Original Article

Evaluation of the cytotoxic, anti-proliferative, anti-metastatic and pro-apoptotic effect of aqueous leaf extract of *Annona muricata* on oral tongue squamous cell carcinoma cell line (SCC-15): An *in vitro* study

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Abstract

Background: Oral cancer still represents the leading cause of mortality in India. Due to the drawbacks of current treatment options, a safe, low-cost therapy is the need of the hour. Recently, novel plant extracts with anti-cancer properties have gained greater attention. One among them is Annona muricata and its leaf extract, which has been studied for its anti-cancer effect against various cancers. However, studies on oral cancer cells are very much limited and hence the study.

Aims: To evaluate the cytotoxic, anti-proliferative, anti-metastatic and pro-apoptotic effect of aqueous leaf extract of Annona muricata (ALEAM) against SCC-15 cell lines through *in vitro* assays.

Materials and Methods: *In vitro* assays such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], colony formation and wound healing assays were performed. Furthermore, to evaluate the underlying mechanism, gene and protein expression analysis of apoptotic/anti-apoptotic marker genes Bax, P53 and Bcl2, were done using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analysis. Student's *t*-test has been performed for analysis of experimental data.

Results: The results showed that ALEAM exhibited significant cytotoxic activity in a dose-dependent manner as well as inhibited colony formation and cell migration. The pro-apoptotic properties were affirmed by a highly significant drop in Bcl-2 gene expression and a highly significant rise in P53 and Bax genes in the study group compared to the control ($P < 0.05$).

Conclusion: The current study provides evidence that ALEAM has the potential to be developed as a novel anti-cancer drug for the treatment of SCC after further clinical studies.

Keywords: Annona muricata, anti-cancer, cell lines, *in vitro*, oral squamous cell carcinoma

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INTRODUCTION

Across the globe, oral cancer remains a public health issue, especially in developing nations. There are significant geographic differences in occurrence, with some areas on each continent (such as India, Pakistan and Sri Lanka in Asia) being particularly impacted. Despite the oral cavity being widely accessible for inspection, it is reasonably a regular finding in the scientific literature that roughly half of the oral malignancies are discovered at advanced stage.[1]

Unfortunately, the significant therapeutic advancements employed in recent decades have not sufficiently offset the detrimental impact of the disease with no notable gains in overall survival for this neoplasm. Furthermore, in recent years, apart from the known risk factors, such as tobacco use, alcohol consumption and HPV infection, the involvement of lesser-known hazards, such as BMI (body mass index), food, oral health, socioeconomic position, occupation and family history (genetics), may also contribute to the development of this illness.[2]

Conventional treatment options for advanced-stage oral cancer include surgery and radiotherapy with or without chemotherapy. They always have an impact on normal healthy tissues leading to deleterious effects.[3] Also, the concept of field cancerization states that there is about 20% chance of the development of a second primary tumour in cases even after histopathologically clear surgical margin.[4]

The natural process through which cells die, called apoptosis, is a possible target for cancer treatment. The apoptotic pathway is often suppressed in cancer through a variety of mechanisms. The up-regulation of anti-apoptotic B-cell leukaemia/lymphoma 2 protein (Bcl2) and down-regulation of Bcl-2-associated X protein (Bax) and/or Bcl-2 antagonist/killer (Bak) proteins are the ways by which cancer cells evade apoptosis.[5]

Indigenous medicine serves as the representative in determining their potential as a hotspot for future pharmaceuticals. *Annona muricata*, otherwise known as a prickly custard apple, is one among these plant species that grow in our backyard and has received a lot of attention recently due to its many medicinal properties, particularly anti-cancer activity.[6]

Previous studies have shown that *Annona muricata* leaves had the most legitimate cytotoxic effect against a variety of cancer cell lines such as breast, prostate, pancreas, lungs, colon, liver, head and neck that triggered apoptosis via different mechanisms.[7-9]

However, studies on oral squamous cell carcinoma have been remarkably few. To the best of our knowledge based on the literature, the current research is the first to evaluate the gene and protein expression levels of ALEAM-treated SCC-15 cell lines.

