Antiproliferative Effects of *Pancratium Maritimum* Extracts on Normal and Cancerous Cells

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Abstract

**Background:** Plants are an important natural source of compounds used in cancer therapy. *Pancratium maritimum* contains potential anti-cancer agents such as alkaloids. In this study, we investigated the anti-proliferative effects of *P. maritimum* extracts on MDA-MB-231 human epithelial adenocarcinoma cell line and on normal lymphocytes in vitro.

**Methods:** Leaves, flowers, roots, and bulbs of *P. maritimum* were collected and their contents were extracted and diluted to different concentrations that were applied on MDA-MB-231 cells and normal human lymphocytes in vitro for different intervals. Cells viability, proliferation, cell cycle distribution, apoptosis, and growth were evaluated by flow cytometry and microscopy. Parametric unpaired t-test was used to compare effects of plant extracts on treated cell cultures with untreated control cell cultures. IC₅₀ was also calculated.

**Results:** *P. maritimum* extract had profound effects on MDA-MB-231 cells. It inhibited cell proliferation in a dose- and time-dependent manner. The IC₅₀ values were 0.039, 0.035, and 0.026 mg/ml after 48, 72, and 96 hours of treatment with 0.1 mg/ml concentration of bulb extract, respectively. Those values were 0.051 and 0.03 mg/ml after 72 and 96 hours for root extract, respectively, and 0.048 mg/ml after 96 hours for flower extract. There were no significant effects of *P. maritimum* bulb extracts on normal lymphocytes proliferation.

**Conclusion:** *P. maritimum* extract has anti-proliferative effects on MDA-MB-231 cell line in vitro. The effects imply the involvement of mechanisms that inhibits cell growth and arresting cells at S and G₂/M phases. Cyclin B1, Bcl-2, and Ki67 expression was also affected.

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**Keywords** ● MDA-MB-231 ● *Pancratium maritimum* ● Gene Expression ● Cell Proliferation ● Lymphocytes

Introduction

Cancer is one of the most common devastating disease affecting millions of people per year worldwide. Cancer has been estimated as the second leading cause of death in humans. According to the American Cancer Society,¹ deaths arising from cancer constitute 2-3% of the annual deaths recorded worldwide. Cancer is an uncontrolled proliferation of a cell that might invade and destroy other normal tissues and can eventually lead to patient death.

What's Known

- *P. maritimum* has purgative, acaricidal, insecticidal, and antifungal activities.
- Previous studies on this plant have led to the isolation of a variety of alkaloids belonging to different classes.
- Clinical studies were conducted to investigate the use of amaryllidaceae alkaloids and their synthetic derivatives as a new anti-tumor drugs.

What's New

- We found that *P. maritimum* extract has a profound anti proliferative effect and arrested cancerous cycling cells at S and G₂/M phases. It also affected Cyclin b1, Bcl-2, and Ki67 expression.
Several chemo-preventive agents are used to treat cancer, but many are toxic, which limits their full utilization in treatment regimes. Over the past years, there has been an intense search to find and develop novel anticancer drugs to combat this disease.

More than 60% of currently used anticancer agents are derived in one way or another from natural sources. According to one estimate, about 50% of breast cancer cases and 37% of prostate cancer patients are treated with plant derivative products. The medicinal value of plants lies in some chemical substances usually secondary metabolites, that produce a definite physiological action in the human body. The most important of these bioactive compounds are alkaloids, flavonoids, tannins, and phenolics. The Middle East region comprises about 700 plant species that are known for their medicinal values.

_Pancratium maritimum_ L. (Amaryllidaceae family) grows wild in sandy coastal habitats of the Mediterranean. It has white flowers and very large bulbs. This species was not registered in the Syrian flora until recently where it has been found near Tartaus-Syria by Al-Odat. Previous studies on this plant led to the isolation of a variety of alkaloids. Those studies indicated that alkaloids and flavonoids contained in the bulbs _P. maritimum_ have pharmaceutical properties. Pancratistatin, which is one of those alkaloids, has anticancer properties. _P. maritimum_ extract has purgative, acaricidal, insecticidal, anti-migratory, antiviral, antimicrobial, immunostimulant, analgesic, antimalarial, antitumor, antifungal, and antioxidant activities. More recently, some studies have demonstrated a potent anticancer activity of Lycorine, Pancratistatin and Urginomionorine alkaloids.

Pharmacological effects of Amaryllidaceae alkaloids fueled clinical studies to use them and their synthetic derivatives in developing anti-tumor and anti-Alzheimer’s therapeutics. Another study by Kaya has elucidated that _P. maritimum_ extract has a cytotoxic activity on the brine shrimp (Artemia salina Leach).

