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CYP450–mediated metabolites of aqueous leaves extract of *Moringa oleifera* inhibits absorption of chloroquine in *Plasmodium yoeli yoeli* infection

Segun Solomon Ogundapo*, Olajoju Temidayo Soniran, Joseph Bagi Suleiman, Kerian Chigozie Ngobidi, Nwogo Ajuka Obasi, Victor Uzochukwu Olugbue, Oswald Obinna Chukwunwike and Idowu David Olatunji

Abstract

Background: This study tested an *in vivo* model designed to evaluate the potential of HDI of chloroquine and aqueous leaf extract of *Moringa oleifera* to overcome multi-drug resistant *Plasmodium yoeli yoeli* infection in albino mice.

Methods: Using Rane's test, 40 male albino mice were randomized into 10 groups of four mice each. Nine groups inoculated with multidrug-resistant strains of *Plasmodium yoeli yoeli* were treated with water, 150 mg/kg of *Moleifera oleifera* aqueous leaf extract, 10mg/kg chloroquine (CQ), CQ co-administered with same leaf extract at intervals of 60, 30 and 0 minutes and artemeter lumefantrin at a dose of 1.14/6.85 mg/kg, while an uninfected tenth group was treated with water. All treatment commenced from day 3 and continued to day 5. Antimalarial activity was determined from thin tail blood smear on day 3 just before treatment and on day 5, 6 and 9. In addition to the parasitaemia chemosuppression, changes in plasma lipid parameters, liver marker enzymes and kidney function parameters were determined from blood collected by ocular puncture using standard methods. Phytochemical screening of aqueous extract of *Moringa oleifera* leaves was done using standard methods.

Results: Time dependent significant increase ($p<0.05$) in percentage chemosuppession in all treated groups were observed with total parasite clearance comparable to ACT treatment observed on day 9 for the group treated with extracts 30 minutes after CQ administration. Blood analysis revealed that the HDI of this additive interaction was not associated with any significant changes in lipid, kidney and liver parameters when compared to the ACT treated control.

Conclusion: The finding of this study suggests that Co-administration of aqueous leaf extract of *M. oleifera* with CQ has potentials for antagonistic drug-drug interactions.

Keywords: *Moringa oleifera*; HDI; *Plasmodium yoeli;* antiplasmodial activity; CYP-450.

*Correspondence: *Phone: +23480394646644; Email: solohitachi@yahoo.com;* ORCID: *https://orcid.org/0000-0003-0230-4430 (Ogundapo Segun.Solomon)*
Department of Science Laboratory Technology, Akanu Ibiam Federal Polytechnic Uwana Afikpo Ebonyi State, Nigeria

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Background

Malaria is a life threatening parasitic infection caused by the acomplexan plasmodium species (*P.falciparum*, *P. ovale*, *P. malariae* and *P.vivax*) and the most virulent to man being *Plasmodium falciparum* [1]. *Plasmodium falciparum* is responsible for about 80% of all malaria cases and about 90% of the death from malaria [2]. Malaria is a disease with highly mortality and morbidity especially in children and pregnant women. The disease which is endemic in sub-Saharan Africa with children under the age of five and pregnant women at risk is among the top three deadly communicable disease and it is the most deadly in the tropics despite various efforts towards its control [3]. The provision of effective chemoprophylaxis and treatment of malaria is still a major problem in tropical countries. How**e**ver, vaccines would have been the ultimate and effective control but none is available for use. Several vaccines candidate such as RTS, S/ASO2A and VAR2CSA are being tested and evaluated. Hence vector control and chemotherapy remain the major interventions for the control of malaria [4].Several drugs particularly chloroquine has been used to treat malaria. However, most of the drugs that we use today are becoming less effective because of the problem of drug resistance [5].

This has led to the development of newer drugs of which artemisinin derivatives have been widely accepted. Artemisinin antimalaria drugs derived from the extract of a Chinese herb *Quinhaosu* used for the treatment of fevers have in the past three decades been reported to be efficacious in clinical management of chloroquine resistant malaria [6]. The level of confidence placed on herbal preparation is encouraging their used along with prescribed orthodox drugs and over the counter (OTC) drug for various disease conditions [7].

