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Research Article

In-Vivo Evaluation of Trypanocidal Efficacy of *Phyllanthus amarus* Root Extracts in Mice Experimentally Infected with *Trypanosoma congolense*

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Abstract

Trypanosoma congolense is debilitating to livestock and invariably fatal if left untreated. Chemotherapy, the main means of controlling this disease has been reported to be toxic and unreliable due to reports of parasite resistance. Thus, the study was aimed at evaluating root extract of *Phyllanthus amarus* for trypanocidal activity in mice experimentally infected with *Trypanosoma congolense*. Root of *Phyllanthus amarus* was extracted using methanol and aqueous solvents. Forty five mice weighing between 25-35g were divided into 9 groups (group 1- 9) of 5 mice each. Mice were inoculated with 0.2 ml of blood containing 2.0 x 10^6 parasites intramuscularly/mouse and were treated with various concentrations of 100, 250 and 500mg/bw of methanolic and aqueous root extracts of *Phyllanthus amarus*. Diminazine (Nozomil®) at standard concentration of 3.5mg/bw was used as control. Data collected were analyzed using analysis of variance (ANOVA) at P<0.05 level of significance. The result of the study showed that treatment with methanolic and aqueous root extract of *Phyllanthus amarus* exhibited anti-trypanosmal properties which was concentration dependent thus resulted in significant reduction (P>0.05) in parasitaemia and extension of survival days of the treated mice for additional 22 days against the infected untreated group. There was also a corresponding higher PCV with a fluctuating mean weight values in groups of mice infected but treated with the root extract of *Phyllanthus amarus*, this was however, statistically significant (P>0.05) when compared with the infected untreated group. Root extracts of *P. amarus* have shown a successful trypanocidal activity based on suppressed parasitaemia in the treated mice, it can however be used in optimal concentrations to sustain animals infected with animal trypanosomiasis in the absence of synthetic chemical drugs to prevent death of animals infected with *Trypanosoma congolense*.

Keywords: *Phyllanthus amarus*; Weight; PCV values; Parasitaemia; Trypanosomiasis; *Trypanosoma congolense*.

Introduction

African animal trypanosomiasis (AAT) is a disease of vertebrate animals. The disease is caused by trypanosomes of several species in the genus *Trypanosoma: T. b. brucei, T. congolense, T. vivax, T. evansi* and *T. equiperdum.*. *Trypanosoma congolense* is considered the most important cause of AAT in East Africa, and *T. vivax* in West Africa [1]. The disease is most significant in cattle but can cause serious losses in pigs, camels, goats, and sheep. Infection of cattle by one or more of the three African animal trypanosomes, results in sub-acute, acute, or chronic disease characterized by intermittent fever, anaemia, occasional diarrhea, and often terminates in death [2].

Phyllanthus amarus is a plant of the family Euphorbiaceae, it is commonly called Carry me Seed or Stone-Breaker or Wind Breaker. *P. amarus* have been reported to contain a wide range medicinal bioactive component however; plant with these values may provide an alternative in the treatment of trypanosomiasis in which synthetic drugs are resistant. This study was aimed at evaluating root extracts of *Phyllanthus amarus* for in vivo

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of trypanocidal efficacy in mice experimentally infected with *Trypanosoma congolense*

Materials and methods

Collection and identification of plant materials

Mature P. amarus plant were collected from their natural habitat in Ikot Nkim, Ibesikpo Asutan Local Government Area, Akwa Ibom State, Nigeria. The plant was identified by Taxonomist of Botany Department, University of Uyo, Akwa Ibom State, Nigeria.Voucher number: Udo, UUH3810 (Ikot Nkim Ibesikpo) was given and kept in Herbarium unit, Department of Botany University of Uyo, Akwa Ibom State, Nigeria. The collected plant specimens were taken to Federal University of Agriculture, Department of Biological Sciences' Laboratory for extraction and further experiments.

Preparation of methanolic and aqueous extracts

Fresh leaves of P. amarus were washed thoroughly using distilled water and air dried on laboratory bench for two weeks. Methanolic and aqueous solvents were used for the extraction using cold maceration technique as described by Evans (2002) and Sofowora (2006) [1,2]

Experimental animals

Fifty mice of both sexes were obtained from The Laboratory Animal Unit of Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Plateau State, Nigeria. They were maintained based on the Guide for the Care and Use of Laboratory Animals (DHHS, NIH Publication No. 85-23, 1985) and allowed to acclimatize the environment for 7 days before the commencement of the experiments. The animals were allowed to have access to clean drinking water and feed (Vital Feed) ad libitum before and during the experiments.

