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ORIGINAL ARTICLE

Root extract of *Prunella vulgaris* inhibits *in vitro* and *in vivo* carcinogenesis in MCF-5 human breast carcinoma cells via suppression of angiogenesis, induction of apoptosis, cell cycle arrest and modulation of PI3K/AKT signalling pathway

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Summary

Purpose: *In this study we examined the anticancer effects of methanolic root extract of Prunella Vulgaris (PVE) against the MCF-5 breast cancer (BC) cell line along with its mode of action.*

Methods: *The proliferation rate of the MCF-5 cells was assessed by MTT assay. Apoptosis was confirmed by acridine orange (AO)/ethidium bromide (EB) and annexin V/propidium iodide (PI) staining. DNA damage was checked by comet assay. Cell cycle analysis was performed by flow cytometry. Protein expression was determined by western blotting. In vivo evaluation of the extract was carried out in xenografted tumor mice models.*

Results: *PVE inhibited the growth of the MCF-5 cells and exhibited an IC_{50} value of 25 μg/ml. The investigation of underlying mechanism revealed that PVE triggered apoptotic cell death of the MCF-5 cells which was also associated with enhancement of the expression of Bax and decrease in the expression of Bcl-2. PVE also caused arrest of the cells in the G2/M phase of the cell cycle and also exerted the anti-angiogenic effects. In vivo evaluation of PVE showed that it could inhibit the tumor weight and volume, suggestive of the anticancer potential of PVE.*

Conclusion: *The root extract of Prunella vulgaris in this study was shown to exert potent anticancer effects in MCF-7 human BC cells both in vitro and in vivo, accompanied with apoptosis induction, inhibition of angiogenesis, cell cycle arrest, and modulation of PI3K/AKT signaling pathway.*

Key words: *angiogenesis, apoptosis, breast cancer, cell cycle arrest, prunella vulgaris*

Introduction

Breast cancer (BC) is the most commonly detected cancer among women, causing significant morbidity and mortality [1]. In women it is the most common cause of death, with more than 0.4 million deaths annually [2]. It has been reported that BC constitutes around 14% of all cancer-related deaths in females, with an increasing trend in incidence [3]. The main obstacles to treatment of BC include late diagnosis, lack of reliable biomarkers and the therapeutic targets, and the limited availability of efficient drugs [4,5]. Since synthetic drugs are associated with side effects, the natural products may prove their value as a very useful source in the treatment of BC [6]. Plants and microbes have provided humankind with a diversity of drugs for the treatment of severe diseases and they are likely to continue to serve as source of more important drugs [7]. Plants are specialized to produce metabolites to fight environmental stresses. Such metabolites, commonly referred to as sec-

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ondary metabolites, have been employed for the treatment of diseases including cancer [8]. *Prunella vulgaris* is an important medicinal herb which has been reported to possess a diversity of bioactivities [9]. Antioxidant, anti-inflammatory and anticancer activities have been reported for different *Prunella vulgaris* extracts [10]. However, to date, the anticancer activity of *Prunella vulgaris* root extract has not been reported. In the present study, the anticancer effects of the methanolic root extract of *Prunella Vulgaris* (PVE) were evaluated against the MCF-5 BC cells.

Methods

Cell line and culture conditions

The BC cell line MCF-5 was obtained from American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium in CO_2 incubator (Thermo Scientific, Waltham, Mass, USA) at 37°C in 5% CO_2 and 98% humidity.

Proliferation assay

The proliferation rate of the MCF-5 cells was assessed on BC cell line by MTT assay, as previously described by Mosmann [11], at concentrations ranging from 0-100 µg/ml. The BC cells were subjected to treatment with varied concentrations of PVE and the proliferation rate was determined by monitoring the absorbance at 570 nm via spectrophotometer. Colony formation assay was carried out as described previously [12].

Apoptosis assay

The evaluation of whether or not PVE leads to induction of apoptosis was investigated by AO/EB staining as described previously [13]. In brief, BC cells (0.6×10^6) were grown in 6-well plates. Following 12 hrs of incubation, the cells were subjected to PVE treatment for 24 hrs at 37°C. The cell cultures were then centrifuged and the pellets were washed with phosphate buffered saline (PBS). After staining with AO/EB, the cells were centrifuged and washed again with PBS. Finally, the stained cells were examined by fluorescence microscopy. The percentage of apoptotic cells was estimated by annexin V/PI staining, as previously described [14]. Comet assay was carried out as previously described [15].

