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Antidotal Assessment of Hydroethanol Root Extract of *Curcuma longa* L. (Turmeric) against Chemically-induced Hepatic Neoplasm in Wistar Rats

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ABSTRACT

Curcuma longa L. (Family: Zingiberaceae; turmeric) is cultivated extensively worldwide, popularly used as spice and folk medicine but is native to Southeast Asia. Following urbanization, chemical exposure is incorporated into our daily living, threatening our health and ecosystem. Thus, the quest for ameliorative substances becomes imperative and continues. Study was to evaluate the hepatoprotective/ antitumour potentials of hydroethanol root extract of *Curcuma longa* (CLE) against chemically-induced hepatic neoplasm in rats. Acute toxicity was determined in Wistar rats using Up-and-Down Procedure. Rats (24) were randomly divided into 4 groups (6 each) and treated thus: Group-I received food and water *ad libitum*. Group-II received 200 mg/kg b.wt diethylnitrosamine (DENA, i.p.), followed by 3 mL/kg b.wt CCl$_4$ (i.p.) and 0.05% phenobarbital (PB) (in drinking water). Group-III received 100 mg/kg b.wt silymarin (p.o) daily. Groups-IV received 1000 mg/kg b.wt of extract one week prior to the induction of hepatotoxicity and subsequently for 90 days. After 90 days, rats were euthanized under diethyl ether anaesthesia and sacrificed. Blood samples were collected via cardiac puncture for biochemical analysis. From each euthanized animals, the liver was eviscerated, blotted and fixed in 10% buffered formalin for histopathological examination. Extract was safe on acute exposure with LD$_{50}$ >5000 mg/kg b.wt. Results showed significant (P<0.001) reduction in DENA/CCl$_4$/PB-induced biochemical alteration compared to control. Rats pre-treated with CLE prevented the formation of hepatocellular carcinomas (HCCs) and preserved hepatic microarchitecture similar to that of silymarin. Findings suggests the antitumour/antidotal properties of turmeric root extract against chemically-induced liver damage/tumourigenesis in rat model.

Keywords: Antineoplastic, diethylnitrosamine, hepatocellular carcinoma, hepatotoxicity, liver cancer, phenobarbital, turmeric.

1. Introduction

Today, anthropogenic activities threaten our very existence and ecosystem. In the sub-Shaharan African region, hardly does a day go by without humans being exposed even at lower doses to harmful chemicals, physical and/or biological agents in food, air, water, drugs, cosmetics, automobiles, phones and other electronics as well as from all toxic dump sites near homes, offices, schools and walkways. Chemicals are everywhere and are with the inherent capacity to biomagnify and bioaccummulate in biological systems as well as impairing normal physiological processes, leading down to several diseases like cancers, cardiovascular diseases, diabetes, infertility, obesity, memory loss, early aging amongst others.

In humans, the liver is the second most largest bodily organ, and its pivotal role in metabolism and detoxification of xenobiotics predisposes it to harm and/or injury due to inhaled or ingested chemicals (including drugs etc.). Liver diseases like cirrhosis, chronic hepatitis and non-alcoholic fatty liver are risk factors for primary liver cancer (e.g. hepatocellular carcinoma) [1]. Hepatocellular carcinoma (HCC) is a type of cancer characterised by uncontrolled multiplication, dedifferentiation, loss of function, invasiveness and metastasis (spread) of abnormal forms of the hepatocytes [2]. Hepatocellular carcinoma emerges the second most common cause of cancer-related mortality the world over [3] and is predominantly (80-90%) found in patients with liver cirrhosis [1]. Despite the understanding of the pathology of cancer and the advancement in its therapy, there lies a germane need for newer agents with selective toxicity to cancer cells only and not other rapidly dividing cells like the hair and nail cells, and chemoprevention, especially as treatment outcomes with chemotherapy besides its non-selective toxicity remains dissatisfactory. The isolation, characterization and evaluation of chemicals from plant origin remains the mainstay for the discovery of newer pharmaceuticals and is with tremenduous/impressive outcomes.