Trypanosome stock

Trypanosoma congolense stabilate was obtained from Department of Vertrinary Parasitology and Entomology Faculty of Veterinary Medicine Ahmadu Bello University (ABU), Zaria, Nigeria. The parasites were inoculated into rats. The inoculated rats were monitored daily for parasitaemia by examining microscopically at x 40 magnification from a drop of blood collected from the tail.

Evaluation of P. amarus extract for Antitrypanosomal Activity in T. congolense infected mice

Infection of mice

Forty five mice weighing between 20-35g were divided into 9 groups (group 1- 9) of 5 mice each. Group 1 to group 8 (G1-8) were infected with *Trypanosoma congolense* parasites intraperitoneally with 0.2 ml of blood containing a specified number (2.5×10^6) of trypanosomes / mouse. By 7 days post infection, parasitaemia was established in all the infected mice.

Groupings and treatment of mice

By 9 days post infection when parasitaemia was approximately log 7.8 (63×10^6 parasites/ml) treatment with graded doses of both methanolic and aqueous root extract of *P. amarus* (100, 250 and 500 mg/kg respectively) were administered for 5 consecutive days to 6 different groups of mice (groups 1 - 6) orally. Diminazene diaceturate (Nozomil®) was given to mice in group G7 at a dose of 3.5 mg/kg intraperitoneally. Mice in group 8 were infected untreated controls while those in group 9 were uninfected.

Detailed groupings were as follows:

Group 1: *T. congolense* infected mice were treated with 500mg/kg of aqueous root extract of *P. amarus*

Group 2: *T. congolense* infected mice were treated with 250mg/kg of aqueous root extract of *P. amarus*

Group 3: *T. congolense* infected mice were treated with 100mg/kg of aqueous root extract of *P. amarus*

Group 4: *T. congolense* infected mice were treated with 500mg/kg of methanolic root extract of *P. amarus*

Group 5: *T. congolense* infected mice were treated with 250mg/kg of methanolic root extract of *P. amarus*

Group 6: *T. congolense* infected mice were treated with 100mg/kg of methanolic root extract of *P. amarus*

Group 7: *T. congolense* infected mice were treated with Diminazene Diaceturate (Nozomil®) at standard dose (3.5 mg/kg) given intraperitoneally.

Group 8: Infected mice - non treated (positive control).

Group 9: Non infected – non treated (negative control).

Determination of live weight

Mice in all the groups were weighed in grams (g) on a 3 day interval using weighing balance (sensitive electronic weighing balance with Model number: Labtech® BL2OOO1). Mean weights from each replication was also recorded to establish the growth rates against the expected normal growth curve of a mouse.

Determination of body temperature

Body temperature was determined per rectum by the use of a digital clinical thermometer (MODE: GF-MT502).

Quantification of parasitaemia

A drop of mouse tail blood was examined first at x10 then x40 magnification using a table microscope. The number of trypanosomes in each field was counted. Each counting per field was matched with log figures obtain from the reference table. The log figures were converted to antilog values which were subsequently converted to absolute numbers. This gave the *number of trypanosomes per milliliter[5]*.

Packed cell volume (PCV) determination

The microhaematocrit method of Coles (1968) [6] was used for determination of PCV. Briefly, mice tail blood was collected into sodium heparinized micro-haematocrit capillary tubes to one third of the tube. The unfilled end of the tube was sealed with plastiseal by pushing the capillary tube down into it. The capillary tubes were then centrifuged in a microhaematocrit centrifuge at 10, 000 revolutions per minute (rpm) for five minutes. The PCV was read off using a haematocrit reader.

Analysis of data

Data were entered and managed using Microsoft Excel (version 2007). Statistical analysis was done using SPSS version 21. Values of the data obtained was summarized and expressed as mean ± standard deviation. The significant differences of the mean of parasitaemia, body weight, packed cell volume of mice from the negative control group, Diminazene treated group and the extracts treated groups at different dosages were compared by One Way ANOVA at p values less than 0.05 being considered significant.