Cell cycle analysis

The distribution of the BC cells in different cycle phases was performed by flow cytometry. In brief, the BC cells were grown in 6-well plates and treated with PVE for 24 hrs. The cells were then collected and washed with PBS, followed by fixation in ethanol (70%). After overnight incubation at 4°C, the cells were subjected to PI staining and flow cytometry.

Western blotting

Following the lysis of the BC cells in RIPA lysis buffer, the protein content of each lysate was estimated by bicinchoninic acid (BCA) assay. The samples were then loaded on SDS-PAGE. The gels were then transferred to nitrocellulose membranes and subjected to treatment with primary antibody at 4°C for 24 hrs. After this, the membranes were incubated with HRP-conjugated secondary antibody (1:1000) for 50 min at 25°C. Enhanced chemiluminescence reagent was used to visualize the protein bands.

In vitro angiogenesis assay

The *in vitro* angiogenesis assay was carried out by using an Angiogenesis kit (Kurabo, Japan). In brief, human umbilical vein endothelial cells (HUVECs) and fibroblasts were co-cultured in 24-well plates and MCF-5 cells were cultured in the upper chamber and separated from lower chamber by a membrane. The upper cham-

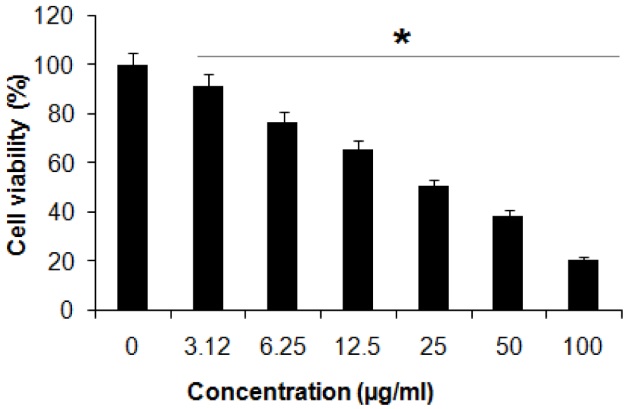

Figure 1. Effect of PVE on the viability of the MCF-5 breast carcinoma cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.01).

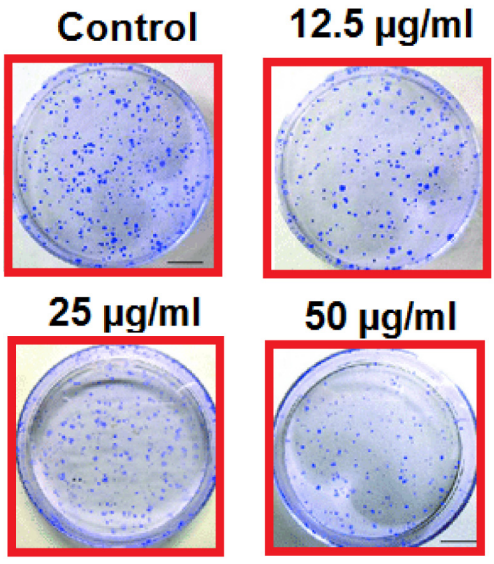

Figure 2. Effect of PVE on the colony formation of the MCF-5 breast carcinoma cells showing that PVE inhibits the colony formation of the MCF-5 cells in a concentration-dependent manner. The experiments were performed in triplicate.

ber was removed after the 7th day and HUVECs were subjected to staining with anti-CD31 antibody. Formation of the tubes was evaluated by counting 10 random fields.

In vivo study

The *in vivo* anticancer effects of the PVE were evaluated in the xenografted mice models in accordance with National Institutes of Health standards. Herein, around 4-week-old female immunodeficient nude mice, obtained from the Tumor Hospital of Qingdao, Qingdao, China, were used. In brief, the mice were injected with 5×10^6 MCF-5 cells subcutaneously at flanks. As the tumors became apparent (~5 mm after about 2 weeks), the mice (n=5) were injected intraperitoneally with DMSO (0.1%) dissolved in PVE and diluted with 100 µL normal saline at 25 mg/kg body weight. PVE was administered to the mice three times a week, while the control mice were administered DMSO (0.1%) in normal saline only. At the end of the study, the mice were euthanized and tumors were harvested for estimation of tumor growth and other investigations.