This has necessitated the exploration of HDI of readily available medicinal plants and cheap and affordable monotherapies for the management of drug resistant malaria. However HDI is one of the most important clinical concerns in the concomitant consumption of herbs and prescription drugs [8].

Moringa oleifera, a member of the family *Moringaceae* is highly reputed in traditional practice in Nigeria. The leaves are rich in essential and non-essential amino acids, vitamins and minerals and aqueous extracts of *Moringa oleifera* leaves is used locally in Nigeria as anti-malaria and anti-helminthic agent [9]**.** The aqueous extract of the leaves have been reported to boost haematological parameters and as well show antiparasitic effect on *Plasmodium yoeli* infected mice [10]. To determine the potential of HDI to overcome drug resistance, we tested an *in vivo* model designed to evaluate the potential of HDI of chloroquine and aqueous leaf extract of *Moringa oleifera* to overcome multi-drug resistant *Plasmodium yoeli yoeli* infection and associated changes in liver, kidney function parameters and lipid profile in mice. Preliminary findings from our study will serve as baseline data for further studies investigating the feasibility of developing a cost effective treatment of drug resistant malaria using a combination of herbs and monotherapies especially in poor resource settings.

Methods

Drugs

The drugs administered for control and treatment group was chloroquine (DANA, Nigeria) and artemether lumefantrin (CIPLA, India). This was dissolved in physiological saline to obtain fixed doses as used in the assay.

Plant Material

Fresh leaves of *M. oleifera* with flowers and fruits still attached was collected randomly from tress in Afikpo area of Ebonyi state, Nigeria in May and authenticated by Mr Alfred Ozioko a plant taxonomist at Bioresources Diversity and Conservation Programme (BDCP), Nsukka, Enugu State, Nigeria. It was assigned a voucher number: INTERCEED/501.

Parasite

The parasite that was used for this study was chloroquine resistant *Plasmodium yoeli* strains obtained from the American type culture collection (ATCC) Manassas, VA 20110-2209, USA. A donor mouse having parasitaemia of about 35% was sacrificed and bled by cardiac puncture. The parasitaemia was adjusted downward using physiological saline and each experimental albino mice was inoculated intraperitoneally with approximately $1.0x10^7$ parasitized erythrocyte in volumes 0.2 mL.

Animals

Forty (40) in-bed male Swiss albino mice weighing between 22.7 g and 37.7 g (mean 30.2 g) obtained from animal house of department of veterinary medicine, University of Nigeria, Nsuka were used for this study. They were kept in well ventilated rodent cubicles under 12hours light/dark cycles and fed with feed and water *ad libitum*. The caring and experimental use of the mice was according to the guidelines of National Institute of Health guidelines for cares of laboratory animals. They were acclimatized for 14 days before being randomized into the experimental groups.

Experimental design

A total of 40 male mice were used for the study and were randomized into the following groups (n=4):
Group1 ; Consist of plasmodium passaged mice to serve as a negative control, given 0.2ml of water.
Group 2: Consist of *plasmodium* passaged mice to serve as a positive control treated with chloroquine at a dose of 10mg/kg bw CQ
Group3: Consist of *plasmodium* passaged mice treated with 150mg/kg of leaf extract co-administered with 10mg/kg bw CQ.
Group4: Consist of *plasmodium* passaged treated with *Moringa oleifera* leaf extract 30 minutes before treatment with 10 mg/kg body weight chloroquine.
Group5: Consist of *plasmodium* passaged mice treated with *Moringa oleifera* leaf extract 60 minutes before treatment with 10 mg/kg body weight chloroqunie.
Group6: Consist of *plasmodium* passaged mice treated with *Moringa oleifera* extract 30 minutes after treatment with 10 mg/kg body weight chloroquine.
Group7: Consist of *plasmodium* passaged mice treated with *Moringa oleifera* leaf extract 60 minutes after treatment with 10 mg/kg body weight chloroquine.
Group8: consist of *plasmodium* passaged mice treated with standard and unknown concentration of Artemisinin combination therapy.
Group9: Consist of *plasmodium* passaged mice treated with aqueous leaf of *Moringa oleifera*.

Group 10: consist of uninfected mice that serve as a control orally administered water.

