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EVALUATING IN-VITRO ANTIOXIDANT AND ANTICANCER POTENTIAL OF AN INDIGENOUS PLANT OF MEGHALAYA

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ABSTRACT

Objective: A perennial herb of Meghalaya origin, Anemone rivularis Buch.- Ham. ex DC., belongs to the family Ranunculaceae, was traditionally used for the treatment of inflammation and cancer. This study aims to evaluate the in-vitro antioxidant and anticancer property of Anemone rivularis methanolic extract through free radical scavenging assays and anticancer activity against ovarian cancer cell line.

Method: The previously dried leaves were processed for extraction by using maceration and hot percolation technique, followed by 2,2-diphenyl-1-picryl hydrazyl radical scavenging activity (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity **(**ABTS),Total Flavonoid Content (TFC)and Total Phenolic Content (TPC), and cell viability by 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay (MTT) and apoptosis study using flow cytometry analysis.

Result: The results revealed that the plant is a high source of antioxidants as it showed very potent inhibition towards free radicals. The anticancer effect by MTT assay were evaluated on the viability of the human ovarian cell line (PA-1). The results showed that as the concentration of the extract increases, the percentage of cell viability of the PA-1 cell line decreases. Further apoptosis study using flow cytometry method revealed that methanolic extract dose dependently induced apoptosis in PA-1 cell. Methanolic extract treatment significantly promoted early apoptosis when compared with control.

Conclusion: These results suggest that A. rivularis possess antioxidant and anticancer properties and thus justify the traditional use for the treatment of anticancer diseases. However, research on in-vivo methods along with toxicity study is recommended to validate the safety for society.

KEYWORDS: Anemone rivularis; Antioxidant; Anticancer; Extraction; Apoptosis

INTRODUCTION

Natural products, which are renowned for their distinctive chemical variety and bioactivity, have continued to provide prototypes for the creation of new therapeutic scaffolds. However, natural products refer to secondary metabolites created by any living thing include the byproducts of overflow metabolism caused by nutritional constraint, defense mechanisms, or regulator molecules. They are frequently restricted to a small set of species in a phylogenetic group. They seem to play an important role in plant defense against and other interspecies defense mechanisms. Natural products continue to be used widely to treat a variety of disorders, since they have played a significant part in ancient traditional medicine systems including the Chinese, Ayurveda, and Egyptian systems.

Cancer is a serious health disease and the second-leading cause of death worldwide. Traditional medicinal plants have a vital influence in the development of anticancer medications and largely being utilized in developing nations. In comparison to synthetic drugs, ethnomedical plants have demonstrated better pharmacological effects. Breast, lung, skin, blood, and liver cancers are among the most prevalent forms of cancer. Currently, radiotherapy, chemotherapy, and malignant surgery are used to treat cancer. Compared to synthetic drugs, ethnomedicines are generally better tolerant and more effective. Commonly utilized anticancer plants throughout the world which further leads to pharmaceutical industries for manufacture of unique anticancer medications from traditional plants [1].

A serious cancer of the female reproductive system, ovarian cancer is currently the eighth most common cancer diagnosed worldwide. Given the modest nature of early symptoms, the hidden location of ovarian cancer in the body, and the lack of effective early screening methods, the majority of patients are usually diagnosed with advanced-stage illness at presentation. However, advancements in imaging and liquid biopsy technologies should increase the likelihood of ovarian cancer being detected early and offer more options for therapy [2].

The genus Anemone has more than 150 species of flowering plants belonging to the family Ranunculaceae and native to the temperate zones of both Northern and Southern hemispheres. The Species are characterized with rhizomes, basal leaf blades from entire to ternate, tepals which are sparsely pubescent (sometimes glabrous),

achenes without projections, glabrous or subglabrous. Anemone rivularis has as many as 20 triterpenoid saponins showing anti-inflammatory, antitumor activity making it a key asset of saponin compounds with pharmacological properties in traditional medicine [3]. The roots of the plant have been used as an antitumor agent in Chinese traditional medicine [4]. Over the last few decades, ethnobotanical field surveys and pharmacological studies have been conducted to know more about the medicinal properties of A. rivularis viz. antioxidant, antibacterial, antitumor, anti-inflammatory properties of the species. The species exhibit an extensive medicinal applications including cancer, rheumatoid arthritis, diabetes, malaria, snakebite, dysentery and wound healing [3].