Curcuma longa L. (Family: Zingiberaceae; turmeric) is cultivated extensively worldwide and is popularly used as spice and folk medicine by numerous ethnic groups of the world, but is native to Southeast Asia [4]. Turmeric is a known spice that gives numerous Asian dishes their characteristic yellow colour [5]. It has a diverse pharmacological spectrum including but not limited to anti-inflammatory, anthelmintic, antioxidant, anticlotting, carminative, antinfective, and anticholesterolemic properties. The chemicals - carbon tetrachloride (CCl_4) and diethylnitrosamine (DENA) are renowned hepatotoxicants known to induce liver cirrhosis and HCC respectively. As earlier reported by Yoshiji *et al*. [6], DENA is biotransformed to some reactive electrophilic species. These metabolites of DENA then disrupts the DNA structure, with resultant chromosomal aberrations [7] and alkyl DNA adducts formations. Phenobarbital (PB) is a known anticonvulsant agent that promotes cell/tissue proliferation (tumours) in rodents especially when administered in quick succession to a primary hepatocarcinogen like DENA. Thus, PB promotes or enhances hepatocarcinogenesis in rodents. The present study aimed at determining the antidotal (antitumour and hepatoprotective) properties of hydroethanol root extract of *C. longa* (CLE) against hepatotoxicity/tumourigenesis chemically induced by CCl_4, DENA and PB in Wistar albino rats.

2. Materials and methods

2.1 Chemicals

Carbon tetrachloride, ethanol (99%), diethylnitrosamine and phenobarbital were all procured from Bristol Scientific Company Limited, Lagos, Nigeria (authorized agent for Sigma-Aldrich Co. Germany).

2.2 *Plant collection, identification and extraction*

The plant material with flowers was collected from the Faculty of Pharmacy Medicinal Plants Reserve, University of Uyo, Nigeria for botanical identification and voucher specimen referencing. It was identified and authenticated as *Curcuma longa* by Prof (Mrs) Margret Bassey, a taxonomist in the Department Botany and Ecological studies, University of Uyo, Nigeria. Thereafter, the sample was conserved at the Faculty of Pharmacy Herbarium under the reference number UUPH 80(b). It was thoroughly rinsed and allowed to drain.

About 5045.0 g of fresh turmeric rhizomes was weighed, cut into bits, reduced to a paste using a pestle and mortar and macerated in hydroethanol (50% $^V/_V$) for 72 h. The solution was vigorously shaken for 15 min prior to its filtration using Whatman filter paper No. 42. The filtrate obtained was concentrated and evaporated to dryness *in vacuo* at 40 °C using rotary evaporator. The extract was stored in a tight capfitted container at -4 °C until when used for the experiment.

2.3   Experimental animals

Healthy adult female Wistar albino rats (120 – 160 g) were obtained from and kept at the Department of Pharmacology & Toxicology Animal House of the University of Uyo, Uyo, Nigeria. The animals were maintained under standard environmental conditions, were fed with standard pelleted feed obtained from Livestock Feed, Nigeria Ltd. and given water *ad libitum*. All animals were kept at room temperature in cross-ventilated rooms, without illumination at night to achieve 12 h light/ 12 h dark period. The animals were acclimatized to the laboratory condition for at least 7 days prior to the experiment, during which they were allowed free access to food and water *ad libitum*. The care and use of animals was conducted in strict adherence to the National Institute of Health Guide for the Care and Use of Laboratory Animals and ethical approval for use of lower animals in research was obtained from the Experimental Ethics Committee on Animal Use of the Faculty of Pharmacy, University of Uyo, Nigeria.

2.4   Acute toxicity study

The median lethal dose (LD_{50}) of the root extract was determined using the preliminary limit test dose of the Up and Down (OECD 425) testing procedure [8]. This involved the oral administration of 5000 mg/kg b.wt CLE to five young adult female Wistar rats which were starved overnight. The animals were observed closely for manifestation of physical signs of toxicity such as writhing, decreased motor activity, decreased body/limb tone, decreased respiration, somnolence and mortality within 48 h post-administration. They were further observed for 14 days for any delayed toxic outcome and mortality.