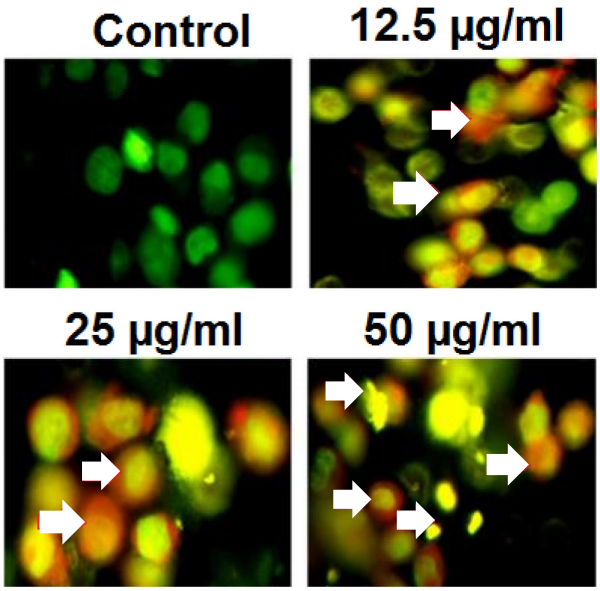

Figure 3. PVE triggers apoptotic cell death of the MCF-5 cells as depicted by AO/EB staining. Arrows show apoptotic cells. The experiments were repeated in triplicate.

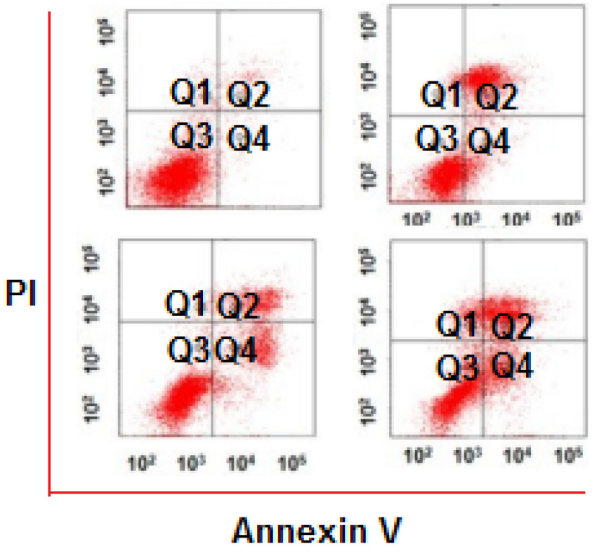

Figure 4. Determination of the percentage of the apoptotic cells by annexin V/PI staining. The Figure reveals that the apoptotic cell percents increase in parallel with the increase of PVE concentration. The experiments were repeated in triplicate.

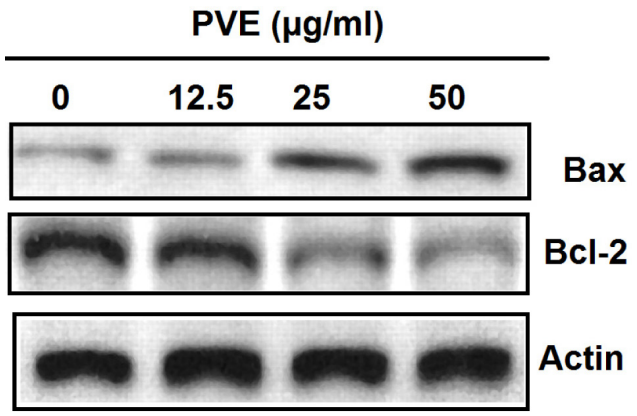

Figure 5. Effect of PVE on the expression of Bax and Bcl-2 in the PVE-treated MCF-5 cells by western blotting. The Figure shows that PVE increases the Bax expression and decreases the Bcl-2 expression. The experiments were repeated in triplicate.