Plant Profile

Kingdom: Plantae
Class: Magnoliopsida
Order: Ranunculales
Family: Ranunculaceae
Genus: Anemone
Species: rivularis Buch.-Ham. ex. DC.

MATERIALS AND METHODS:

I) Extraction: The leaves of the plant A. rivularis were washed with tap water to remove all foreign debris and finally with filter water and then cut and dried. After that the dried leaves were extracted with methanol by using Maceration Technique and Hot Continuous Percolation method by Soxhlet Technique for 72 hours and 18hours respectively. After collection of both the extract it was stored at 4℃ for further purpose. [6]

II) Determination of in-vitro antioxidant activity:

A) Determination of 2,2-diphenyl-1-picryl hydrazyl radical scavenging activity (DPPH): The free radical scavenging activity of the plant sample and ascorbic acid as positive control was determined by using the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl). 1 mg of the powdered sample dissolved in 1 ml of solvent (methanol). From the stock solution (1 mg/ml) different concentrations 10, 150, 350 and 500 µg/ml were prepared by using methanol for dilution. 1.5 ml of each diluted sample was taken in different test tubes followed by addition of 1.5 ml DPPH. The final volume of all the test tubes was made up to 3 ml with methanol. After that all the test tubes were incubated for 5 minutes at room temperature in dark and absorbance for all the tubes were measured at 517 nm. The percentage inhibition was calculated using equation: % DPPH scavenging activity = 100 x (A control – A sample)/A control)

B) Determination of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)radical scavenging activity (ABTS): The free radical scavenging capacity of the sample and ascorbic acid was determined by reduction of ABTS radicals. 1 mg of the powdered sample dissolved in 1 ml of solvent (methanol). From the stock solution (1 mg/ml), different working solutions of 10, 150, 350 and 500 µg/ml concentrations were prepared by using methanol for dilution. After that test sample about 300 µl was mixed with ABTS around 3 ml and the final volume of all the test tubes was made up to 4 ml with methanol. After that the decrease in absorbance in all the test tubes were measured exactly one minute after mixing the solution, then up to 6 min. Finally absorbance for all the tubes was measured at 745 nm. The percentage inhibition was calculated according to the formula:

Scavenging effect (%) = (A control – A sample)/A control) x 100

C) Determination of total phenolic content (TPC): The total phenolic content was determined by using Folin-Ciocalteu phenol reagent method. From the stock solution (1mg/ml), 1 ml was taken in test tubes (in triplicate), then 1.5 ml of 10 % Folin-Ciocalteu reagent was added to all the test tubes, 1 ml of 7.5% Na_2CO_3 was added to the solution and mixed well by vortexing making the final volume of the tubes to 3 ml with methanol. After that all the test tubes were incubated for 15 minutes at 45 ºC in the water bath. Finally, absorbance for all the tubes were measured at 765 nm using UV-VIS spectrophotometer. In the same manner, different concentrations of Standard Gallic acid 25, 50, 100 and 200 µg/ml were prepared. The absorbance of the solutions was taken against the blank at 765 nm using UV-Visible spectrophotometer. The total phenol content was expressed in GAE (mg/g) of extract that calculated as given in equation -

$$T = \frac{C \times V}{M}$$

Where, T = Total phenolic contents, mg/g of the test sample in Gallic Acid Equivalent (GAE), C = Concentration (mg/ml) of Gallic acid obtained from the calibration curve, V= Volume of the test sample(ml), M=Weight (g) of test sample.