Extraction of plant materials

Exactly 100 g of freshly collected *Moringa oleifera* leaves were weighed, washed and blended with 1000 mL of distilled water using an electric blender. It was sieved with a muslin cloth and filtered with Whatman 42 filter paper. The extract dose was prepared from this crude stock with distilled water according to the design and was used immediately.

Parasite density determination inoculation preparation

Standard inoculum was prepared from donor mouse innoculated with *P. yoeli* parasitized erythrocyte infected blood by cardiac puncture after being anesthetized with chloroform as described by Iwalokun [11]. Parasitaemia was established by microscopic examination of a thin blood film under oil immersion at x100 magnification and measured as mean percentage of infected erythrocyte in three fields. Each mouse was infected with standard inoculums of the $1.0x10^7$ parasitized erythrocyte suspension in physiological saline (0.9%) from the donor mouse that was prepared based on percentage parasitaemia and number erythrocyte count per micro litre of blood. The inoculated mice were randomized into nine groups of four mice per cage and fed with commercial animal feed (Top feeds, Nigeria) and water *ad libitum.*

Phytochemical screening

Chemical tests were carried out on the aqueous extract of leaves of *M. oleiefera* to determine the presence of alkaloids, tannins, flavonoids, saponins, glycosides, phenols and other phytochemicals using standard procedures to identify the constituents as described by [12,13,14].

Antimamarial assay

The seven days curative method (Rane's test) describe by [11] was used with mordifications. The animals were divided into 9 groups of 4 mice each and treated according to the experimental design. Treatment commenced on day 3 post inoculation (4^{th} day after inoculation) and continued on day 4, 5 and 6 via oral route using steel canula. Tail blood films of the infected animals were prepared, fixed in methanol, stained with Giemsa and examined microscopically under oil immersion (x100) on day 8 post inoculations. Parasite density and percentage reduction in parasitaemia relative to chloroquine and the ACT control on group by group basis were calculated.

Determination of percentage parasite suppression

Percentage of parasitaemia and percentage of suppression were calculated using a modified formular proposed by [15].

$$\% \text{ suppression} = \frac{(\text{parasitaemia on day 3}) - (\text{parasitaemia on day 6 and 9})}{\text{Parasitaemia on day 3}} \times 100$$

$$\% \text{ parasitaemia} = \frac{\text{No of uninfected RBCS} - \text{No of infected RBCS}}{\text{No of infected Red Blood Cells}} \times 100$$

Biochemical assays

The following biochemical parameters were assessed: Liver function (aspartate aminotransferase (AST), alanine aminotransferase (ALT) by the method of [16], alkaline phosphatase (ALP) kidney function, plasma creatinine, urea as described by [17] and uric acid by method described by Vijayalakshmi *et al*., [18]. Lipid profile (HDL, LDL, VLDL, triacylglycerols and total cholesterol were determined as described by [19]

Statistical analysis

Data obtained was analyzed and expressed as mean standard error of mean (SEM) and significance between means of groups determined using one way analysis of variance (ANOVA) at 95% confidence interval ($P < 0.05$).

Results and discussion

The result of the phytochemical screening of the aqueous extract of *Moringa oleifera* leaves used in this study is presented in Table 1. It reveals strong presence of Flavonoids, phenols, terpenoids and tannins but the presence of alkaloids, steroids and glycosides were moderately represented while saponin was not detected. These observations are consistent with reports of phytochemical screening as reported [20, 21]. Differences in the compositions of the bioactive constituents are mostly due to extraction methods, the stage of maturation of the plant and geographical distribution. Plant flavonoids, phenolics terpenoids and alkaloids have been reported to have antiplasmodial activity and potent antiplasmodial compounds from these classes of phytochemicals have been isolated and characterized [22, 23]. Flavonoids are recognized for their antioxidant activity while alkaloids which are nitrogen-containing naturally occurring compounds, are reported to have antiprotozoal and antimicrobial properties due to their ability to intercalate with DNA of the organisms [24].