2.5.   Experimental design

Wistar rats (24) were randomly divided into 4 groups (n = 6) and treated as follows: Group 1 (normal control) received nothing besides food and water *ad libitum* throughout the 90-days test duration. Group 2 (induction control), received a single dose of 200 mg/kg b.wt diethylnitrosamine (i.p.) followed by 3 mL/kg b.wt CCl_4 (i.p.) and 0.05% phenobarbital (in drinking water throughout the test duration) to induce hepatocarcinogenesis and liver cirrhosis. Group 3 (positive control) were daily treated with 100 mg/kg b.wt silymarin (p.o) post-induction of liver damage and tumourigenesis. Group 4 was administered via oral gavage, 1000 mg/kg b.wt CLE one week prior to induction of carcinogenesis/hepatotoxicity and subsequently throughout the 90-days test period. At the expiration of 90 days, the animals were euthanized under diethyl ether anaesthesia and sacrificed. Blood samples were collected via cardiac puncture in different sample bottles for biochemical analysis. From each euthanized animals, the liver was eviscerated, blotted and fixed in 10% buffered formalin for histopathological examinations.

2.5.   Biochemical analysis

Following the protocol earlier described by Bessey *et al*. [9], serum alkaline phosphatase (ALP) was determined at 405 nm. While serum alanine aminotransferase (ALT) and aspartate amino transferase (AST) were determined at 340 nm using Reitman and Frankel [10] method. Serum cholesterol levels were determined by Parekh and Jung [11] method, while free fatty acids (FFA) and serum triglycerides were estimated using the methods of Hron and Menahan [12] and Foster and Dunn [13] respectively. Also, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) were estimated. All these determinations

were done colorimetrically using Randox analytical kits according to standard procedures of manufacturer's protocols.

2.6. Histological studies

From all the diethyl ether euthanized rats, the liver were eviscerated, freed from adventitia, blotted with tissue paper, weighed, sectioned and fixed in 10% buffered formalin for histological studies. Fixed sections were passed through xylene, alcohol and water to ensure that the tissue was totally free of wax and alcohol. Each section was then stained with haematoxylin and eosin for photo-microscopic assessment using light microscope at a magnification of 400.

2.7. Statistical analysis

Using Graph Pad Prism software (version 6), the results were statistically analysed by one-way of variance (ANOVA) followed by Newman–Keuls as post hoc test and p values less than 0.05 ($p<0.05$) were considered statistically significant. The results were expressed as mean ± SD.

3. Results and discussion

3.1. Acute toxicity study

Result from the acute toxicity study in Wistar rats showed that oral administration of the hydroethanol turmeric extract at 5000 mg/kg body weight produced no toxic effects nor mortality. The LD_{50} of CLE was determined to be greater 5000 mg/kg body weight (p.o). The median lethal dose (LD_{50}) as well as the lethal concentration dose (LC_{50}), no observed adverse effect level (NOAEL), lowest observed adverse effect level (LOAEL), derived no-effect level (DNEL) etc are all toxicological dose descriptors used to identify the relationship between the specific effect of chemicals and the dose at which such occurred. In safety pharmacology, they are useful tools used for risk assessment and the determination of globally harmonized system (GHS) hazard classification – a system that defines, classifies and communicates the health and safety information on hazards of chemical products and mixtures. Thus, the reported LD_{50} (> 5000 mg/kg) suggests the relative safety of the extract on acute exposure, especially as the female strain may be more sensitive to toxic substances. This is in agreement with earlier toxicity studies on animals [14, 15]. Futhermore, with respect to the human population, the Food and Drug Administration (FDA) classifies tumeric among substances Generally Recognized as Safe (GRAS).

3.2. Effect of extract on liver enzyme activity and lipid profile of experimental rats

Table 1 shows the effect of CLE on liver enzyme activity of the experimental rats. Result presented significant ($P <0.001$) reduction in the enzymatic activities of the liver transaminases of rats pre-treated with CLE compared to the DENA+CCl$_4$+PB treated rats (toxic control group). Administration of 1000 mg/kg b.wt CLE had potent effect on liver transaminases than the 100 mg/kg b.wt silymarin (positive control group). Result presented significant ($P < 0.001$) increase total cholesterol, serum triglycerides, free fatty acid, low density lipoproteins (LDL), very low density lipoproteins (VLDL) but a decrease in high density lipoproteins (HDL) compared to the normal control rats. Rats pre-treated with CLE showed no significant change in the lipid profile compared to normal control. However, CLE treated rats showed considerably low levels of free

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fatty acids, cholesterol, triglycerides, low density lipoproteins (LDL), very low density lipoproteins (VLDL) as well as an impressive increase in high density lipoproteins (HDL) compared to the toxic control rats (Table 2).

