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Original Article

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In vivo anti-acetylcholinesterase, antioxidant activities and acute toxicity of the alkaloid fraction from *Hippeastrum reticulatum*

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ABSTRACT

Background: *Hippeastrum reticulatum* (HR) belongs to the *Amaryllidaceae*, a well-known ornamental family. **Objectives:** This study was designed to reveal *in vivo* anti-acetylcholinesterase (AChE) and antioxidant activities and evaluate the acute toxicity of the alkaloid fraction (AF). **Methods:** The D-galactose-induced aging model and the scopolamine-induced cognitive-deficit model were used for the *in vivo* tests. The median lethal dose (LD_{50}) of the AF was determined using the Litchfield and Wilcoxon method. **Results:** On the aging model, the AF at 10 and 15 mg/kg significantly improved the antioxidant markers including superoxide dismutase, glutathione peroxidase, and malonyl dialdehyde. Upon the scopolamine-injected mice, the AF at 10 and 15 mg/kg notably downregulated cerebral AChE in mice. The LD_{50} of the HR AF is 90.2 mg/kg. **Conclusion:** These findings provide strong evidence for the safety and advantages of utilizing HR in treating Alzheimer's disease.

Key words: Acute toxicity, alkaloid, anti-acetylcholinesterase, antioxidant, *Hippeastrum reticulatum*

INTRODUCTION

Alzheimer's disease (AD), a breakdown in brain cell function, is the primary factor contributing to approximately 60–70% of dementia cases, resulting in diminished cognitive abilities and disruptions in daily personal activities. The worldwide incidence of dementia is projected to grow from 50 million in 2010 to 113 million by the year 2050.[1] The incidences of dementia in general and cognitive impairment in particular increase with age and age is also its most important risk factor.[2] As an adjunct to the officially approved AD medications, which encompass cholinesterase inhibitors and N-methyl D-aspartate antagonists, antioxidants emerge as a promising avenue for the prevention and management of AD.[3] Several medicinal plants and natural compounds were reported with both antioxidant and anti-acetylcholinesterase (AChE) activities, indicating that they can be used as drugs or function food for AD.[4-7]

Hippeastrum reticulatum (HR) (L'Hér.) is one of the members of the *Hippeastrum* genus belonging to the *Amaryllidaceae*, a well-known ornamental family.[8] The *Hippeastrum* genus is known for its diversity of pharmacological activities such as anti-cancer, anti-protozoan, AChE inhibition…[9] These effects are largely attributed to its alkaloids, particularly galantamine, which has gained FDA approval for addressing mild to moderate AD.

In Vietnam, the *Hippeastrum* genus has two species, *Hippeastrum equestre* and HR. The initial screening results of the research group showed that both *H. equestre* and HR have AChE inhibitory effects with IC_{50} values of 59.09 ± 2.43 and 39.85 ± 0.94 µg/mL, respectively. Thus, the HR species has better biological activity. Therefore, this species was included in further research on biological activity.

In 2017, the chemical composition of 12 compounds found in the HR was initially documented, which included galanthamine. *In vitro* and *in silico* testing showed AChE inhibition activity of some potential molecules among

them.[9] In 2020, Hoang *et al*. reported seven additional alkaloids, including three new compounds, some of which had *in vitro* AChE inhibition activity. Furthermore, the alkaloid compound demonstrated beneficial activities on memorial and cognitive impairment in mice with scopolamine-induced memory deficits.[10] These findings suggested that the alkaloid fraction (AF) from HR could be a promising option for AD treatment. The issue of concern is whether the AF is toxic and what are the mechanisms of its activities as an AD remedy.

This study aimed to evaluate the antioxidant and anti-AChE properties of the AF extracted from HR using a range of biochemical parameters in both the aging model induced by D-galactose and the cognitive-deficit model induced by scopolamine. In addition, the study assessed the acute toxicity of the AF in mice.

MATERIALS AND METHODS

The experiments on mice adhered to The Animal Center Guidelines for the Care and Use of Laboratory Animals at the Vietnam Military Medical University, in accordance with the EU Directive 2010/63/EU.[11] Approval for this study was granted by the Committee for Animal Experiments and Ethics at the Vietnam Military Medical University (Approval number: IACUC-053/20).