The potential of the herb-drug interaction (HDI) of aqueous extract of *Moringa oleifera* leaves and chloroquine for synergistic antiplasmodial effect against multi-drug resistant strain of *Plasmodium yoeli yoeli* was determined in this study. To evaluate how this HDI affect the pharmacokinetics or pharmacodynamics of chloroquine, we administered same concentration of aqueous extract to the animals 60 and 30 minutes before administering chloroquine in some groups. The extracts were concurrently co-administered with the drug to another group. Significant increase ($p<0.05$) in percentage chemosuppression was observed in the groups treated with the extract and CQ (Table 2) at intervals when compared with the CQ treated group but when these are compared with the extract treated group, a non significant decrease ($p>0.05$) was observed that seemed to suggest an antagonistic HDI that actually resulted in a reduction in the efficacy of the of the extract. The reason for this observation is not immediately clear but it may not be unconnected with decreased absorption and bioavailability of the CQ and aqueous extract. We suggest that the mechanism of reduced absorption is by way of formation of less absorbable adducts formed from the products of intestinal CYP enzymes metabolism of the leaf extract and CQ. This probably is why the concurrent administration of CQ and the extract achieved significantly higher chemosuppression ($p<0.05$) than those observed in the groups administered the extract 60 and 30 minutes before CQ. More studies are needed to confirm and elucidate a mechanism for this. When CQ was

administered 30 minutes before the extract, we observed that the HDI resulted to a significant antiplasmodial effect comparable to that of the ACT treated standard control with total clearance of parasitaemia by day 9 post inoculation. The treatment with the extract 60 minutes after CQ also achieved significant chemosuppression but failed to clear parasitaemia by day 9 post inoculation which may be due to rapid excretion of the bioactive metabolites. This observations while lending credence to our earlier supposition that the CYP enzyme mediated metabolic products of the extract inhibited the absorption and bioavalaibility of CQ or its metabolite desethylchloroquine, also suggest that the synergistic HDI antiplasmodial activity decreased with increase in time interval between administration of CQ and extract. The potentiation of antiplasmodial activity of the HDI may be attributed to the modulation or inhibition of multidrug resistant protein 1a transporter located on surface membrane bound vessicles on the multi-drug resistant parasite surface membrane by bioactive components of the extract and CQ [25, 26]. This may have led to increased parasite sensitivity to CQ coupled with the antiplasmodial activity of the extract as previously reported [21, 27]. Even though the mechanism of the antiplasmodial activity of the extract is poorly understood, our observations suggest that the HDI of the administration of the extract after CQ has potentials for significant synergistic antiplasmodial activity**.**

The synergistic HDI decreased mean plasma total cholesterol, triglycerides and LDL (Figure 1) when compared to the ACT treated control. This suggests a hypolipidaemic effect which may not be unconnected to plasmodium induced impaired-ability of the liver to produce lipoproteins when compared to the ACT treatment. Changes in serum lipid profile of patients presenting malaria has been attributed to increased haemolysis in malaria pathogenesis and is related to severity of infection [28].

The HDI of the administration with the significant antiplasmodial activity was observed to be associated with significant increase ($p < 0.05$) in the activity of the aminotransferases (Figure 2) and bilirubin concentration (Figure 3) when compared to ACT treatment. This we attribute to the hepatic metabolism of the CQ and the extract secondary metabolites and the consequences of hepatic schizogonic of the plasmodial pathogenesis.

The significantly elevated mean plasma bilirubin concentration in the untreated negative control is thought to be due to excessive heamolysis characteristic of malaria pathogenesis. We also observed that even though the HDI of the administration of the extract before the CQ did not significantly reduce parasitaema, it was characterised by milder hepatoxicity.

The HDI of the administration with significant synergistic antiplasmodial activity was observed to be associated with insignificantly ($p > 0.05$) elevated kidney function parameters when compared to that of the ACT treatment. The co-administration of the extract with CQ significantly increased the plasma urea and creatinne concentrations when compared to the values observed for the groups treated with ACT and CQ and extract administrated at different intervals.. This suggests that plasmodium induced kidney damage was exercabated by the HDI of CQ administration and HDI of CQ extract co-administration. The HDI of the CQ and extract at time intervals significantly ameliorated the plasmodium induced kidney damage. This we propose is due to the nephro protective properties if moringa leaf extract as reported by [29]. The sustained impairment of kidney function by the co-administration may not be unconnected with reduced absorption of the extract by the CQ extract HDI limiting its nephroprotective activity.