Table 1
Result of the up-and-down procedure of acute toxicity testing of CLE in Wistar rats

Test sequence	ID	Dose (mg/kg)	STR (48 h)	LTR (14 days)
1	T_1	5000	O	O
2	T_2	5000	O	O
3	T_3	5000	O	O
4	T_4	5000	O	O
5	T_5	5000	O	O

O = Survival; ID = Identification number; STR = Short term result; LTR = Long term result; LD_{50} = > 5000 mg/kg body weight CLE (rat, p.o)

Table 2
Effect of extract on liver enzyme activity of experimental rats.

Treatment	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Distilled H_2O*	135.00 ± 3.21	106.0 ± 0.57	181.33 ± 0.88
DENA+CCl_4+PB**	630.33 ± 1.85[c]	506.33 ± 3.71[c]	489.33 ± 3.52[c]
Silymarin+DENA+CCl_4+PB	352.33 ± 3.84[c]	279.66 ± 1.76[c]	298.33 ± 1.45[c]
CLE+DENA+CCl_4+PB	298.75 ± 1.49[b]	173.25 ± 2.72[c]	150 ± 11.06[a]

Data expressed as mean ± SD, Significant at [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ compared to control. Student t-test comparisons were made between all groups; (n = 6). *Normal control group; **Toxic control group; DENA – diethylnitrosamine; PB – phenobarbital; CCl_4 – carbon tetrachloride.

CCl_4-iduced liver damage is an often used model for the evaluation of drugs/substances with hepatoprotective potentials [16]. The increase in serum levels of AST, ALT, ALP and cholesterol found in this study is attributed to the distorted and/or damaged structural integrity of the liver following exposure to the hepatotoxicants used and is in accordance to the findings of Sallie *et al*. [17]. CCl_4 is metabolized by CYP-450 group of enzymes, generating a trichloromethyl free radical ($.CCl_3$) in the process. In the presence of oxygen, the generated free radical induces lipid peroxidation chiefly by combining with cellular lipids and proteins [18, 19] with dire consequencies on the structural integrity of the hepatic cellular orientation as well as the deactivation of several metabolic and biochemical pathways, potentiating liver injury [20]. Most hepatotoxicants causes the parenchymal accumulation of abnormal fat (chiefly the triglycerides) secondary to liver injury [21]. As earlier reported by Jiang *et al.* [22], hepatic carcinogenesis induced by diethylnitrosamine and phenobarbital result in a marked accumulation of serum cholesterol, triglycerides and free fatty acids in rats. Our present findings are in harmony with such report. Also, significant increase in LDL and VLDL but decrease in HDL levels seen in the toxic control group of rats is a clear indication of hyperlipidemia associated with exposure to hepatotoxicants/carcinogens. Treatment of

rats with 1000 mg/kg b.wt CLE showed appreciable ameliorative effects on DENA+CCl$_4$+PB-induced biochemical alterations similar to that of silymarin. The reduction in these parameters and increase in HDL level gives evidence to the hepatoprotective as well as the hypolipidemic properties of turmeric extract in lower animals. These beneficial effects observed in the present study are attributed to the bioactive compounds found in turmeric. For example, curcumin – an active phytochemical constituent of turmeric has been extensively studied and reported to have a broad pharmacological spectrum including but not limited to anti-inflammatory, antioxidant, antiperoxidative and antitumour properties.