In the present study, we investigated the anti-tumor effects of _P. maritimum_ extracts on MDA-MB-231 human breast cancer cells, provided by Professor P. Bécuwe from the Cancer Research Unit (EA SIGRETO, Nancy, France), were aseptically cultured in 125 ml cell culture flasks containing RPMI-1640 complete medium (supplemented with 10% fetal bovine serum (FBS)), 50 U/ml penicillin/streptomycin, and 2 mM L-glutamine. Cell cultures were kept under optimum culturing conditions (37 °C, 85% humidity, 5% CO₂). Determined concentrations of the plant extracts were added to the cell cultures when the density of cells reached 40-50%, as evaluated using Olympus converted microscope. The tested concentrations of extracts were 0.1, 0.02, 0.01, 0.001, or 0.0001 mg/ml. Treated cells were harvested for subsequent analyses after 24, 48, 72, and 96-hour intervals in culture. In addition, we investigated the prolonged effects of the _P. maritimum_ extract on MDA-MB-321 by treating three cell cultures with 0.1 mg/ml of bulb extract for 9 days.

Materials and Methods

**Plant Material**

Samples of _P. maritimum_ (leaves, flowers, roots, and bulbs) were collected in August and September 2011 during the flowering season from its natural environment near Tartaus on the Syrian Mediterranean Seacoast. Voucher specimens of this plant were deposited in the herbarium of plant biotechnology department at the Atomic Energy Commission of Syria (AECS). Plant identity (voucher sample number 1116), was approved by Al-Odat (plant taxonomist).

**Preparation of the Plant Extract**

Fresh plant tissues (100g leaves, 100g flowers, 50g roots, and 50g bulbs) were cut into small pieces and extracted with 95% EtOH three times (48h each) at room temperature. The ethanol extracts were passed through Whatman filter paper (No.1) in order to remove debris and then concentrated with a rotary evaporator (BUCHI Heating Bath B-490). Concentrated extract was lyophilized with a freeze-dryer. The dried matter was stored at −20 °C until use. The yield of dried extracts from starting crude materials was 4.48g, 1.49g, 7.44g, and 7.43g (w/w) for the leaves, roots, bulbs, and flowers, respectively.

To study the anti-proliferative and the cytotoxic effects of the extract, lyophilized extracts were dissolved in dimethyl sulfoxide (DMSO) and then different dilutions of the resultant solution were applied on cell cultures.

**MDA-MB-321 Cell Line Cultures**

MDA-MB-321 human breast cancer cells, were aseptically cultured in 125 ml cell culture flasks containing RPMI-1640 complete medium (supplemented with 10% fetal bovine serum (FBS)), 50 U/ml penicillin/streptomycin, and 2 mM L-glutamine. Cell cultures were kept under optimum culturing conditions (37 °C, 85% humidity, 5% CO₂). Determined concentrations of the plant extracts were added to the cell cultures when the density of cells reached 40-50%, as evaluated using Olympus converted microscope. The tested concentrations of extracts were 0.1, 0.02, 0.01, 0.001, or 0.0001 mg/ml. Treated cells were harvested for subsequent analyses after 24, 48, 72, and 96-hour intervals in culture. In addition, we investigated the prolonged effects of the _P. maritimum_ extract on MDA-MB-321 by treating three cell cultures with 0.1 mg/ml of bulb extract for 9 days.
**Human Lymphocytes Cell Cultures**

Normal human whole blood samples were cultured in a complete ready to use chromosome P medium with or without the presence of a phytohemagglutinin as a mitogenic stimulant. Culturing was conducted under sterile conditions and cell cultures were kept under optimum growth conditions. Plant extracts were then added to lymphocytes cultures at the same concentrations used for MDA-MB-231 cells. Treated cells were then harvested for subsequent analyses after 24, 48, 72, and 96-hour intervals in culture.

**Preparing Cells for Analysis**

Treated cells were harvested from flasks by a brief treatment with trypsin and then transferred into separate 50 ml conical centrifuge tubes. After centrifugation for 5 min at 300×g, supernatant was discarded and cells were resuspended in 1 ml cell culture medium. A cell count was performed manually using a hemacytometer and cell concentration was adjusted to ~5×10⁶ cells/ml. Aliquots of those cells were then subjected to subsequent assays.

**Cell Viability Assay**

Cell viability of treated and untreated MDA-MB-231 cells was evaluated by BD cell viability kit as indicated in the company product datasheet. Cell samples were analyzed directly using BD FACSCalibur flow cytometer. Absolute cell numbers of live and dead cells were calculated as instructed in the product datasheet using Cellquest pro software.