Table 1. Qualitative Phytochemical composition of aqueous extract of *Moringa oleifera* leaves

Phytochemicals	**Result**
Alkaloids	+
Steroids	+
Flavonoids	+ + +
Phenols	+ + +
Glycosides	+
Terpenoids	+ +
Saponins	ND
Tannins	+ +

Table 2. Mean antimalarial percentage chemosuppression for the different groups.

GROUPS	DRUGS		ANTIMALARIA ACTIVITY			
		DOSE	% **ELEVATION**/CHEMOSUPRESSION			
		mg/Kgbw	Day 3	Day 5	Day 6	Day 9
GRP 1	H_2O	0.1ML	0	**3.98±0.88**	**5.67±1.16**	**10.83±0.88**
GRP 2	CQ	10	0	30.00±1.03	44.31±1.20	58.61±2.21
GRP 3	EXTRTCQ	150	0	29.17±1.15	63.92±0.88	83.33±0.58
GRP 4	EXT+CQ	150+10	0	59.74±0.88	64.92±0.58	77.90±0.33
GRP 5	EXT+CQ	150+10	0	63.79±0.66	68.96±0.58	77.60±0.67
GRP 6	CQ+EXT	10+150	0	52.85±0.58	92.84±0.88	100
GRP 7	CQ+EXT	10+150	0	52.43±0.00	91.47±1.20	93.89±0.88
GRP 8	ACT	1.14/6.85	0	70.21±0.33	80.68±0.33	100
GRP 9	EXTRCT	150	0	43.33±0.33	77.79±0.33	81.96±0.33
GRP 10	H_2O	0.1	0	0	0	0

N = 4.Mean values ± SEM. P is significant at < 0.05.
GROUP 1 (untreated control); **GROUP 2**(CQ treated control); **GROUP 3** (treated with 10 mg/kg CQ co-administered with150 mg/kg dose of extract); **GROUPS 4 and 5 (**treated with 150mg/kg dose of extract 30 and 60 minutes before CQ administration respectively); **GROUPS 6** and **7 (**treated with10 mg/kg CQ 30 and 60 minutes before 150 mg/kg dose of extract administration respectively); **GROUP 8** (ACT treated control); **GROUPS 9** and **10)** (aqueous extract treated and the uninfected water treated control respectively).

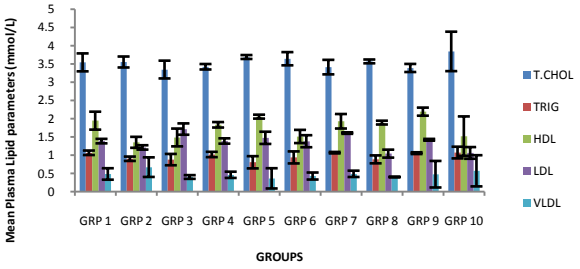

Figure 1. Mean plasma lipid panel parameters of the different groups

N = 4.Mean values ± SEM. P is significant at < 0.05.
GROUP 1(untreated control); **GROUP 2** (CQ treated control); **GROUP 3** (treated with 10 mg/kg CQ co-administered with150 mg/kg dose of extract); **GROUPS 4 and 5 (**treated with 150 mg/kg dose of extract 30 and 60 minutes before CQ administration respectively); **GROUPS 6** and **7 (**treated with10 mg/kg CQ 30 and 60 minutes before 150 mg/kg dose of extract administration respectively); **GROUP 8** (ACT treated control); **GROUPS 9** and **10)** (aqueous extract treated and the uninfected water treated control respectively).

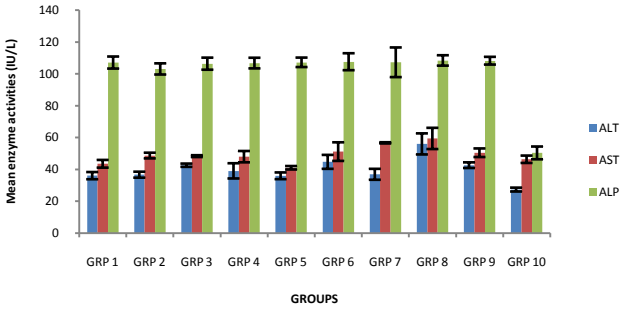

Figure 2. Mean serum activities of liver function enzyme biomarkers of the groups

N = 4.Mean values ± SEM. P is significant at < 0.05.
GROUP 1(untreated control); **GROUP 2** (CQ treated control); **GROUP 3** (treated with 10 mg/kg CQ co-administered with150 mg/kg dose of extract); **GROUPS 4 and 5 (**treated with 150mg/kg dose of extract 30 and 60 minutes before CQ administration respectively); **GROUPS 6** and **7 (**treated with10 mg/kg CQ 30 and 60 minutes before 150mg/kg dose of extract administration respectively); **GROUP 8** (ACT treated control); **GROUPS 9** and **10)** (aqueous extract treated and the uninfected water treated control respectively).