Results and discussion

Effect of treatment on daily live weight of mice infected with T. congolence and treated with different concentrations of aqueous and methanolic root extracts of P. amarus

The result of weight of mice infected with *T. congolense* and treated with root extract of *P. amarus* is presented in Table 1. Mice in groups did not show significant drop ($P < 0.05$) in their various body weight on Day 4 through Day 15 post infection days (7 days post treatment) when compared with the control group (group9). But there was a significant high ($P < 0.05$) weight values in the infected untreated group (group14). However, Mice in the treated groups, (group 1 – 6) exhibited a statistically insignificant decrease ($P < 0.05$) in their live body weight on day 8 of the post experimental infection days.

There was however an increase in live body weight of mice on day 12 through Day 19 though the difference was not statistically significant ($P < 0.05$) when compared to the control group. On day 22 of the experimental days, the mean live weight of the treated mice was seen to increase in group 2, 3, and deminazene (group7) treated group 7 with mean values of 26.60±1.77, 29.43±1.41 and 30.10±1.35 respectively and the difference was statistically significant ($P < 0.05$) when compared with the infected not treated group. The mean value was however seen to reduce on 29 through day 34 of the experimental days.

On the other hand, mice in group 1 were seen to exhibit a reduction in their various live weight though this was not statistically significant ($P >0.05$) when compared to the uninfected untreated group 9 on day 22 through the remaining experimental days.

There was however, a continuous reduction in the mean values of mice in group 8 which served as the infected not treated group from day 8 till the end of the experimental days.

In contrast, there was a progressive increase in live body weight of mice all through the experimental days in group 9 which served as the uninfected control group.

Table 1. Mean live weight (grams) (Mean±SD) of mice infected with *T. congolense* and treated with different concentrations of aqueous and methanolic root extracts of. *P. amarus*

	DAY 1	DAY 4	DAY8	DAY 12	DAY 15	DAY 19	DAY 22	DAY 29	DAY 34
GROUP 1	26.67±4.15	26.33±4.18	30.67±4.07	31.00±3.90a	27.97±3.11a	31.80±5.37a	31.40±5.23a	30.29±5.78a	29.10±6.79a
GROUP 2	27.53±2.75	27.03±2.65	26.47±2.81	27.13±3.07	25.90±2.50a	26.60±1.77a	28.00±1.27a	25.00±0.00a	-*b
GROUP 3	25.70±2.55	25.10±2.40	24.57±2.55	25.30±2.60	28.80±3.56a	26.07±2.40a	27.50±1.98a	-*b	-*b
GROUP 4	28.67±3.58	27.97±3.68	27.10±3.70	27.87±3.79	28.90±3.45a	29.43±1.41a	30.10±1.87a	33.22±3.63a	28.98±3.93a
GROUP 5	27.37±3.29	27.00±3.87	27.97±3.72	27.60±4.08	28.33±3.65a	27.73±3.73a	29.03±4.19a	31.30±3.38a	29.04a
GROUP 6	25.23±3.68	25.30±3.44	24.63±3.56	25.17±3.60	25.50±56a	26.70±1.21a	27.40±1.27a	-*b	-*b
GROUP 7	28.57±1.92	28.57±1.82	27.80±1.70	28.63±1.61	29.36±1.60a	30.10±1.35a	30.90±1.27a	31.86±1.14a	32.63±1.40a
GROUP 8	26.63±0.96	26.33±0.55	25.46±0.50	23.83±0.87	-*b	-*b	-*b	-*b	-*b
GROUP 9	25.27±4.42	26.33±4.42	27.20±4.56	28.03±4.34	28.90±4.10a	29.60±3.96a	30.37a	31.67±3.61a	32.80±3.68a

Values are presented as mean ± standard deviation (SD).Values indicated by asterisk down the group are statistically different compared to the Non- infected control (GRP9) at P<0.05 whereas all values indicated by the superscript (a) down the group are statistically different compared to the Infected control group (GRP8) and values indicated by superscript (b) are statistically different compared to the Dimi group (GRP7).
Key: - represent complete mortality of mice in the group.