In light of this context, the current work was proposed to assess ALEAM's anti-cancer capacity by evaluating the cytotoxic, anti-proliferative and anti-metastatic potential and to determine its probable mode of action executed through the apoptotic pathway. Therefore, the current study might serve as a starting point for the discovery of future state-of-the-art anti-cancer agent.

MATERIALS AND METHODS

The study was approved by the Institutional Ethical Committee of Sree Mookambika Institute of Medical Sciences, Kulasekharam, Tamilnadu dated 25/03/2021.

Study design

In vitro experimental study

Plant material

Fresh leaves of *Annona muricata L.* were harvested (8°21'32.7"N and 77°15'56.7"E), and a voucher specimen (reference: BSI/SRC/5/23/2021/Tech/301) was maintained at the institution after being authenticated by Scientist-E and Head of Office, Botanical Survey of India. The leaves were completely cleaned in fresh water, shade-dried for three weeks, and then pulverized to fine powders that were then kept at ambient temperature until used.

Extraction of *Annona muricata* aqueous leaf extract

ALEAM was prepared as reported in previous studies.[10] Separately weighed 100 grams of plant leaf powder were added to 1000 ml of double-distilled water, and the mixture was left at 25°C for 72 hours with periodic stirring. The final mixtures were filtered and sieved through Whatman N° 1 filter paper. The procedure was carried out three times, and after each 72-hour interval, the filtrates were concentrated by rotary evaporation under decreased pressure, and the aqueous extracts were lyophilized. In order to attain stable weights, the concentrates were air-dried. The extract residues were collected and kept at 4°C for storage. It was solubilized with 50 µl of dimethyl sulfoxide (DMSO) to make the stock solution.

Cell culture

The SCC-15 cell line was selected and purchased from the American Type Culture Collection (ATCC CRL-1623). The cells were grown in T25 (25 cm^2) cell culture flasks

coated with polystyrene. The culture medium used was Dulbecco's modified Eagle's medium (DMEM). This growth media was supplemented with 10% foetal bovine serum (Sigma-Aldrich, St. Louis, Mo, USA), phenol red (Thermo Scientific, New Delhi, India) and 1% penicillin-streptomycin (Sigma, New Delhi, India) and were maintained at 37ºC in a humidified atmosphere containing 95% air and 5%CO_2.

Cytotoxicity assay

MTT assay was used to evaluate the cytotoxic effects of ALEAM. Different concentrations of the extract-dilutions taken, i.e. 10, 20, 100, 200, 500 and 1000 µg/ml, were exposed to the 96-well plate containing the monolayer of cells for about 24 hrs. Next, the media was aspirated with phosphate-buffered saline (PBS) and the cells were washed. To each well, 0.5 mg/ml of MTT solution was added and the plate was incubated at 37°C for 4 h in the dark. The plate was shaken for 5 min at 150 rpm, and then, an enzyme-linked immunosorbent assay reader was used to record the absorbance at 570 nm. The result was calculated in triplicate, and the percentage of cell inhibition was calculated as mentioned in previous studies.[11]

Clonogenic assay

The effect of ALEAM on colony formation was evaluated in parallel with its control. In 12-well plates, SCC-15 cells were seeded and then treated with ALEAM. The treated cells were maintained in a humidified incubator at 37°C and 5% CO_2. After culturing for 14 days, the medium was discarded, and cells were washed with PBS. Next, 4% paraformaldehyde is used to fix the cells at room temperature for 30 min and then stained with 0.1% crystal violet for 30 min. Following this, the crystal violet was removed and the plates were air-dried. Clones with more than 50 cells were counted under a microscope (Labomed, USA). The experiment was done in triplicate.

Wound healing assay

To detect the effect of ALEAM on the migration of the SCC-15 cells, we performed a wound healing assay. SCC-15 cells were seeded into 6-well plates; a wound was made through the monolayer using a 200-µL pipette tip. Then, control and ALEAM SCC-15-treated cells were maintained in a humidified incubator at 37°C and 5% CO_2 for 24 hours. All cellular debris was removed by washing with PBS. Images of the scrape line were taken at both 0 and 24 hours. Then, the wound closure rates between the control and treated groups were compared using ImageJ analysis software and the percentage of wound closure relative to the initial wound size was calculated.