D) Determination of total flavonoid content (TFC): About 0.25 mg of dried powdered extract was taken in a test tube and 1.25 mL of water was added (done in triplicates). 750 µl of sodium nitrate was added and mixing them using by vortexing. All the test tubes were placed in dark for 6 minutes. Then, 150 µl of 10% aluminium chloride was added into the test tubes and again incubated for 5 minutes in dark for the complete reaction to occur. Finally, 500 µl of 5% sodium hydroxide and 275 µl water was added to the test tubes, making the final volume of all the tubes to 3 ml using methanol. The absorbance was measured for all the tubes at 510 nm. Quercetin was used as standard and prepared in a concentration of 1 g/ml in methanol in a total volume 10 ml. It was diluted by adding methanol to prepare working standard as 25, 50, 100 and 200 µg/ml concentration for use. The total flavonoid content of the test sample was measured by utilizing the given following equation-

$$T = \frac{C \times V}{M}$$

Where, T = Total flavonoid content, mg/g of the test sample, C = Concentration (mg/ml) of quercetin obtained from the calibration curve, V= Volume of the test sample(ml), M=Weight (mg) of test sample.

III) In-vitro anticancer activity of methanolic extract against ovarian cancer cell line:

A. Cell line and culture: Human Ovarian Cancer cell line (PA-1) was obtained from National Center for Cell Sciences (NCCS), Pune. The cells were cultured in Minimum Essential Medium (MEM, HiMedia), which was supplemented with 10% Fetal Bovine Serum (Gibco), 1% Sodium Bicarbonate, 1% Sodium pyruvate (HiMedia) and 1% MEM Nonessential amino acids (HiMedia). The cells were maintained in a humified 5% CO_2 atmosphere at 37°C.

B. In -vitro anticancer activity by 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay: PA -1 cells were seeded (2×10^2 cells per well) in 96 well plate. The cells were incubated for 24 hours and the cells were treated by methanolic extract (100-1000 µg/ml concentration). The cells were treated with sample drug and incubated for 24 hours. The 100µl of complete media contain MTT (0.5mg/ml) was added to each well and the cells were incubated for 4 hours and the media was carefully removed and the formazan crystals were dissolved in 70µl DMSO for 30mins. The absorbance was then obtained on a microplate reader at 570 nm and the IC_{50} value was calculated.

C. Apoptosis study: To quantify the number of apoptotic and necrotic cells, PA-1 ovarian cancer cells (2×10^5) were seeded in each well of 6 well plates and grown in DMEM media for 24 hundred standard cell culture conditions. The cells were then treated with the IC_{50} of methanolic extract, while untreated cells served as control. The further analysis was performed by using Annexin V- FITC Apoptosis Detection Kit I and the protocol was followed based on manufacturer instructions. The fluorescent labelled cells were then analyzed in flow cytometry (DxFLEX, Beckman Coulter). Unlabelled cells were used to negate theauto fluorescence produced by the cells.

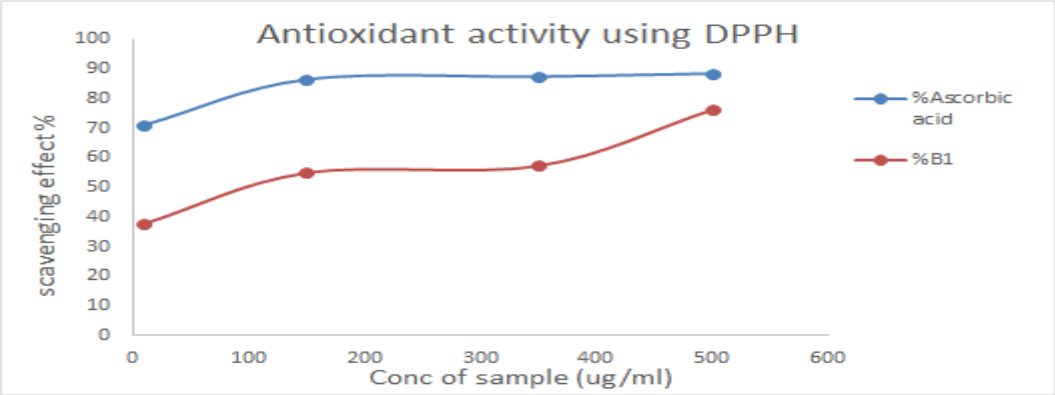
RESULTS

I) Extraction: The methanolic extraction was carried out by using maceration and hot percolation (Soxhlet techniques). The percentage yield for maceration and hot percolation were found to be 66.67% and 53.33% respectively.