Mean group temperature (°C) (Mean±SD) of mice infected with T. congolense and treated with different concentrations of aqueous and methanolic root extracts of P. amarus

The result of Temperature of mice infected with *T. congolense* and treated with concentrations of root extract of *P. amarus* is presented in Table 2. An increase in mice body temperature was recorded in the mean values of mice across the infected group on day 8 of experimental days and the difference was statistically significant (p<0.05) when compared with the uninfected control group (group 9). However, this continued through day 5 of the experimental days.

On Day 19, a decrease in mice body temperature ranging from (38.40±36.20±0.98) was recorded across the treated group, though this was only statistically significant (p<0.05) in group, 1, 2 and 7 with mean values 37.65±0.07, 36.76±1.49 and 36.20±0.98 respectively (Table 2). Group 3 and group 6 recorded a significant decrease (P < 0.05) with mean values 35.50±0.63, 35.65±0.63 and 35.65±0.66 of temperature respectively on day 22.

A continuous drop in body temperature of the treated mice across the groups was recorded from day 22 through the experimental days though was not statistically significant (p<0.05) when compared with the uninfected control groups (group 9). In group 8 which served as the infected untreated group, mice in the group exhibited a rise in their body temperature which was statistically significant (P < 0.05) on day 8 and this was however followed by a statistically significant drop (P < 0.05) in body weight on day 12 of the experimental period. Mice in group 9 did not show any difference in their body temperature all through the period of the experiment.

Mean PCV (%) (Mean±SD) of mice infected with T. congolense and treated with different concentrations of aquoeus and methanolic root extracts of P. amarus

The result of PCV of mice infected with *T. congolense* and treated with root extracts of *P. amarus* is presented in Table 3. PCV of Mice in group 1 – 8 decrease steadily from day 8 ranging from 50.40 ± 0.78 to 48.93±0.60. This was however statistically significant (P < 0.05) on Day 13 when compared with group 9 (uninfected control group).

Among all the treated groups, increase in PCV values was recorded in group 7 with mean value of 49.20 respectively. However, all the extract treated group (1, 2, 3, 4, 5 and 6) on the other hand recorded a progressive decrease in PCV values from day 8 through 21 days of the experiment though this was significantly higher than the infected untreated control group (group 9). However, there was a progressive decrease in PCV values of the treated group from Day 29 through the experimental days. There was however, a progressive increase in PCV values recorded in the deminazene treated group from day 21 through the experimental days. Mice in the infected untreated group 8 exhibited a progressive decrease in PCV values from say 8 through the experimental days, the difference was however, statistically significant (P < 0.05) when compared with group 9. Mice in group 9

which served as the uninfected control group did not show any variation in their PCV values all through the period of the experiment.

Table 2. Mean group Temperature (^{o}C) (Mean±SD) of mice infected with *T. congolense* and treated with different concentrations of aqueous and methanolic root extracts of *P. amarus*

	DAY 1	DAY 4	DAY8	DAY 12	DAY 15	DAY 19	DAY 22	DAY 29	DAY 37
GROUP 1	38.10±0.10	39.60±0.17*	38.60±0.50	38.93±0.15	37.90±0-.10a	37.65±0.07a	37.60±0.56a	38.05±0.35a	38.10±0.28a
GROUP 2	38.10±0.10	39.13±0.25*	39.43±0.20*	39.06±0.37	37.93±0.95a	36.76±1.49s	36.56±1.28a	37.05±0.07a	-*
GROUP 3	38.06±0.11	39.16±0.64*	39.23±0.41*	38.93±0.15	37.63±0.32a	37.43±0.49a	35.65±0.63*ab	-*b	-*
GROUP 4	38.20±0.00	39.36±0.50*	39.23±0.40*	38.70±0.17	37.83±0.55a	39.00±0.26a	38.23±0.60a	38.05±0.35a	36.95±0.35a
GROUP 5	38.16±0.20	39.26±0.56*	38.66±0.47	38.86±0.15	37.33±0.92a	37.56±0.75a	38.33±0.25a	37.05±0.07a	36.90±0.42a
GROUP 6	38.20±0.20	39.36±0.23*	38.90±0.20	38.76±0.80	37.46±1.42a	36.20±0.98*ab	35.65±0.66*a	-*b	-*
GROUP 7	38.33±0.15	39.56±0.11*	39.20±0.26	149.00±191	38.76±0.15a	38.20±0.20a	38.50±0.30a	38.26±0.25a	38.06±0.11a
GROUP 8	38.06±0.11	39.43±0.50*	39.43±0.30*	36.15±0.49	-*b	-*b	-*b	-*b	-*
GROUP 9	38.16±0.15	38.00±0.10ab	38.03±0.05ab	38.10±0.10	38.06±0.11a	38.16±0.15a	38.10±0.17a	38.20±0.20a	38.20±0.20a