Plant Materials

The HR bulb was obtained from Thua Thien Hue province in Vietnam, specifically at coordinates 16°44′30″N 107°23′48″E in August 2018. Dr. Vu Tien Chinh from the Vietnam National Museum of Nature, Vietnam Academy of Science and Technology, confirmed its identity. A specimen (TTH-01) has been archived at the Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Vietnam.

The bulbs of HR are cleaned, roots removed, cut into small pieces, and dried at 55°C after harvesting. The dried herb is then ground into coarse powder and stored in a dry place.

Preparation of the AF

30 g of the AF were prepared from 12.5 kg of the bulbs with Diaion HP-20 column chromatography as previously described.[10]

The dried bulbs of HR (12.5 kg) were pulverized and subjected to three rounds of methanol extraction (20 L each) under ambient conditions. Subsequently, the solvent was evaporated under reduced pressure, yielding a crude extract. The total extract is dispersed in 2 L of distilled water and then applied to a Dianion HP-20 column (15 cm in diameter, 50 cm in length). Pass 10 L of water through the column (at a rate of 10 mL/min) to remove water-soluble components. Subsequently, elute the compounds with 2 L of methanol, resulting in the isolation of the methanol extract (150 g). Subsequently, the methanol extract was suspended in a 2% HCl solution and subjected to ethyl acetate washing (3 times, 1 l each). The remaining acidic solution was neutralized to a pH of 10 using NH_3 and subjected to partitioning with CH_2Cl_2 (3 times, 1 each) to produce the AF (AF, 30 g).

Experimental Animals

Adult male Swiss mice (19–23 g) were employed for the study. The mice were kept in a controlled environment at 25 ± 2°C under a 12-h day/night cycle, with unrestricted access to both food and water. The studies on acute toxicity, anti-aging effects in mice induced with aging by D-galactose, and *in vivo* AChE inhibitory activity were conducted over 4, 10, and 5 weeks, respectively. The mice were randomly assigned to groups, each group containing 10 mice.

Acute Toxicity

The acute toxicity study was implemented in compliance with the Guidelines of the OECD and Vietnam's Ministry of Health.[12] In the test assessing acute toxicity, the AF was suspended in normal saline. Ninety adult Swiss mice were randomly allocated to nine batches of 10 mice. These groups received varying oral doses, specifically 30, 55, 70, 90, 120, 160, 200, 250, and 300 mg/kg, respectively, as a single administration. The tested doses were determined after a preliminary test with a start of 0.2 mL/20 g of body weight, once every 24 h, at a concentration of 0.5 g/mL, and then with a dose increment within 30% of the extract concentration. A control group using normal saline was used for the detection of any abnormal parameter.

Daily monitoring of all the animals for unusual behavior and mortality was conducted for a period of 72 h. Monitored abnormal behaviors of the mice during the observation period including reduced activity, slow movement, decreased food and water intake, pale skin and mucous membranes, slightly erect fur, the appearance of diarrhea, huddling in one place, and death.

The median lethal dose (LD_{50}) of the AF of HR was determined using the Litchfield and Wilcoxon method.

In vivo Antioxidant Activity

The aging model was established by administering a subcutaneous D-galactose injection at 100 mg/kg daily for a duration of 4 weeks, following established procedures.[13] Following the 4-week period, 50 mice were divided into five groups of ten mice through random allocation, comprising an untreated group, three treatment groups, and a positive control group. Moreover, there was a normal control group consisting of healthy mice that had received saline injections over the same 4-week period. The treatment groups were orally administered using a syringe with the AF suspended in saline at the daily dose of 5 mg/kg, 10 mg/kg, and 15 mg/kg, respectively. The dosage was determined after our preliminary studies on a wide range of doses up to 25 mg/kg. The fifth group was treated with Vitamin E (Sigma Chemical Co., Cat. No: NC1846560, MO, USA) at doses of 50 mg/kg/day for the subsequent 4 weeks. At the same time, both the untreated group and the normal group received oral administration of an equivalent volume of the vehicle solution. The administration was performed at 8 am every day.

After 4 weeks of treatment, 500 µL blood was collected from the mice's eyeballs using a microcapillary into EDTA tubes. Plasma was acquired through centrifugation at 3000

revolutions per minute for 10 min at a temperature of 5°C and subsequently stored at −20°C.