II) Determination of in-vitro antioxidant activity :

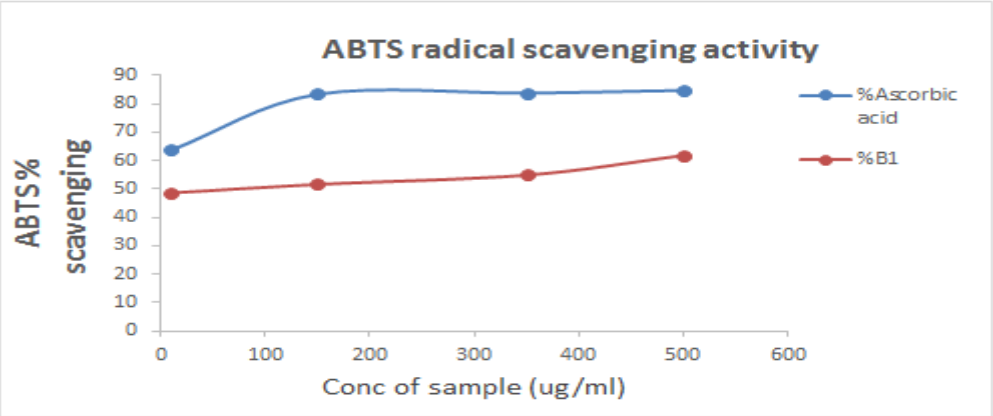
A) Determination of 2,2-diphenyl-1-picryl hydrazyl radical scavenging activity (DPPH): DPPH is a free radical which is stable at room temperature and this method is often employed to evaluate the antioxidant activity of many plant extracts. The concentration in µg/ml of the sample to scavenge 50% of the DPPH radical is called IC_{50} and lower IC_{50} values indicate higher DPPH activity. The IC_{50} of A. rivularis extract is shown in the following fig 1 as compared to standard (ascorbic acid). The result indicates that A. rivularis exhibit DPPH activity, however, the standard ascorbic acid showed significantly higher anti-oxidant activity. The graph below shows the concentration of 500 µg/ml had high antioxidant activity as compared to the other concentration.

Fig: 1 Graph showing antioxidant activity exhibited by scavenging DPPH radical



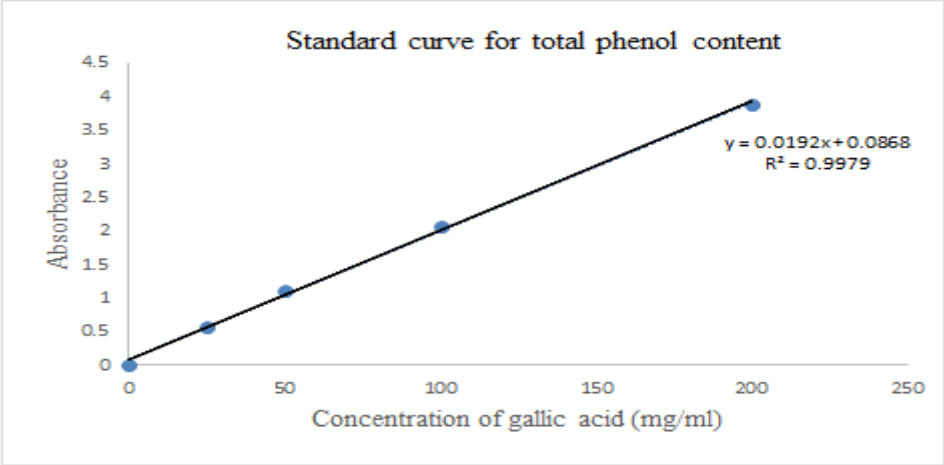
B) Determination of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid radical scavenging activity (ABTS): The ABTS assay showed the antioxidant activity of methanolic extracts by scavenging the ABTS generated in aqueous phase and the values are expressed as IC_{50} when compared with ascorbic acid as standard. The graph below represented that the concentration at 500 µg/ml showed high activity than the other concentrations. The value of IC_{50} were shown in the fig 2 as compared to the ascorbic acid (standard).

