral patterns (alertness and positioning of animals).

Sub-chronic toxicity

Repeated-dose oral toxicity was achieved according to OECD guidelines 407¹³. The animals were divided into three groups of 6 each. The first group (group 1) received distilled water and served as control group. The second (group 2) and the third (group 3) groups received, respectively, the extract at 500 mg/kg and 1000 mg/kg body weight.

The extract was administrated daily for 28 days at the same time and the animals were observed at least twice daily for morbidity and mortality. All groups were administrated 1 mL/100 g body weight of solution. The body weights were recorded every day. The rats were observed for aggressiveness, mobility, diarrhea, appetite, and answer to

sound stimulation. On the 29th day, after 12 hours of fast, the rats were anesthetized first by ether. Blood samples were collected from the retroorbital sinus in dry tubes for biochemical analyses and in EDTA tubes for haematological analyses. Samples for biochemical analyses were centrifugated at 2500 rpm for 15min and the serums were collected. Biochemical parameters such as serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), alkaline phosphatase (ALP), creatinine, urea, creatine phosphokinase (CPK), cholesterol total, triglycerides, protein C reactive, and glucose were performed. Sodium (Na⁺), Potassium (K⁺), Chloride (Cl⁻) and Calcium (Ca⁺⁺) ions were measured in the serum. Haematological parameters achieved were white blood cell count (WBC), red blood cell count (RBC), haematocrit (HCT), haemoglobin (HB), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), platelet count (PLT) and mean corpuscular volume (MCV). Then the animals were euthanized with ether and necroscopy of all rats was carried out and some organ weights (kidney, liver, spleen, heart and abdominal fat) were recorded.

Statistical analysis

Results were expressed in mean ± standard error of the mean (SEM). Statistical analysis was performed by analysis of variance (ANOVA) with Tukey test to evaluate the difference between two groups. Values of p<0.05 were considered significant. The statistical package GraphPad Prism 6.01 was used to carry out all statistical analyses.

II. RESULTS

Phytochemical screening and extraction of plant materials

The evaporation allowed us to obtain a yield of 12.91%. The results of the phytochemical screening have shown the presence total phenols, flavonoids, condensed tannins, terpenes, cardiac heterosides and coumarin like seen in table 1.

Table 1: Qualitative phytochemical screening of chemical groups of *Xylopiya aethiopic* dried fruit extract.

Researched Groups	Extract
	<i>X. aethiopic</i>
Alkaloids (<i>Bouchardât</i>)	-
Alkaloids (<i>Mayer</i>)	-
Alkaloids (<i>Dragendorff</i>)	-
Total Phenols	+
Flavonoids	+
Condensed Tannins	+
Hydrolyzable Tannins	-
Sterol	-
Terpenes	+
Anthracenes	-
Saponins	-
Cardiotonic Heterosides	+
Coumarin	+

+: presence; -: absence

The quantitative phytochemical screening of cardiotonic glycosides allowed us to obtain the concentration of 7,47 ± 0,7 µg of Digoxin / mg of *X. aethiopic*.

Brine shrimp toxicity screening

According to figure 1, the LC₅₀ values of *X. aethiopica* was 0.64 ± 0.13 mg/mL. The result is expressed in terms of

concentration lethality using linear regression curves ($Y = 2,8484\ln(x) + 9,3717$ with $R^2 = 0,9361$)

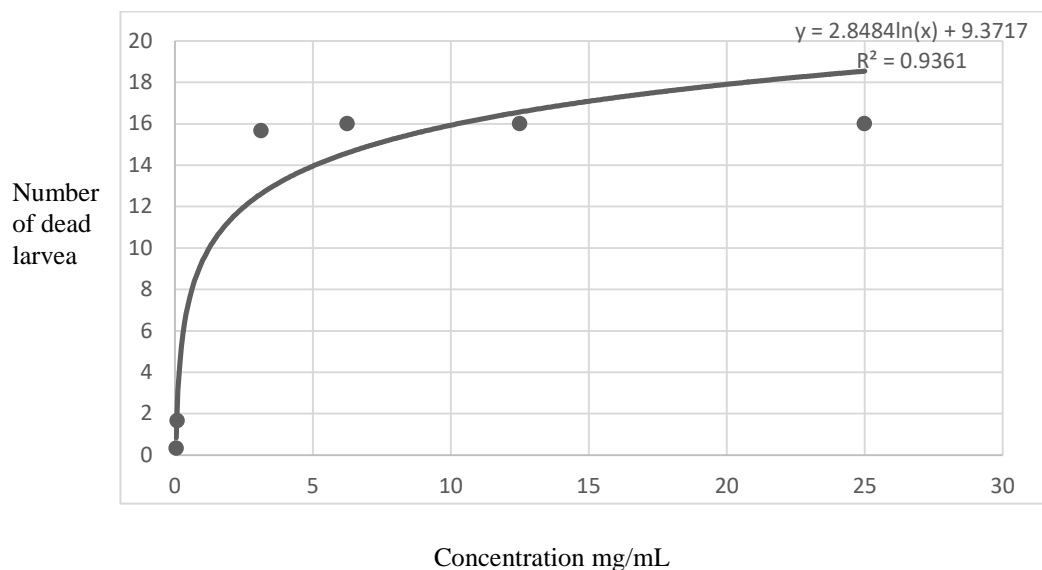


Figure 1: *In vitro* cytotoxicity of *X. aethiopica* leaves on *Artemia salina* larvae.