The levels of malonyl dialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) in the plasma were assessed using commercially available kits, which included a MDA (Cat. No: EKMOU-0347); GSH-PX (Cat. No: EKMOU-0565) and SOD (Cat. No: EKMOU-0348) supplied by Melsin Medical Co., Ltd, China). All experimental procedures were conducted in strict adherence to the manufacturer's provided protocol instructions.

In vivo AChEI Activity

Sixty male Swiss mice were randomly assigned to six groups of 10 mice: Control (saline) (1), 1.5 mg scopolamine/kg/day (2), 5 mg AF/kg/day + 1.5 mg scopolamine/kg/day (3), 10 mg AF/kg/day + 1.5 mg scopolamine/kg/day (4), and 15 mg AF/kg/day + 1.5 mg scopolamine/kg/day (5), positive control (5 mg donezepil/kg/day + 1.5 mg scopolamine/kg/day) (6). The dosage was determined after our preliminary studies on a wide range of doses up to 25 mg/kg. The AF suspended in normal saline was orally and daily administered using a syringe while scopolamine dissolved in saline was intraperitoneally injected. The administration was performed at 8 am every day. After 30 days of treatment, mice were sacrificed by decapitation under ether anesthesia. The brains were promptly extracted and transferred to a chilled plate. In mice, AChE activity is primarily in the hippocampus and frontal cortex.[14] Therefore, they were then isolated, weighed, and homogenized in 0.1M phosphate buffer at pH 8.0, supplemented with 1% Triton. The homogenates underwent a 20-min-centrifugation at 15,000 × g, at 4°C. The transparent supernatant was preserved at −20°C. The total AChE assays were performed in triplicate using portions of brain homogenates. AChE activity was determined using the colorimetric method introduced by Khalil and Abass with slight adjustments to suit the measurement of enzyme activity in the supernatants of brain homogenates.[15]

Statistical Analysis

The experimental results were presented as the mean ± standard error of the mean, and statistical analysis was conducted using the statistical package for the social sciences (SPSS) 20.0 software package (SPSS Inc., Chicago, IL, USA). To assess variations among the batches, the one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were employed. A statistical significance was obtained when $P < 0.05$.

RESULTS

Acute Toxicity

Based on observations and calculations presented in Table 1, the LD_{50} value for the orally administered AF of HR was determined to be 90.2 (with a range of 76.12–106.89) mg/kg body weight. Based on the Hodge and Sterner toxicity scale, the LD_{50} value of the AF from HR falls within the moderately toxic range.[16]

In vivo Antioxidant Activity of the AF

After subcutaneous injection with D-galactose for 4 weeks, the plasma MDA level exhibited a notable rise ($P < 0.05$), while the SOD and GSH-Px levels displayed a significant reduction ($P < 0.01$) in the untreated aging group in comparison to the control group. This result confirms the successful establishment of an aging model using D-galactose.

Using the one-way ANOVA with a non-repeated factor, we found a statistically significant difference in serum MDA concentrations among the groups ($F[5, 59] = 4.741$, $P < 0.01$). Additional *post hoc* examination indicated that the untreated group, which received the subcutaneous D-galactose injection, exhibited markedly elevated serum MDA levels compared to the normal control group (Tukey test, $P < 0.01$). Both the administration of the HR AF and Vitamin E led to a statistically significant decrease in serum MDA levels when compared to the untreated group (Tukey test, $P < 0.05$ and < 0.01, respectively). The data, as shown in Figure 1, illustrates these results.

By the 28th experimental day, the untreated group exhibited a notable decline in serum SOD and GSH-Px levels in comparison to the normal control group (Tukey test, $P < 0.001$ and $P < 0.001$, respectively). However, administering Vitamin E and the HR AF at doses of 10 mg/kg and 15 mg/kg resulted in significant increases in serum SOD and GSH-Px levels (Tukey test, $P < 0.05$ and $P < 0.001$, respectively, for SOD, and $P < 0.01$ and $P < 0.001$, respectively, for GSH-Px). The data showing these findings is depicted in Figures 2 and 3.