Fig: 2 Graph showing antioxidant activity exhibited by scavenging ABTS radical



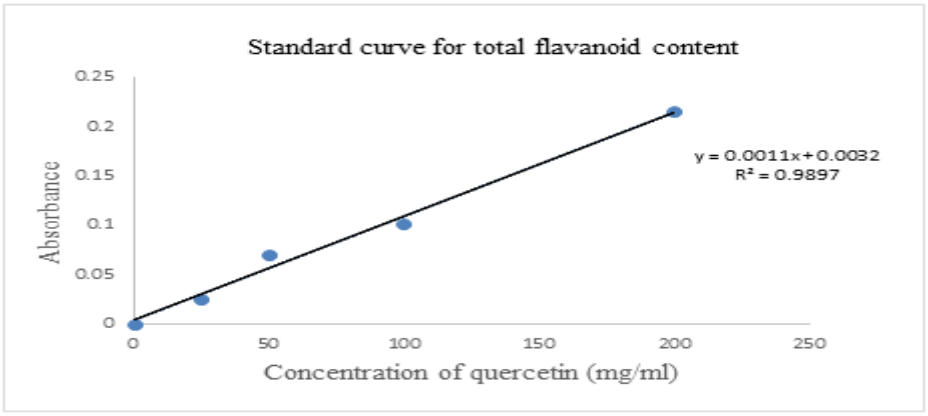
C) Determination of total phenolic content (TPC): The TPC was expressed as mg of Gallic acid equivalent/gm and was determined by the Folin-Ciocalteu method. The high amount of phenolic compound present in plant extract show great potency. The total phenol content was found to be 155.80 mg GAE/gm of extract. The standard gallic acid curve was shown in fig 3.

Fig: 3 Gallic Acid Standard Curve



D) Determination of Total Flavonoid Content (TFC): Flavonoid is an important secondary plant metabolite which is produced by plant for their defense mechanism. The Flavonoid content was found to be 955.27 mg quercetin/gm of extract. The standard quercetin curve was shown in fig 4.

Fig:4 Quercetin standard curve



III) In-vitro anticancer activity of methanolic extract against ovarian cancer cell line:

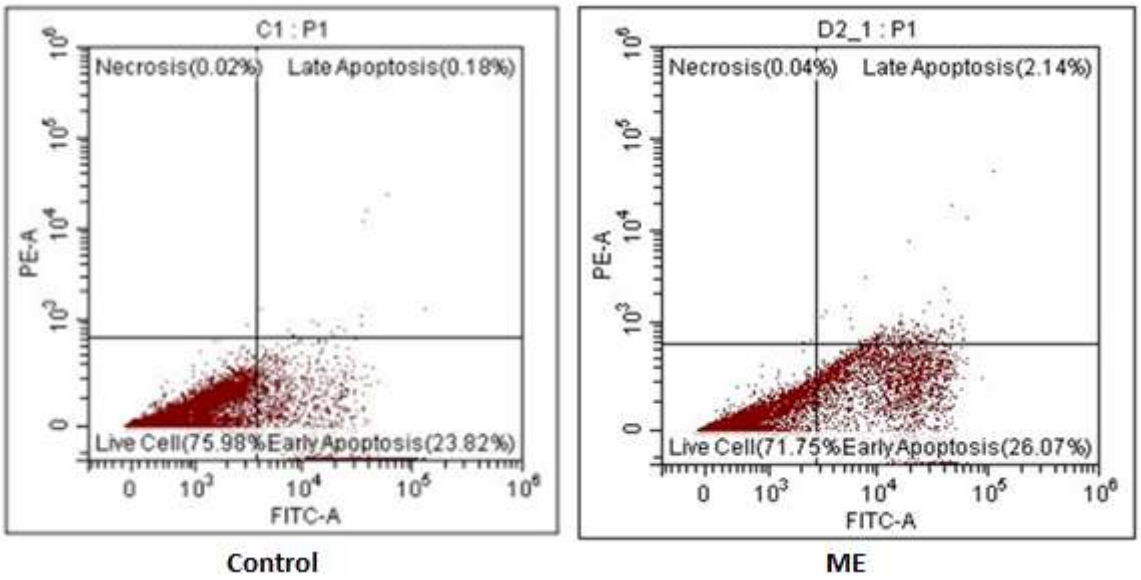
B) In -vitro anticancer activity by 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay: In this study, the anticancer effects of methanolic extract (ME) were evaluated in-vitro on the viability of the human ovarian cell line (PA-1). Various concentrations of extract were ranging from 100 to 1000 µg/ml were used for testing. The results showed that as the concentration of the extract increases, the percentage of cell viability of the PA-1 cell line decreases. The lowest percentage of cell viability was found in 1000 µg/ml. The IC_{50} value was found 670 µg/ml (Table 1).