Acute toxicity

The limit dose 5000 mg/kg of *X. aethiopica* didn't lead to mortality or acute toxic effects of the three rats dosed. The LD₅₀ was then over 5000 mg/kg.

Sub-chronic toxicity

In the clinical evaluation, no behavioural changes and death were observed at the end of treatment. Similarly, no significant differences in body weight were observed between the control and treated groups during this period (Table 2).

Table 2: Effect of hydroethanolic extract of *X. aethiopica* on rat body weight after 28 days of experiment.

Week	Control	<i>X. aethiopica</i> extract dose	
		500 mg/Kg	1000 mg/Kg
0	139 ± 5,29	138,5 ± 5,84	137,33 ± 12,35
1	142,5 ± 4,95	144,83 ± 6,67	135,16 ± 7,1
2	147,83 ± 4,92	152,5 ± 6,23	142,66 ± 7,7
3	150,66 ± 5,45	154,5 ± 4,97	145,83 ± 10,01
4	156,66 ± 6,8	159,83 ± 6,51	153,83 ± 16,51

Each value is expressed as mean ±SEM; ANOVA followed by Tuckey test; n=6.

No significant differences in organs ratio were observed between the control and treated groups during this period (Table 3).

Table 3: Effect of hydroethanolic extract of *X. aethiopica* on rat's organ ratio weight after 28 days of experiment.

Organs	Control (%)	<i>X. aethiopica</i> extract	
		500 mg/Kg (%)	1000 mg/Kg (%)
Kidney	0,65 ± 0,06	0,67 ± 0,02	0,68 ± 0,01
Heart	0,41 ± 0,02	0,41 ± 0,02	0,46 ± 0,03
Spleen	0,44 ± 0,11	0,43 ± 0,11	0,32 ± 0,05
Liver	3,35 ± 0,21	3,50 ± 0,14	3,38 ± 0,15
Abdominal fat	2,83 ± 0,5	2,91 ± 0,42	2,64 ± 0,88

Each value is expressed as mean ±SEM; ANOVA followed by Tuckey test; n=6.

Tables 4 and 5 show the haematological and biochemical parameters, respectively. For hematological parameters, we observe a significant decrease of platelets (500 and 1000 mg/Kg. No changes in biochemical parameters were observed apart from a significant increase on the one hand of Alkaline Phosphatase (1000 mg/Kg), Serum glutamic oxaloacetic transaminase (500 and 1000 mg/Kg) and a significant

decrease on the other hand in platelet number (500 and 1000 mg/Kg) and Creatine phosphokinase (500 and 1000 mg/Kg) when compared with controls. Biochemical others parameters such serum glutamic pyruvic transaminase, glycemia, urea, creatinine, total cholesterol, triglycerides and C-reactive protein were not significantly changed when compared with control

Table 4: Effect of hydroethanolic extract of *X. aethiopica* on haematological parameters after 28 days of experiment.

Parameters	Control (n = 6)	<i>X. aethiopica</i> extract (n = 6)	
		500 mg/Kg	1000 mg/Kg
WBC	9,08 ± 1,31	8,01 ± 1,53	4,98 ± 0,33
RBC (×10 ⁶ /μl)	6,95 ± 0,99	7,03 ± 1,31	7,54 ± 0,84
HB (g/dl)	15,58 ± 1,76	14,75 ± 2,61	17,66 ± 1,98
HCT (%)	42,9 ± 3,99	41,01 ± 5,55	47,01 ± 4,22
MVC (fl)	52,58 ± 3,54	54,51 ± 4,83	51,43 ± 3,53
MCH (pg)	18,68 ± 0,82	18,43 ± 0,53	19,01 ± 0,94
MCHC (g/dl)	35,85 ± 0,96	34,83 ± 2,19	37,26 ± 0,85
PLT (×10 ⁵ /μl)	758,33 ± 31,01	486 ± 54,77****	628,66 ± 13,86****

WBCs: White Blood Cells; RBCs: Red Blood Cells; HB: Hemoglobin; HCT: Hematocrit; MVC: Mean Corpuscular Volume; MCHC: Mean Corpuscular Hemoglobin Concentration; MCH: mean corpuscular hemoglobin; PLT: Platelets.