Table 1: Results of the acute toxicity test

Groups	Dose (mg/kg)	Number of animals	Number of dead mice	Mortality rate (%)
1	300	10	10	100
2	250	10	10	100
3	200	10	10	100
4	160	10	10	100
5	120	10	8	80
6	90	10	6	60
7	70	10	2	20
8	55	10	0	0
9	30	10	0	0

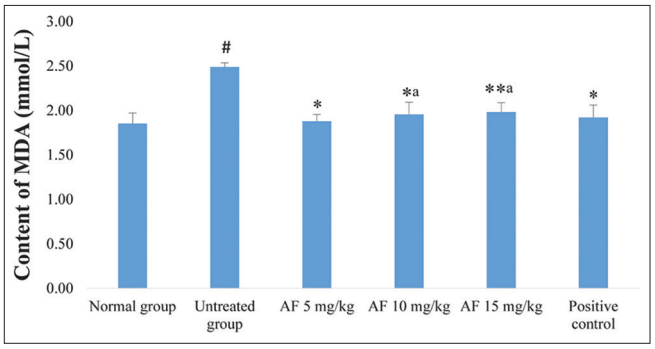

Figure 1: Effect of alkaloid fraction on the content of malonyl dialdehyde in serum in aging mice. (#): $P < 0.01$ versus normal control; (*): $P < 0.05$ versus untreated mice; (**): $P < 0.01$ versus untreated mice; (a): $P > 0.05$ versus positive control

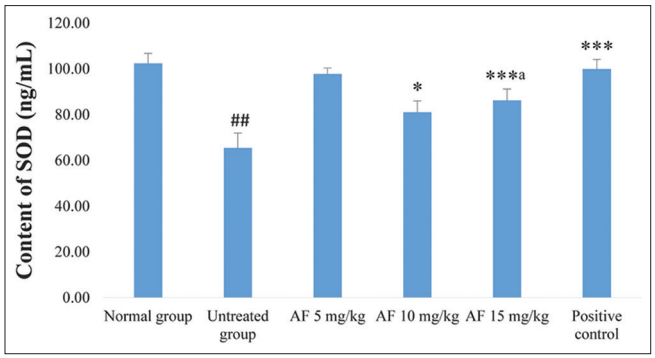

Figure 2: Effect of alkaloid fraction on the content of superoxide dismutase in serum in aging mice. (##): $P < 0.001$ versus normal control (*): $P < 0.05$ versus untreated mice; (***): $P < 0.001$ versus untreated mice; (a): $P > 0.05$ versus positive control

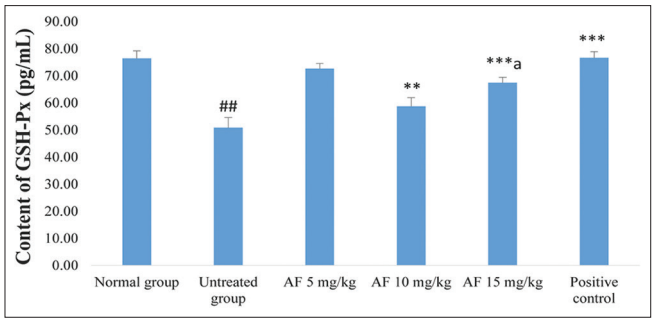

Figure 3: Effect of alkaloid fraction on the content of glutathione peroxidase in serum in aging mice. (##): $P < 0.001$ versus normal control; (**): $P < 0.01$ versus untreated mice; (***): $P < 0.001$ versus untreated mice; (a): $P > 0.05$ versus positive control

In vivo Inhibition of AChE Enzyme

Using the one-way ANOVA with a non-repeated factor, we observed a statistically significant variation in AChE activity in the hippocampal region of mouse brains among the study groups ($F[5, 59] = 12.223$, $P < 0.001$). Further *post hoc* analysis indicated that the untreated group (used scopolamine at 1.5 mg/kg/day) showed significantly higher AChE activity compared to the normal control group (Tukey test, $P < 0.001$). Furthermore, administering Donepezil at 5 mg/kg and the HR AF at 10 mg/kg and 15 mg/kg, led to remarkably reduced AChE activity compared to the untreated group (Tukey test, $P < 0.001$). The data illustrating these results are presented in Figure 4.