Cancer is a debilitating condition characterised by abnormal cellular proliferation, invasion and metastasis. The disease, its progession, prevention as well as therapy continues to challenge all those with an interest the biology and pathobiology of cancer. Diethylnitrosamine is a potent and renowned hepatocarcinogen used to induce carcinogenesis in experimental animals [23]. Carbon tetrachloride is a strong hepatotoxicant that induces cirrhosis of the liver in experimental animals. The latter amongst others, is a risk factor for hepatocellular carcinomas. The antiepileptic drug, phenobarbital especially when administered in tandem to an inducer promotes tumour formation. Thus, the combination of DENA+PB is a known model for the estimation of the antitumour properties of numerous drugs and chemicals in experimental animals [24]. The escalated and concurrent cirrhosis of the liver and hepatocellular carcinomas observed in the toxic control group shows an established relationship between liver cirrhosis and HCCs. The study couldn't pinpoint the exact reason and possible mechanism for this realtionship. However, our hypothesis is similar to that earlier reported by Zalatnai and Lapis [25] which suggested the involvement of a modulated drug-metabolizing enzyme system. The drug, Silymarin® has been reported severally to possess anti-inflammatory [26], antioxidant [27], and antifibrotic [28] effects in chronic liver disease and has been extensively studied for preventing hepatocellular carcinomas in several experimental models [29]. Ramakrishnan *et al.* [30] reported silymarin to exhibit inhibitory effects on proliferation and induced apoptosis in the human HCC cell line HepG2. Several studies have reported the beneficial effects of silymarin on hepatotoxicity as well as carcinogenesis [31, 32]. Findings of the present study showed CLE to compete effectively with silymarin in the preservation of the hepatic microarchitecture of DENA+CCl$_4$+PB exposed Wistar rats. The hindered formation of hepatocellular carcinomas observed in both the positive control and CLE treated groups is indicative of the antitumour, antiproliferative and antifibrotic properties of the said agents. This observed pharmacological action of CLE may be due to the inherent bioactive phytochemicals found in tumeric rhizomes. The authors are of this mental disposition chiefly as review of available literature shows curcumin (chemical name: diferuloylmethane), a polyphenol constituent of *C. longa* rhizomes to be a potent antiproliferative agent against a variety of tumours *in vitro* [33 – 35]. Its probable mechanism of action may be via the suppression of nuclear factor-nB (NF-nB), inhibition of angiogenesis and promotion of the tumour suppressor genes (p53, p21). Additionally, curcumin has been shown to heighten the antineoplastic effects of most anticancer drugs, including cis-platinum, doxorubicin and paclitaxel synergistically [33 – 35].

Table 3

Effect of extract on lipid profile of experimental rats

Treatment	Cholesterol (mg/dL)	Triglyceride (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)	FFA (mg/dL)
Distilled H_2O*	162.13 ± 3.51	208.33 ± 0.84	45.63 ± 2.02	56.99 ± 0.01	15.66 ± 0.05	27.31 ± 1.98
DENA+CCl_4+PB**	335.10 ± 4.75[c]	318.40 ± 0.28[b]	20.20 ± 1.06[a]	98.88 ± 0.01[c]	39.28 ± 0.01[c]	48.85 ± 3.26[c]
Silymarin+DENA+CCl_4+PB	158.03 ± 2.15[a]	230.84 ± 0.18[a]	38.26 ± 3.11[a]	70.64 ± 0.01[a]	20.36 ± 0.01[c]	25.81 ± 2.03
CLE+DENA+CCl_4+PB	164.70 ± 2.98[c]	233.12 ± 0.08[c]	41.01 ± 4.12	55.73 ± 0.99	21.42 ± 0.02[b]	36.47 ± 2.77[c]

Data expressed as mean $\pm$ SD, Significant at [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ compared to control. Student t-test comparisons were made between all groups; (n = 6). *Normal control group; **Toxic control group; DENA – diethylnitrosamine; PB – phenobarbital; CCl_4 – carbon tetrachloride.