Gene expression analysis using qRT-PCR

TRIzol (Invitrogen, Waltham, MA, USA) was used to extract total RNA from treated and untreated cells. The total extracted RNA (1 g) was reverse transcribed using Takara's (Japan) Prime Script 1st Strand cDNA Synthesis kit. Real-time PCR was carried out in the Bio-Rad CFX96 Real-Time PCR Machine using the IQ SYBR Green PCR master mix kit (Thermo Fischer Scientific, India). The primer sequences were tabulated [Table 1]. The procedure for RT-PCR was as follows: 40 cycles of 94°C for 30s and 55°C for 30s were performed following 95°C for 5 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as an internal control in the calculation of the fold changes in gene expression between the control and treated samples.

Protein expression analysis using western blotting

After treatment of SCC-15 cells with ALEAM, the cells were collected and lysed with radio immune precipitation assay (RIPA) buffer (Sigma-Aldrich, USA). Protein concentration was measured using a Bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, India) with standard protein concentrations of Bovine Serum Albumin (BSA). The experiment was done according to the manufacturer's instructions. Finally, a chemiluminescent substrate was used (ECL, Bio-Rad) and the protein bands were visualized using a chemi-doc imaging system (Bio-Rad). The protein bands were analysed, and the protein expression levels were quantified using Image Lab Software (Bio-Rad).

Statistical analysis

The results were expressed as mean ± standard deviation using Statistical Package for Social Sciences (SPSS) 20.0 software. All graphs represent the mean and standard

Table 1: The sequences of primers used for amplification

Primers	Sequences	
	Forward primer	Reverse primer
Bcl-2	5'-TAGCGCTTCAGGCAGCCACA-3'	5'TTCCAAACCCGGTGAGGAGGCT-3'
Bax	5'- AGCGAGTGTCTCCGGCGAAT-3'	5'-ACGCGGCCCCAGTTGAAGTT-3'
P53	5'-TGCGTGTGGAGTATTTGGATG-3'	5'-TGGTACAGTCAGAGCCAACCTC-3'
GAPDH	5'-CGCTTCCTTACCTGGTTGAT-3'	5'-GAGCGACCAAAGAACCATA-3'

variations of at least three independent experiments, and Student's *t*-test has been performed for experimental data. Statistically significant difference was considered with *P* value ($P < 0.05$).

RESULTS

Cytotoxic effects of ALEAM on SCC-15 cell line

MTT assay results revealed that ALEAM showed significant cytotoxic effects with an IC_{50} value of 857.90 µg/ml, which was observed through dose-dependent inhibition of the growth of SCC-15 cells [Table 2]. Linear regression curve is used to determine the IC50 value using the straight-line equation y = mx + c (y denotes % inhibition of SCC-15 cells, m represents the slope of linear regression curve, x is concentration of *ALEAM,* and c is the Y-intercept). [Figure 1].

Anti-proliferative and anti-metastatic effect of ALEAM on SCC-15 cell line

Figures 2 and 3 show the photomicrograph and bar chart representing the inhibition of colony formation and cell migration of SCC-15 cells following treatment with ALEAM compared to those of control cells.

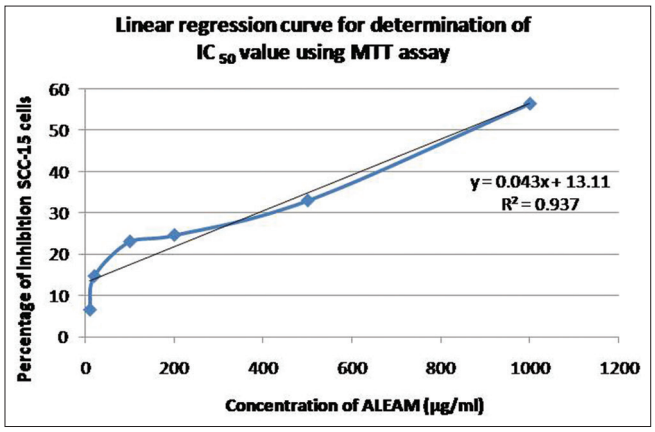

Figure 1: Extrapolation of the linear regression curve at half-maximal inhibition of SCC-15 cells using the straight-line equation, y = mx+c. Correlation coefficient (R^2) value of 0.937 implies a strong positive correlation between % inhibition of SCC-15 cells and concentration of *ALEAM*