Values are presented as mean ± standard deviation (SD).Values indicated by asterisk down the group are statistically different compared to the Non- infected control (GRP9) at P<0.05 whereas all values indicated by the superscript (a) down the group are statistically different compared to the Infected control group (GRP8) and values indicated by superscript (b) are statistically different compared to the Dimi group (GRP7).
Key: - represent complete mortality of mice in the group.

Table 3. Mean PCV (%) (Mean±SD) of mice infected with *T. congolense* and treated with different concentration of aqueous and methanolic root extracts of *P. amarus*

	DAY 1	DAY8	DAY 13	DAY 21	DAY 29	DAY 35
GROUP 1	50.10±3.55	47.90±2.89*	43.40±5.11*a	33.17±7.59*ab	26.75±4.60*ab	13.50±0.00*ab
GROUP 2	52.43±0.51	50.13±4.35	42.80±3.21*a	28.85±5.16*ab	20.85±0.91*ab	16.80±0.00*ab
GROUP 3	51.16±1.04	49.13±0.90	41.03±2.16*a	26.50±2.12*ab	19.60±0.00*ab	-*b
GROUP 4	50.56±0.81	48.93±0.60	41.87±2.60*a	37.26±11.11*a	31.20±0.50*ab	19.85±2.90*ab
GROUP 5	50.03±0.05	48.53±0.75	40.70±2.14*a	28.57±0.00*ab	22.30±1.56*ab	-*bb
GROUP 6	51.60±1.34	50.20±0.95	38.90±2.01*a	29.00±2.83*ab	25.10±0.00*ab	-*bb
GROUP 7	51.93±0.81	50.40±0.78	46.00±1.32*a	49.20±0.55a	47.66±0.90*a	49.03±1.26*a
GROUP 8	52.33±0.28	50.03±1.02	30.83±1.35*b	-*b	-*b	-*b
GROUP 9	52.20±0.26	52.26±0.37	52.54±0.25ab	52.20±0.26a	52.46±0.35ab	52.13±0.15a

Values are presented as mean ± standard deviation (SD).Values indicated by asterisk down the group are statistically different compared to the Non- infected control (GRP9) at P<0.05 whereas all values indicated by the superscript (a) down the group are statistically different compared to the Infected control group (GRP8) and values indicated by superscript (b) are statistically different compared to the Dimi group (GRP7).
Key: - Represent complete mortality of mice in the group.

Effect of treatment on parasitaemia of mice infected with *T. congolense* and treated with different concentrations of aqueous and methanolic root extracts of *P*. amarus

The parasitaemia of mice infected with *T. congolense* and treated with *P. amarus* is presented in Table 4. Parasitaemia was established in all the infected group on 7th days post infection days (Day 7). There was a decrease in Parasitaemia level across the treated group from Day 13 through Day 19 with significant reduction (p<0.05) achieved on Day 19 across the treated groups. Group 4 achieved highest (39.10±11.22) reduction in mean Parasitaemia value.

However, group 8 (infected untreated group) recorded a significant higher (280.10±30.43) parasitaemia on Day 13 when compared with other groups. A complete reduction in parasitaemia was recorded on Day 19 in the Dimmazene treated group (Group 7). However, there was a relapse in parasitaemia across the extract treated group from day 21 which increase steadily, till the end of the experiment. No relapse was seen in the Deminazene treated group (group 7).