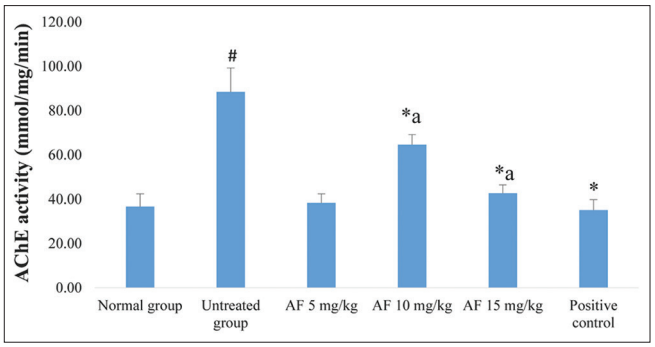

Figure 4: Effect of alkaloid fraction on acetylcholinesterase activity in aging mice. (*): $P < 0.01$ versus the scopolamine untreated mice; (a): $P > 0.05$ versus the positive control

DISCUSSION

At present, AChE inhibitors are widely recognized as the primary pharmacological approach for alleviating symptoms in cases of mild to moderate AD.[17] Many potent AChE inhibitors are sourced from natural origins, with a majority falling within the alkaloid chemical class, such as galantamine, extracted from *Amaryllidaceae* species. The search for natural compounds or extracts with AChE inhibition activity from the *Amaryllidaceae* family for AD treatment thus has continued, resulting in the discovery of several potential candidates.[9,10,18,19]

The *in vitro* and *in silico* anti-AChE activity of some alkaloids from the HR was reported but we have known little about *in vivo* effects of such alkaloids on AChE activity. In the study by Hoang *et al*., the AF of HR improved performance in spontaneous alternation tasks of mice in the Y-maze and shortened the exploring- novel-objects time at 15 mg/kg. In addition, it reduced the escape latency and swimming distance in the Morris water maze in scopolamine-induced memorial impairment mice at 10 and 15mg/kg.[10] Our study's findings on the HR AF align closely with the results from the article by Hoang *et al*. Both studies report significant improvements in memory and cognitive functions, as well as reductions in AChE activity, following administration of the AF.

The current study is the first to reveal the *in vivo* mechanisms of the AF of HR. In the same model of scopolamine-injected mice, our results showed that the AF, at 10 and 15 mg/kg, notably inhibited cerebral AChE activity. The cholinergic system is recognized for its significant contribution to preserving the integrity of brain networks and, consequently, its role in neurodegeneration.[20] Cholinergic signaling has been reported to inhibit the inflammation of neurons which is associated with impaired memory in AD. Moreover, the cholinergic system influences hippocampal memory processes by impacting neuroimmune functions.[21] The AChE downregulation increases the amount of acetylcholine in the synapse, resulting in improved attention, memory, and cognitive.[2] Therefore, our findings on the *in vivo* anti-AChE activity of the AF of HR in the scopolamine model explain its amelioration effects on memory and cognition observed in Hoang's study.

Our study demonstrated that the AF from HR significantly inhibited AChE activity in the scopolamine-induced cognitive deficit model in mice, with effects comparable to donepezil, a well-established AChE inhibitor used clinically for AD treatment. Specifically, the AF at doses of 10 mg/kg and 15 mg/kg significantly reduced AChE activity, and the inhibition efficacy at these doses was comparable to that of donepezil, highlighting its strong potential as a natural therapeutic agent.

When compared to other natural AChE inhibitors, such as galantamine (also derived from the *Amaryllidaceae* family), our findings align closely with the literature, which supports the significant AChE inhibitory effects of alkaloids from this botanical family. Galantamine has been extensively studied and is FDA-approved for treating mild to moderate AD,[22] suggesting that the HR AF may share similar pharmacological properties.

In contrast to some other natural AChE inhibitors, such as those derived from herbs, such as *Ginkgo biloba* or *Bacopa monnieri*, which exhibit moderate AChE inhibition,[23,24] the AF from HR demonstrates more robust efficacy in the specific models tested. This difference may be attributed to the specific alkaloid content in HR, particularly the presence of potent AChE inhibitors similar to galantamine.