Pro-apoptotic effect of ALEAM on SCC-15 cell line

The expression levels of apoptosis-related genes and proteins Bax, Bcl2 and P53 were examined to determine the molecular pathways of ALEAM-induced apoptosis in SCC-15 cells. The anti-apoptotic Bcl-2 gene was shown to be negatively regulated, whereas the pro-apoptotic genes, Bax and p53, were found to be up-regulated when treated with ALEAM [Figure 4]. Additionally, Western blot analysis was performed to confirm the relative protein levels of Bax, Bcl2, P53 and their cleaved fragments. The results were statistically significant with a *P* value of < 0.05 as compared with the control [Figure 5].

DISCUSSION

Natural plant products have made an enormous influence on drug development and, consequently, on the society as a whole. One such outstanding example is the discovery of vinca alkaloids from the leaves of the plant Madagascan periwinkle (Catharanthus roseus L.) to treat haematological malignancies.[12]

It has been demonstrated that *Annona muricata* leaf extract obtained from various solvents exhibited cytotoxic effects on different cancer cells attributed to the presence of

Table 2: MTT assay showing dose-dependent percentage inhibition of SCC-15 cells

Plant used	ALEAM Concentrations (µg/ml)	OD at 540 nm	Percentage of Inhibition (SCC-15 cells)
ALEAM	Control	0.525	0.00
	10	0.491	6.48
	20	0.448	14.66
	100	0.404	23.04
	200	0.396	24.58
	500	0.352	32.95
	1000	0.229	56.38

ALEAM – Aqueous leaf extract of Annona muricata; OD – Optical density; SCC – Squamous cell carcinoma

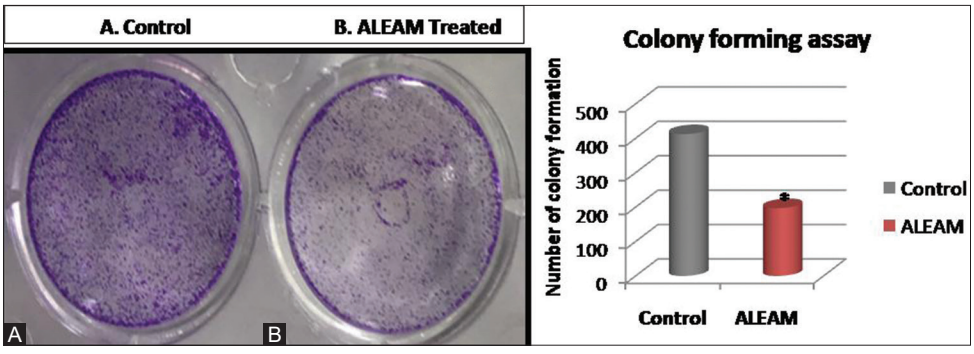

Figure 2: Representative photomicrographs and bar graph of SCC 15 cell colonies at 14 days after treatment with control (A) and *ALEAM* (B). All experiments were repeated for three times (*$P < 0.05$)

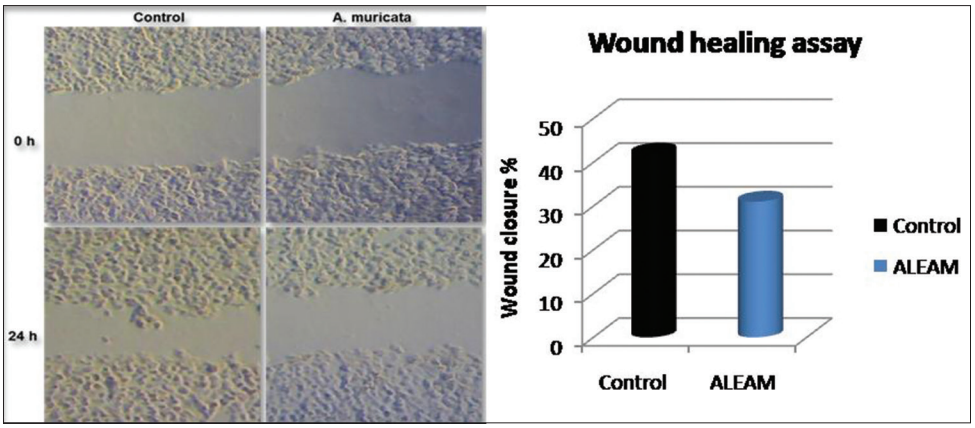

Figure 3: Wound healing (cell migration) assay in SCC-15 cells. Photomicrograph and bar chart represents that treatment with *ALEAM* significantly decreased SCC-15 cell migration compared with their control cells at 0 and 24 hr