Table 1: Anticancer effect of methanolic extract on PA-1 cell line

Concentrations (µg/ml)	Average value	% cell viability
Control	0.5296	100
100	0.4819	90
200	0.46767	88
300	0.433	81
400	0.39543	74
500	0.34967	66
600	0.2848	53
700	0.26183	49
800	0.2253	42
900	0.2132	40
1000	0.1944	36

C) Apoptosis Study: Elucidation of apoptosis contribution to the growth inhibitory effect of PA-1 cell was exposed to methanolic extract at their respective IC_{50} doses for 24h. The cells were stained with Annexin V- FITC Apoptosis Detection Kit I staining and examined by flow cytometry as shown in fig 5. The results indicated that methanolic extract dose dependently induced apoptosis in PA-1 cell. Methanolic extract treatment significantly promoted early apoptosis when compared with control.

Fig.5: Flow cytometry analysis of apoptosis in sample drug treated in PA-1 Ovarian cancer cell line



DISCUSSION

The methanolic leaf extract of A. rivularis exhibited potent antioxidant activity, confirmed by DPPH, ABTS radical-scavenging effects and elevated total phenolic (TPC) and flavonoid (TFC) content which was accompanied by in vitro cytotoxicity studies. These results, along with the identification of phytoconstituents such as phenolic compounds, flavonoids, triterpenoids, saponins, tannins and steroids highlight the promising potential of A. rivularis for application as an anticancer agent. The results for free radical scavenging assays (DPPH and ABTS) suggest that A. rivularis exhibit free radical scavenging activity when compared with standard ascorbic acid.

The methanolic extract further provide evidence on the anticancer efficacy of the plant species. The methods used were MTT assay against human ovarian cell line (PA-1) and apoptosis study through flow cytometry method. The results suggest that these methanolic extracts could be used for management of cancerous diseases in ovary. The extract (MTT assay) showed that as the concentration of the extract increases, the percentage of cell viability of the PA-1 cell line decreases. The IC_{50} value was found to be 670 µg/ml. For apoptosis study the results indicated that methanolic extract dose dependently induced apoptosis in PA-1 cell. The treatment of methanolic extract significantly promoted early apoptosis when compared with control.

These findings validate the use of A. rivularis as an anticancer drug and traditional medicine. The finding of this study reported potent anticancer effect of methanolic leaf extracts of A. rivularis on human ovarian cell line (PA-1). This justified the traditional use of the plant for the treatment of cancerous disease. The literature survey done on the plant species elicited no previous research work on the anticancer activity of the crude extracts of leaves of A. rivularis from the Northeastern state Meghalaya. As such, this could be the first report on such activity and could be a start point for future drug research on different cancerous cells.

CONCLUSION

This study highlights the effectiveness of the methanolic extract of the leaves of A. rivularis, which were collected based on existing literature and has justified the ethnomedicinal properties that were used by traditional practitioner in China for the treatment of inflammation and cancer related condition. Traditional knowledge remains a valuable foundation for drug discovery, and this research provides scientific support for such applications. The extract demonstrated notable free radical scavenging activity and was rich in bioactive compounds, as indicated by antioxidant assays and the quantification of total phenolic and flavonoid content. In vitro cytotoxicity analysis showed that the organic extracts possess significant anticancer activity, inducing apoptosis in cancer cells. The IC_{50} value against the PA-1 cell line was recorded at 670 µg/ml. Overall, these findings support the ethnomedicinal use of A. rivularis and suggest its potential as a source for natural anticancer agents. Further in-vivo research is needed to explore its therapeutic potential and societal benefits.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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