Each value is expressed as mean ±SEM; ANOVA followed by Tuckey test; ****: p < 0,00001; n=6.

Table 5: Effect of hydroethanolic extract of *X. aethiopica* on biochemical parameters after 28 days of experiment.

Parameters	Control	<i>X. aethiopica</i> extract	
		500 mg/Kg	1000 mg/Kg
Urea (mg/dL)	71,62 ± 3,44	78,26 ± 2,44	56,53 ± 3,15
Creatinine (mg/dL)	0,55 ± 0,02	0,56 ± 0,02	0,53 ± 0,02
Glycemia (mmol/L)	85 ± 1,52	86,33 ± 2,9	82 ± 3,51
SGOT (UI/L)	139 ± 4,93	176 ± 3,78***	192,33 ± 3,17****
SGPT (UI/L)	43,5 ± 1,5	43,33 ± 0,88	41,66 ± 1,2
ALP (UI/L)	112,5 ± 5,61	117 ± 7,09	189 ± 4,16****
CPK (UI/L)	653 ± 19,85	394 ± 18,14****	308,66 ± 2,87****
TOTAL CHOL (mg/dL)	60,5 ± 1,55	61,33 ± 2,33	62 ± 2,51
TG (mg/dl)	111,5 ± 7,64	110,5 ± 2,87	96,33 ± 0,66
CRP (mg/dl)	2,68 ± 0,15	2,35 ± 0,1	2,45 ± 0,08

SGOT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase; ALP: Alkaline Phosphatase; CPK: Creatine phosphokinase; TOTAL CHOL: Total Cholesterol; TG: Triglycerides; CRP: C-reactive Protein.

Each value is expressed as mean ±SEM; ANOVA followed by Tuckey test; ***: p < 0,0001; ****: p < 0,00001; n=6.

The result of the determination of the main electrolytes in the blood after 28 days of experiments is shown in table 6. No changes in parameters were observed when compared with control.

Table 6: Effect of hydroethanolic extract of *X. aethiopica* on ionogram parameters after 28 days of experiment.

Ion	Control	<i>X. aethiopica</i> extract	
		500 mg/Kg	1000 mg/Kg
Na ⁺ (mmol/L)	141 ± 1,47	138,25 ± 7,45	143,66 ± 4,33
K ⁺ (mmol/L)	6,17 ± 0,32	5,25 ± 0,43	5,7 ± 0,26
Cl ⁻ (mmol/L)	102,25 ± 0,85	103 ± 7,35	95,33 ± 5,92
Ca ⁺⁺ (mg/dL)	9,3 ± 0,34	7,56 ± 0,36	8,06 ± 0,54

Na⁺: Natremia; K⁺: Kaliemia; Cl⁻: Chloroemia; Ca⁺⁺: Calcemia

Each value is expressed as mean ±SEM; ANOVA followed by Tuckey test; n=6.

III. DISCUSSION

In our extract regarding the qualitative aspect on the one hand, the screening revealed the presence of condensed tannins, terpenoids, flavonoids, total phenols, coumarin and cardiac-glycosides while anthracenes, alkaloids, sterols hydrolysable tannins and saponins were absent. Aguru¹⁴ identified the presence of flavonoids, saponins, steroids and alkaloids while tannins and anthracenes were absent. Ezekwesili¹⁵ reported the presence of saponins, flavonoids, tannins and sterols in essential oil.

Okigbo¹⁶ founded tannins, saponins, flavonoids, steroids and alkaloids in methanolic extract.

Variation in the phytochemical content in this current investigation of the dried fruit hydroethanolic extract could be due to planting location, seasonal variation, and extraction variables such as temperature, time and concentration¹⁷.

The various health benefits¹⁸ of medicinal plants are attributed to the presence of secondary metabolites. However, the particular presence of cardiotoxic glycosides in our extract can be a source of toxicity or benefits for the body depending on the dose ingested. Cardiac glycosides due to the very narrow margin between the therapeutic dose and the toxic dose can therefore, at a high dose, cause cardiac arrest.

On the other hand, with regard to the quantitative aspect, the dosage of cardiotoxic glycosides gives us a concentration equal to $8.36 \pm 0.15 \mu\text{g}$ of Digoxin / mg of *X. aethiopica*. Given the use of this plant as a food source, the administration of the hydroethanolic extract of this plant for 28 days has enabled us, through the evaluation of the effects on various biological parameters, to assess the toxicity relating to this concentration.