The result shows that the extract suppressed parasitaemia and extended survival period of infected mice was and was dose-dependent. This revealed a successful *in vivo* trypanocidal activity of root extract of *P.*

amarus. Thus, this can be improved by using a higher dose or by using isolated active compounds in the extract. These findings are in agreement with a previous study which reported that compound with poyphenols exhibit encouraging *in vivo* trypanocidal activity with a reduction in the level of *T. brucei* parasitemia in mice [7]. This anti-trypanosomal effect may be attributed to specific Phenolic compounds and flavonoid constituents of root of *P. amarus.*

Table 4. Mean (Mean±SD) group Parasitaemia (10^6) of mice infected with *T. congolense* and treated with different concentrations of aqueous and methanolic root extracts of *P. amarus*

	DAY 1	DAY 7	DAY 9	DAY 13	DAY 19	DAY 21	DAY 23	DAY 35
GROUP 1	0	48.10±39.10	139.40±50.70*	102.30±27.40*ab	60.70±19.5*ab	190.20±34.40*ab	301.70±43.30*ab	400.10±113.10*ab
GROUP 2	0	33.70±21.50	143.20±44.30*	99.10±24.80*ab	77.00±18.70*ab	198.20±78.40*ab	429.00±60.10*ab	480.60±0.00*ab
GROUP 3	0	49.67±11.03	143.10±26.50*	115.84±14.42*ab	95.54±7.97*ab	257.80±31.60*ab	401.50±0.00*ab	-
GROUP 4	0	14.97±3.13	128.20±31.60*	114.10±28.10*ab	39.10±11.22*ab	99.50±19.80*ab	184.60±28.60*ab	749.00±632*ab
GROUP 5	0	74.50±17.70*	175.20±27.60*	122.10±25.50*ab	47.48±10.91*ab	117.42±12.48*ab	329.30±98.70*ab	-
GROUP 6	0	42.50±21.80	137.20±24.60*	102.01±3.95*ab	61.60±31.70*ab	281.50±99.70*ab	319.60±0.00*ab	-
GROUP 7	0	39.02±7.54	143.10±22.10*	26.23±13.56a	0	0	0	0
GROUP 8	0	28.80±24.3	159.10±86.40*	280.10±30.4*b	-	-	-	-
GROUP 9	0	-	0	0	0	0	0	0

Values are presented as mean ± standard deviation (SD).Values indicated by asterisk down the group are statistically different compared to the Non- infected control (GRP9) at $P<0.05$ whereas all values indicated by the superscript (a) down the group are statistically different compared to the Infected control group (GRP8) and values indicated by superscript (b) are statistically different compared to the Dimi group (GRP7).
Key: - represent complete mortality of mice in the group.

Weight gain was fluctuating in all the groups and may have had nothing to do with infection or treatment. Emaciation is a feature of chronic infections of animals with trypanosomes. Infected animals suffer from anaemia and emaciation and most die if untreated [8].

There was a corresponding rise in temperature and this is also attributed to the intermittent fever which normally occurs in trypanosoma infection and the subsequent low temperature could likely be related to the compromised action of the immune system in the infected mice.

Animals infected with trypanosomes characteristically exhibit fever in addition to other non-specific host defence mechanisms [9]. Zwart *et al*. (1990) documented high body temperature to be detrimental to the trypanosomes[10].

The elevation in body temperature has also been reported to results in an enhancement of the immune response by increased mobility and activity of the white blood cells [10].

Temporary rise in temperature in trypanosomosis is caused by trypanolytic crisis which enhances red blood cells damage and destruction leading to anaemia [11]. This was shown in this study by the concurrence of febrile peaks with onset of parasitaemia and fall in packed cell values. Other workers have also reported anaemia in animals as a result of *T. congolense* infection [12-14].

The continuous occurrence of parasitaemia upon treatment with the extracts indicate that the parasite may have evolved in an amazing mechanism for escaping obliteration by the host's defenses – namely antigenic variation, resulting from the successive dominance of each of a series of variable antigen type (VATs) over time. Remissions result from generation of protective antibodies that destroy the homologous trypanosomes. It has has been reported that, each time a host's antibodies are almost successful in eliminating infection, the trypanosome elude destruction by expressing a new variant-specific surface glycoprotein (VSG), thus becoming a new VAT and then rapidly multiplying [15].

Conclusions

Root extracts of *P. amarus* have shown a successful trypanocidal activity based on suppressed parasitaemia in the treated mice, it can however be used in optimal concentrations to sustain animals infected with animal trypanosomiasis.

Conflict of interest

Authors have declared no competing of interests.

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