An important factor in implementing this *in vivo* model is the selection of an appropriate dose of scopolamine. The study by Flood and Cherkin indicated that a scopolamine dose of at least 0.1 mg/kg body weight is required to begin inducing cognitive and memory impairments.[25] In practice, there is significant variability among authors regarding the scopolamine dose used, ranging from 0.1 to 3 mg/kg body weight. Based on recent publications related to the scopolamine-induced memory impairment model, this dissertation chose a scopolamine dose of 1.5 mg/kg to evaluate the memory and cognitive-enhancing effects of the AF from the species HR.

The imbalance between the production and quenching of free radicals from oxygen species is considered a risk factor for AD.[2] Furthermore, oxidative stress is also an important cause of memory and cognitive decline. Recent research indicates that oxidative stress plays a crucial role in facilitating neurodegeneration within the context of AD.[26] Therefore, the ability of improving memory and cognitive impairment of AD remedies may partly be related to their antioxidant effects. That is the reason for the common use of a simple aging model induced by D-galactose for the testing of neuroprotective herbs.[27-29] In this study, the extended D-galactose administration induced an oxidative stress condition characterized by an excessive generation of MDA, which resulted from the polyunsaturated fatty acids peroxidation, along with a reduction in enzymes that scavenge or neutralize free radicals, such as GSH-Px and SOD. MDA, SOD, and GSH-Px are indicators related to oxidative processes in the body. SOD and GSH-Px play crucial roles in eliminating free radicals, helping to protect the body. Elevated levels of these enzymes indicate effective antioxidant activity. Conversely, MDA is one of the end products of the peroxidation of unsaturated fatty acids in cells. Increased free radicals lead to excessive production of MDA. Elevated MDA levels in the blood are a marker of increased oxidative stress.

Treatment with either the AF at 10 mg/kg and 15 mg/kg or Vitamin E significantly decreased the MDA level and increased the SOD and GSH-Px levels while the effect of the AF at 5 mg/kg was not statistically obvious. Notably, previous studies have demonstrated that Vitamin E and Vitamin C effectively counteract oxidative disorders in aging animal models.[30] Therefore, we used Vitamin E as a positive control for antioxidant treatment. The results showed that the HR AF, especially at a dose of 15 mg/kg, exhibited antioxidant activity comparable to that of Vitamin E at a dose of 50 mg/kg. These findings highlight the significant anti-aging effects of the HR AF in the experimental animal model. This is the first report on the antioxidant activity of HR. However, the *in vitro* antioxidant activity of lycorine from some other *Hippeastrum* species in the DPPH assay has been reported,[31] so our results on the *in vivo* antioxidant of HR alkaloids might have an association with lycorine. There should be further studies on the specific components in the AF that are determinants in the antioxidant activity. In addition to evaluating antioxidant indices in plasma, these indices can also be assessed in brain tissue in further studies to clarify further the effects of the AF extract in treating AD.

This activity of the HR AF pivotally contributes to its beneficial effects on memory and cognition and facilitates its use in patients with AD.

However, an important issue raised when using alkaloids for human use is their potential toxicity. The LD_{50} of 90.2 mg/kg of the HR AF ten folded the dose with *in vivo* anti-AChE and antioxidant effect, assuring its safety for human use. So far, there has been no report on the acute toxicity of HR or its compounds.

Our study has an important limitation in the experiment on D-galactose-induced aging mice, the antioxidant markers were determined in plasma. Further experiments should be done to reveal the *in vivo* antioxidant activity of the HR AF with assays of brain antioxidant markers for the exploration of its specific effects relating to AD. In addition, to translate these results into human use, it's essential to test the HR AF on other Alzheimer's models or neurodegenerative disorders to confirm its benefits.

CONCLUSION

In summary, this is the 1st time the *in vivo* AChE inhibitory effect and the *in vivo* anti-aging effect of the AF from HR have been reported. Our research showcased the *in vivo* AChE inhibitory and antioxidant effects of the alkaloid extract of HR at 10 and 15 mg/kg. In addition, the LD_{50} value for the HR AF was determined to be 90.2 mg/kg. These findings strongly advocate the safety and potential subject of HR for research and development of drugs for the treatment of AD.

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CONFLICTS OF INTEREST

No conflict of interest was declared.

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