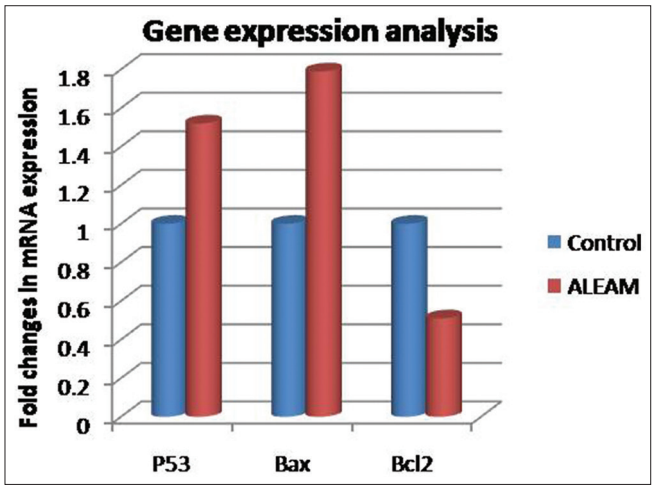

Figure 4: Representative graph showing the fold changes in mRNA expression of P53, Bax and Bcl-2 in SCC-15 cells treated with *ALEAM* compared to the control with significant *P* value ($P < 0.05$)

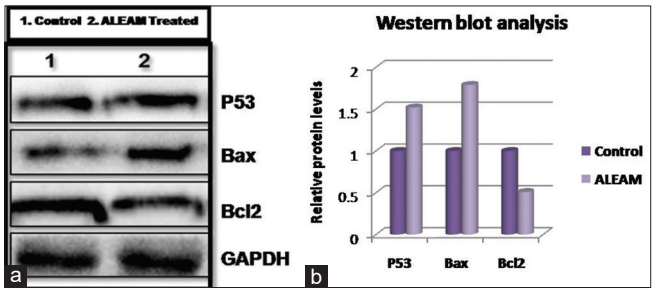

Figure 5: Western blot analysis of protein expression. (a) Protein expression of p53, Bax and Bcl2 in SCC-15 cells treated with *ALEAM* and control. (b) Bar graph of p53, Bax and Bcl2 expression in SCC-15 cells treated with *ALEAM* and control

numerous bioactive compounds in it.[13] However, studies on oral cancer cells are limited.

In a study conducted by Magadi *et al.*[11] in 2015, promising cytotoxic effects were obtained with an IC_{50} value of 12.42 μg/ml in ALEAM-treated SCC-25 cell lines. In another study by Gavamukulya *et al.*,[14] no effect on aqueous leaf extract was shown *in vitro* to tumour cell lines (EACC, MDA and SKBR3). However, the present study showed a good percentage of dose-dependent inhibition of SCC-15 cells after 24 hrs using the MTT assay.

The MTT assay relies on the mitochondria's ability to transform MTT into an insoluble formazan precipitate. Since MTT is reduced by mitochondrial enzymes, changes in metabolism changed the rate at which it was reduced. The half-maximal inhibitory concentration for the SCC-15 cell line was 857.90 μg/ml, and the inhibitory effect was dose-dependent. This demonstrates that varied dosages are needed for various cancer cell types.

Distant metastasis plays a significant role in the treatment and prognosis of patients with oral cancer. There are various theories proposed regarding the mechanism of metastasis.[15] Metastasis results from the combined effect of multiple alterations in the microenvironment of cancer cells and their ability to migrate and invade healthy host tissue. Once invaded, they form colonies in the secondary tumour site.[16]

A study conducted by Torres *et al.*[17] on pancreatic cancer (PC) cells found that *Annona muricata* extract inhibited the migratory capacity of PC cells both *in vitro* and *in vivo* thereby substantiating its anti-metastatic potential.