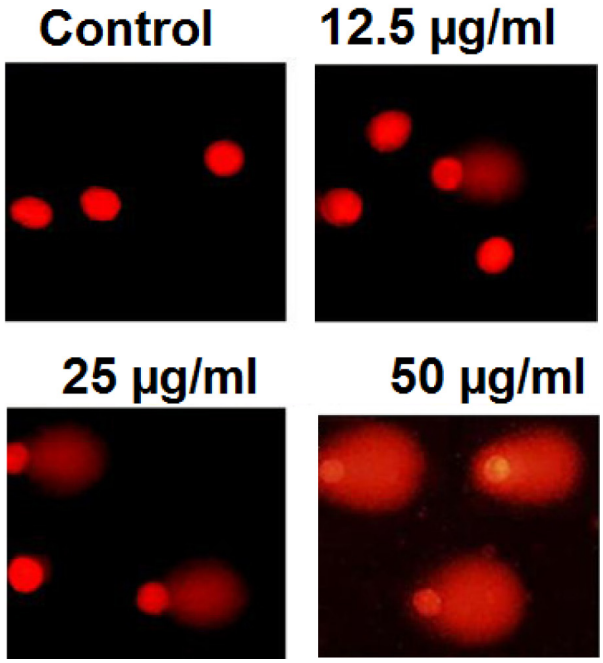

Figure 6. PVE triggers DNA damage on the MCF-5 cells as determined by comet assay. The Figure shows that PVA induces DNA damage in MCF-5 cancer cells concentration dependently. The experiments were repeated in triplicate.

Statistics

All the results are presented as mean ± standard error of the mean from at least three independent experiments. The differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test and $p<0.05$ was considered to indicate a statistically significant difference.

Results

PVE exerts inhibitory effects on the growth of MCF-5 cells

The anticancer activity of PVE on the BC cells was examined by MTT assay. It was revealed that PVE exerts antiproliferative effects on the MCF-5 BC cell line and exhibited an IC_{50} value of 25 µg/ml (Figure 1). In addition, it was found that the PVE could also inhibit the colony-forming potential of the MCF-5 cells in a concentration-dependent manner (Figure 2).

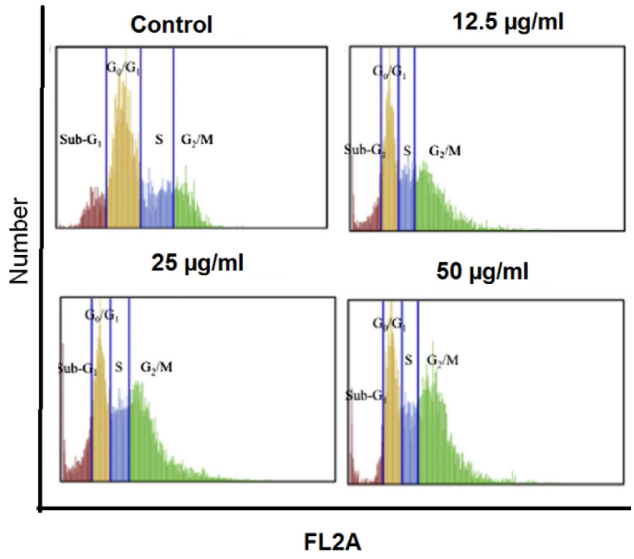

Figure 7. PVE triggers G2/M cell cycle arrest of the MCF-5 cells as depicted by flow cytometry. The experiments were repeated in triplicate.

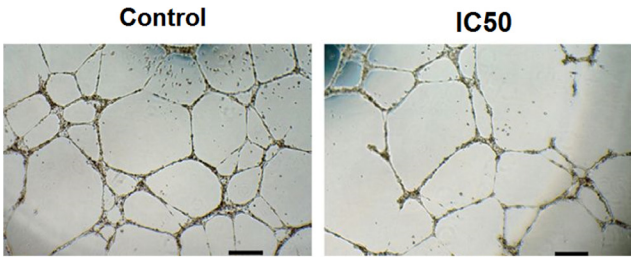

Figure 8. Effect of PVE on the angiogenesis of the cells as depicted by *in vitro* angiogenesis assay. The Figure depicts that PVE inhibits angiogenesis of cancer cells under *in vitro* conditions. The experiments were performed in triplicate.