Histopathological examination of liver sections from normal control presented normal and preserved histoarchitecture. However, the toxic control group showed distorted and/or total loss of hepatic histoarchitecture with kupffer cell hyperplasia, centrilobular hepatic necrosis, vacuolization and congestion of sinusoids, fatty changes, fibrosis and apoptosis (Fig 1). The administration of CCl_4 after initiation of hepatocarcinogenesis exhibited a heightened synergistic effect to DENA especially as hepatic cirrhosis and HCCs occurred concurrently in a large number of animals in the toxic control group. However, rats pre-treated with 1000 mg/kg b.wt CLE prevented the formation of hepatocellular carcinomas and preserved hepatic histoarchitecture similar to that of silymarin (Fig 1).
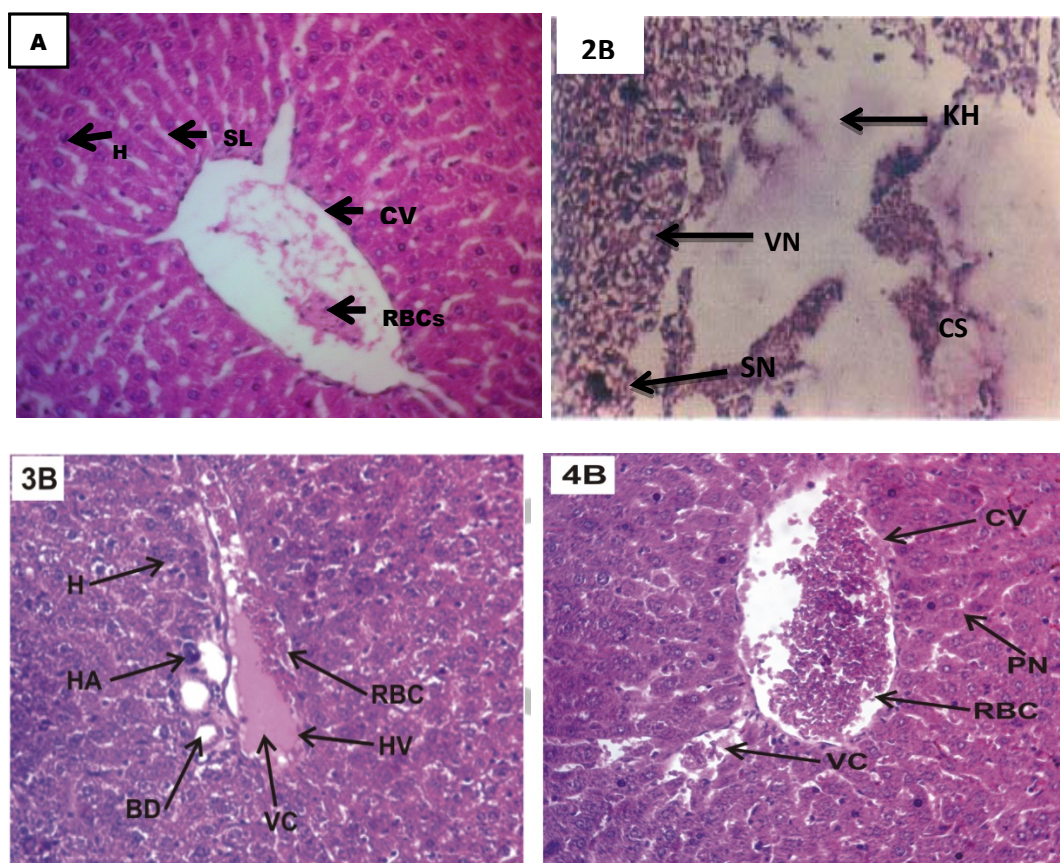

Figure 1. Photomicrographs of typical liver sections from normal control (A), toxic control (2B), positive control [100 mg/kg b.wt silymarin (3B)] and CLE-treated [1000 mg/kg b.wt; (4B)] rats showing normal histoarchitecture, hepatocytes (H), kupffer cells hyperplasia (KH), scar of necrosis (SN), congested sinusoids (CS), vacuolated nuclei (VN), hepatic vein (HV), bile duct (BD), hepatic artery (HA), Sinusoidal Lining (SL), central vein (CV), red blood cells (RBCs), pyknotic nucleus (PN), vascular congestion (VC) at x 400 magnification.

4.   Conclusion

The findings of this study demonstrates the antidotal or hepatoprotective properties of the hydroethanol extract of *C. longa* (turmeric) against DENA+CCl_4+PB-induced hepatotoxicity/tumourigenesis in rats. Thus, the authors recommend that turmeric and its derivatives be exploited as an adjuvant in the therapeutic and chemopreventive management of liver damage, liver cancer and extrahepatic cancers.

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Conflict of interest

The authors have no conflict of interest to declare.

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