In the present study, both the rate of colony formation and the capacity for cell migration were observed to be reduced compared to the control. Thus, ALEAM exhibited significant anti-proliferative and anti-metastatic potential on SCC-15 cell lines.

An important hallmark of cancer is the evasion of apoptosis. Numerous researchers have focused their attention on the development of new anti-cancer agents with significant

apoptotic-inducing properties.[18] *Annona muricata* extracts have shown promising anti-cancer properties through the induction of apoptosis in various cancer cells.[19]

Bax's crucial function in the control of apoptosis has drawn greater interest since its discovery in 1993. While Bax tends to be disorganized in malignant cells, it is essential for maintaining homeostasis. *In vitro* and *in vivo* results have shown promising data on both targeted interaction and activation of Bax as a potential method for cancer therapy. Direct Bax activators have shown a number of benefits and proven to be superior to overcome radio- and chemoresistance. In addition, they selectively cause cancer cell death with minimal harm in healthy cells.[20]

The Bcl-2 protein family contains several distinct anti-apoptotic proteins; though Bcl-2 is the most widely recognized. Both Bax and Bcl-2 have the ability to interact with one another and produce isodimers. Their protein levels directly regulate apoptosis: as Bax levels rise, cell apoptosis will be stimulated.[21]

The results obtained revealed that ALEAM induced apoptosis through a Bax/Bcl-2-dependent mechanism, in accordance with previous studies performed on leaves of *Annona muricata*.[22-24] Furthermore, the p53 gene plays a major role in the carcinogenesis process. It is one of the most commonly mutated genes in human malignancies.[25]

P53 induces apoptosis by targeting one of its main transcriptional targets, the pro-apoptotic protein Bax. Additionally, it has been demonstrated that the p53 protein directly binds to the anti-apoptotic protein Bcl-2 in the mitochondrial outer membrane.[26] All of these details highlight the critical function of the p53 protein in the intrinsic apoptotic process. The gene and protein expression analysis in the current study revealed increased p53, Bax and decreased Bcl2 expression, further demonstrating that the cytotoxicity of ALEAM is in fact apoptosis-triggered [Figure 6].

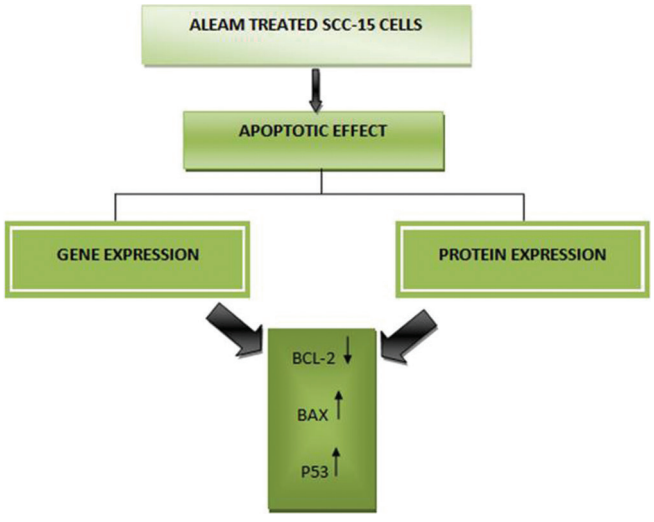

Figure 6: Schematic diagram representing the apoptotic effect exhibited by *ALEAM* on SCC-15 cells

The limitation of the present study is that the particular bioactive compound, which brings about the pro-apoptotic effect, is not elucidated. The method of extraction, specific dosage, effect on normal cells and toxicity need to be evaluated, standardized, and validated further with *in vivo* studies and clinical trials.

CONCLUSION

The findings of the present study demonstrated that ALEAM exhibited potent cytotoxicity, inhibited colony formation and cell migration on SCC-15 cells and accomplished so through apoptotic pathway by inhibiting the expression of anti-apoptotic protein Bcl-2 and activating the pro-apoptotic proteins Bax and p53. Future research on the effects of *Annona muricata* leaf extract on the sensitization of oral cancer cells to chemotherapy may benefit from the fresh insights provided by this work.

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Conflicts of interest

There are no conflicts of interest.

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