PVE induces apoptotic cell death of MCF-5 cells

In order to examine whether PVE induces apoptotic cell death of the BC cells, the cells were subjected to treatment with various concentrations of PVE and then stained with AO/EB. The results of AO/EB assay showed that PVE induced apoptotic cell death in the BC cells (Figure 3). The Annexin V/PI staining showed that the apoptotic cell populations augmented with increase in the concentration of PVE (Figure 4). Analysis of the protein expression of Bax and Bcl-2 showed that the increased expression of Bax was associated with decrease in the expression of Bcl-2 (Figure 5). Furthermore, the comet assay results showed that PVE extract caused significant DNA damage in the MCF-5 BC cells (Figure 6).

PVE causes G2/M arrest of MCF-5 carcinoma cells

The impact of PVE on the distribution of MCF-5 cells in various cell cycle phases was assessed by flow cytometry and the results showed that PVE caused remarkable increase in the percentage of the MCF-5 cells in the G2 phase of the cell cycle. The percentage of MCF-5 cells in the G2 phase increased from 21.11 to 43.16% upon treatment with PVE (Figure 7). These results clearly indicate that PVE induces G2/M cell cycle arrest of the BC cells.

PVE inhibits the angiogenesis of the breast carcinoma

The anti-angiogenesis potential of PVE was investigated by Angiogenesis kit. The results re-

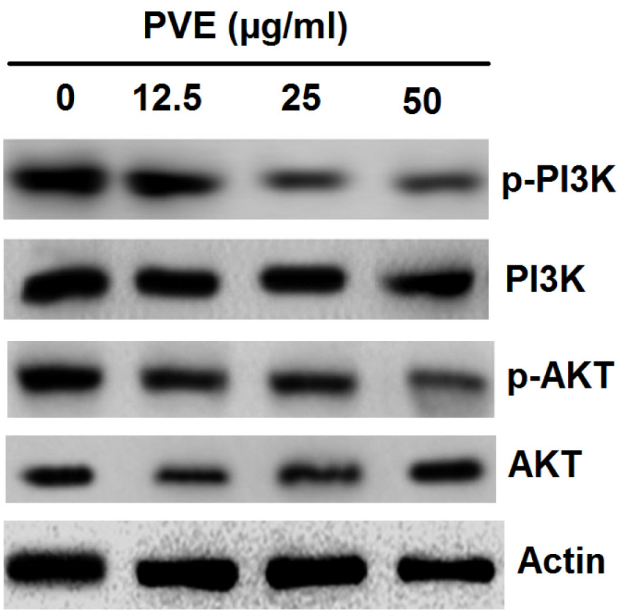

Figure 9. Effect of PVE on the PI3K/AKT pathway as depicted by western blotting. The Figure shows that PVE blocks the PI3K/AKT pathway in a concentration-dependent manner. The experiments were repeated in triplicate.