The LC₅₀ values calculated for the brine shrimp lethality test was $0.64 \pm 0.13 \text{ mg/mL}$. Therefore, the hydroalcoholic extract of *X. aethiopica* leaves is not toxic according to Mousseux scale who classified crude extracts and pure substances into toxic (LC₅₀ value < 0,1 mg/ml) and nontoxic (LC₅₀ value > 0,1 mg/ml). This study is corroborated by that of Ajayi et al. (2017) who found an LC₅₀ of $152.06 \pm 0.38 \text{ mg/mL}$. *Xylopi aethiopica* would therefore not exhibit toxicity on the cells.

The oral acute toxicity study of *Xylopi aethiopica* dried fruit extracts at 5000 mg/kg did not show mortality or any obvious toxic symptom. This signified that the median lethal dose of (LD₅₀) of *Xylopi aethiopica* was higher than 5 g/kg body weight.

This finding was similar with Ekeanyanwu²⁰ and Ayodele²¹ who reported the non-toxic nature of the dried fruit essential oil and ethanolic extract respectively. However, these differences in the LD₅₀ may be due to the age of the plant, geographical location, season and time of harvest, which have been reported to affect the phytochemical compositions of plants and ultimately affect its toxicity²². *Xylopi aethiopica* would not exhibit toxicity following an acute administration of a dose less than or equal to 5 g / kg of body weight.

As for body weight, *X. aethiopica* hydroethanolic extract had not induce significant changes in Wistar rat body weight signifying lack of systemic toxic effect. There were no clinical signs of toxicity and deaths during the experimental procedure.

Moreover, the relative organ weight indicates whether the organ has been exposed to injury or otherwise. The assessment of the weight of organs such as the liver, kidney, spleen, testis, heart, pancreas, brain, and language are very essential in toxicological studies²³.

In the present study the relative organ weights of all treated rats did not differ significantly from those of the control groups. It indicates that the extract did not effect on appetite or adverse effects on the growth of the animals.

The results of the haematological evaluation in this study did not show significant changes in haematological parameters. However, the significant decrease in the number of platelets observed at 500 and 1000 mg/kg may be due to antiproliferative effect on this line in the animals. The decreasing number of platelets by the extract at all doses, implying that the leaf extract has effect on hemostasis, which is controlled by platelets. It would seem likely that the extract also contains some compounds that are capable of blocking the release of a thrombopoietin. No significant changes were observed in total white blood cells and their differentials. All observations indicate the non haematotoxic nature of the extract at the doses used in this study.

AST, ALT, ALP and glucose are serum liver marker enzymes and serum urea, uric acid and creatinine are biomarkers of kidney function. Serum creatinine, uric acid and urea are common biomarkers for prediction of renal dysfunction²².

Aspartate aminotransferase (AST) is found in a variety of tissues, including the liver, brain, pancreas, heart, kidneys, lungs, and skeletal muscles. A significant increase at 1000 mg/kg compared to the control group observed seems that *Xylopi aethiopica* could cause injury one of these organs. This may be due to the presence of compounds in the extract.

ALP is present in the liver, bone, heart, skeletal muscle, kidneys, brain, pancreas, and blood cells. The significant increase in ALP activity respectively at 1000 mg/kg compared to control group may be due to altered hepatobiliary function or may be due to unknown tissue damage (cytolytic effects) at this dose caused by certain compounds present in the extract.

Some biochemical parameters such as CPK, a cardiac injury marker level¹¹, decrease significantly at 500 and 1000 mg/kg compared to control group. This result may be due to the presence of compounds in the extracts. OG would have myo and cardioprotective properties.

Repeated administration of the hydroethanolic extract of dried fruit of *X. aethiopica* had no effect on the lipid profile of the animals.

Estimating levels of electrolytes (Na⁺, K⁺, Cl⁻ and Ca⁺⁺) in serum may be important in assessment of renal function since the outcome of regulatory mechanism of osmotic balance and ionic charges can be determined by the levels of electrolytes in the blood.

Sodium is the major cation of the extracellular fluid where it regulates acid-base equilibrium and protects the body against excessive fluid loss. Potassium is the major intracellular cation with similar role to those of sodium. However, imbalances in these ions may be due to renal failure or renal tubular acidosis or alkalosis. In this study, the serum electrolytes of rats exposed to sub-chronic doses of the extract were no significant.

Xylopi aethiopica may have no significant influence on the electrolyte, acid-base balance and water, at these doses. This is suggestive that kidney function is not compromised.

CONCLUSIONS

In this study, we have evaluated the *in vitro* cytotoxicity of *Xylopi aethiopica* hydroethanolic leaves extract on *Artemia salina* and its acute and subchronic toxicity by oral administration of the extract on male Wistar rats. The results suggest that acute administration of aqueous extract of *Xylopi aethiopica*

aethiopica is associated with no signs of toxicity despite some changes mainly in hematological and biochemical parameters.

Therefore, some caution should be taken when administering *Xylopia aethiopica* leaves for long periods.

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Conflicts of Interest

The authors declare no conflicts of interest involved in this study.

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