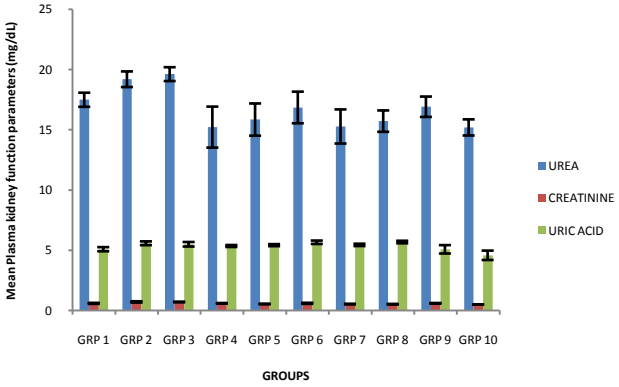

Figure 3. Mean plasma kidney concentrations of biomarkers of the groups

N = 4.Mean values ± SEM. P is significant at < 0.05.
GROUP 1(untreated control); **GROUP 2** (CQ treated control); **GROUP 3** (treated with 10mg/kg CQ co-administered with150 mg/kg dose of extract); **GROUPS 4 and 5 (**treated with 150 mg/kg dose of extract 30 and 60 minutes before CQ administration respectively); **GROUPS 6** and **7 (**treated with10 mg/kg CQ 30 and 60 minutes before 150mg/kg dose of extract administration respectively); **GROUP 8** (ACT treated control); **GROUPS 9** and **10)** (aqueous extract treated and the uninfected water treated control respectively).

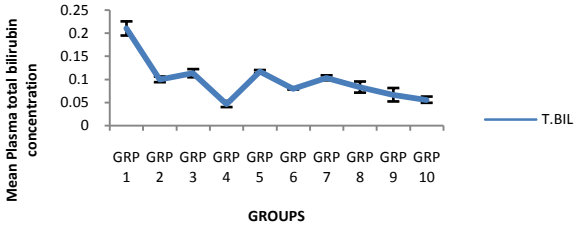

Figure 4. Mean serum total bilirubin concentration of the groups

N = 4.Mean values ± SEM. P is significant at < 0.05.
GROUP 1(untreated control); **GROUP 2**(CQ treated control); **GROUP 3** (treated with 10mg/kg CQ co-administered with150 mg/kg dose of extract); **GROUPS 4 and 5 (**treated with 150 mg/kg dose of extract 30 and 60 minutes before CQ administration respectively); **GROUPS 6** and **7 (**treated with10 mg/kg CQ 30 and 60 minutes before 150mg/kg dose of extract administration respectively); **GROUP 8** (ACT treated control); **GROUPS 9** and **10)** (aqueous extract treated and the uninfected water administered control respectively).

Conclusions

The findings of the study while giving fillip to the much touted possibilities of adverse herb drugs interactions associated with co-administration of herbs with prescription drugs, also seems to suggest beneficial synergistic pharmacodynamic outcome. Our study seemed to suggest that CYP450 mediated metabolic products of *Moringa oleifera* leaves extractsThis, our study has shown may be dependent on the timeline of administration of the extract and the drugs. Our study also seemed to be a plausible model that can be built on for investigation of HDI for beneficial synergistic pharmacodynamic outcomes of plant extract and prescription drugs.

Declaration

Supporting data relating to this publication is available on request from the corresponding author.

Authors' Contribution

OSS conceived the concept and designed the study, SOT, ONA, COO, OVU, SJB, NKC, OID participated in data gathering. OSS wrote the first draft, did data analysis and wrote the discussion and conception of the paper while OSS, SOT and OVU read through the manuscript. All authors read and approved the final article.

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Conflict of interest

The authors declare that they have no competing interests.

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