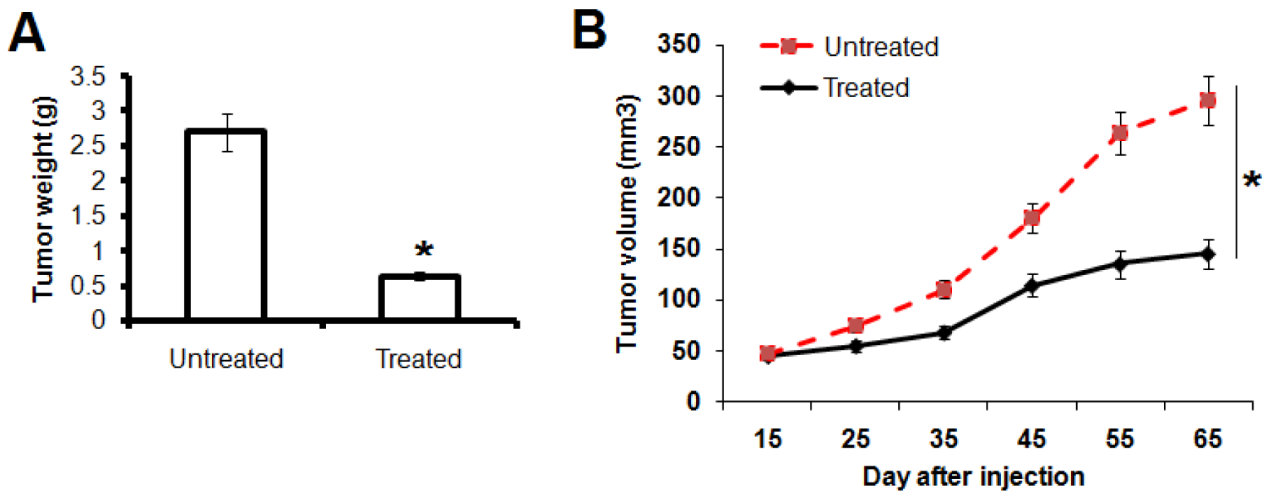

Figure 10. Effect of PVE on **(A)** Tumor weight **(B)** Tumor volume of the xenografted tumors. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.01).

vealed that PVE suppressed the tube formation of the HUVECs cells, suggestive of the anti-angiogenesis effects of PVE (Figure 8). The PVE led to inhibition of tube formation of the HUVECs at the IC_{50} concentrations.

PVE blocks the PI3K/AKT signaling pathway in MCF-5 cells

The effects of PVE were also examined on the PI3K/AKT signaling pathway at various concentrations. It was found that PVE dose-dependently inhibited the expression of p-PI3K and p-AKT, whereas no apparent effect was observed on the expression of PI3K and AKT (Figure 9).

PVE inhibits tumor growth in vivo

PVE exhibited significant anticancer effects *in vitro*; therefore we examined its anticancer effects *in vivo*. The results showed that PVE inhibited the growth of the xenografted tumors at the dose of 25 mg/kg. Additionally, PVE could also decrease the weight and volume in the xenografted tumors (Figure 10A and B).

Discussion

BC is one of the deadly types of cancers and responsible for severe mortality worldwide [16]. The existing anticancer agents create a number of adverse effects which impact negatively the overall patient health [17]. In addition, the existing anticancer agents are a significant sourse of adverse effects on the overall health of patients [17]. In this study, the anticancer effects of PVE, a root extract of a medicinally important plant, were investigated against the BC MCF-5 cell line. PVE concentration-dependently suppressed the growth of the MCF-5 cells, as depicted by the MTT assay. The outcomes of the MTT assay were also complemented by the colony formation assay wherein the PVE was found to inhibit the colony-forming potential of the MCF-5 cells. The anticancer effects of PVE were due to the induction of apoptotic cell death which was confirmed by AO/EB staining. Annexin V/PI staining further showed that the apoptotic cell populations increased with increase in the concentration of PVE, which was also associated with increase in the Bax/Bcl-2 ratio. Apoptosis is one of the imperative cellular processes that causes elimination of defective cells from the body [18]. Studies carried out earlier have shown that many plant extracts induce apoptotic death of cancer cells [19]. In addition to the apoptosis, several anticancer drugs also suppress the growth of cancer cells by causing arrest of the cells at different cell cycle check points [20]. In this study we found that PVE arrests the MCF-5 cells at the G2/M check point, preventing them from completing the cell cycle. PI3K/AKT is an important pathway that has been shown to be activated in cancer cells and it is believed to be an important therapeutic target of anticancer agents [21]. In this study, we found that PVE could inhibit this pathway in a concentration-dependent manner. Furthermore, angiogenesis is an important process in tumorigenesis and subsequent metastasis of cancer [22] and the results of *in vitro* angiogenesis assays revealed that PVE prevents the tube formation. Since interesting results were obtained in the previous *in vitro* studies, the anticancer effects of PVE were also examined in mice xenografted tumor models and showed that PVE decreased the

weight and volume of the xenografted tumors, suggestive of the anticancer potential of PVE.

In conclusion, PVE inhibits the growth of breast carcinoma by induction of apoptosis and cell cycle arrest. It also suppresses angiogenesis and inhibits the growth of the xenografted tumors. Due to these properties PVE deserves more in depth research and the active components of the extract need to be identified.

Acknowledgement

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Conflict of interests

The authors declare no conflict of interests.

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