**Annexin-V Assay**

Annexin-V binding assay was used to evaluate apoptosis by flow cytometry according to the manufacturer's instructions and recommendations indicated in the company product datasheet.

**Proliferation and Cell Cycle Analysis**

Cycletest plus and APC-BrdU Flow kits from BD biosciences were used to analyze proliferation and cell cycle distribution. Cells of treated and control cell cultures were prepared and analyzed as indicated in the manufacturer's products datasheets. Apoptotic cells percentages were determined from DNA content histograms, as the events in the channels below the sub G₁/G₂ peak. Cell samples were analyzed on BD FACSCalibur flow cytometer with doublet discrimination module (DDM) on measuring pulse width versus pulse area of FL2 in linear mode.

**Bcl-2, Cyclin-B1, and Ki67 Expression Analysis**

Fluorochromes conjugated antibodies to Bcl-2 and cyclin-B1 were used to evaluate Bcl-2, Ki67, and cyclin-B1 expression in treated and untreated cells by flow cytometry. For each protein to be detected and for each sample, 100 μl of cell suspension (~1×10⁶ cells) were transferred into a separate 5 ml tube. Cells were washed twice with PBS containing 1% FCS and 0.1% NaCl. Then, fixed and permeabilized with 250 μl of fixation/permeabilization buffer and incubated at 4°C in the dark for 30 minutes. Cells were then washed twice with 1 ml of permeabilization and washing buffer and suspended in 250 μl perm/wash buffer. A 20 μl of the fluorochrome-conjugated antibody was added and the sample was then incubated on ice in the dark for 3 hours, washed with 1 ml PBS containing 1% FCS and 0.1% NaCl, and then suspended in 500 μl of PBS and analyzed on the FACSCalibur. Appropriate isotypes and autofluorescence controls were also included.

**Cell Proliferation Assay**

Bromodioxyuriden (BrdU) at 32.5 mM concentration was added into separate flasks of treated and untreated cell cultures for 30 minutes before cells harvesting. Cells were then washed with PBS containing 1% FCS and 0.1% NaN₃, suspended in 250 μl of fixation/permeabilization buffer for 30 minutes at 4°C in the dark. Cells were then washed twice with 1 ml of permeabilization and washing buffer. They were incubated in 250 μl cytoperm buffer for 10 minutes and then washed. Cells were treated with 250 μl of DNase solution for one hour at 37 °C in the dark and then washed with a permeabilization and washing buffer. A 20 μl of the APC-anti-BrdU antibody was added to each sample and incubated on ice in the dark for 3 hours. Cells were washed with 1 ml of PBS containing 1% FCS and 0.1% NaN₃, and then resuspended in 500 μl of PBS containing 20 μl of 7-AAD. Cells were analyzed on the FACSCalibur.

**Statistical Analysis**

Statistical results were expressed as mean±SD (standard deviation). The parametric unpaired t-test was used to calculate the P value (MS-Excel 2007). The statistical significance of difference (P<0.05) for the treated cultures was determined relative to the control cultures. IC₅₀ for treated cells was calculated using the following equation:²⁰

\[ IC_{50} = \left( \frac{50\% - L o w_{\%}}{H i g h_{\%} - Low_{\%}} \right) \times \frac{L o w_{\%}}{H i g h_{\%}} + \frac{L o w_{\%}}{H i g h_{\%}} \]

Where:

- Low_{\%} / High_{\%}: % inhibition directly below/above 50% inhibition
- Low_{Con}/High_{Con}: Corresponding concentrations of test compound

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Results

P. maritimum extract has profound effects on MDA-MB-321 human breast cancer cells. It inhibited cell growth and proliferation in a dose- and time-dependent manner. It also arrested cycling cells at S and G2/M phases and affected cyclin B1, Bcl-2, and Ki67 expression.

Effect of Extract on Cell Viability and Growth

Bulb or leaf extract of P. maritimum (at a concentration of 0.1, 0.02, 0.01, 0.001, or 0.001 mg/ml) inhibited the growth of MDA-MB-231 cells. These inhibitory effects of both extracts were time- and concentration-dependent. The bulb extract was more effective (P=0.002) than leaf extract (P=0.004) (figure 1A). The anti-proliferative effects of the bulb extract were more evident at 0.1 and 0.02 mg/ml concentrations compared with the low concentrations (0.001 and 0.0001 mg/ml) (figure 1B).

The concentration of 0.1 mg/ml was most effective (P=0.011) and caused growth inhibition by about 42%, 55%, 63%, and 68% after 24, 48, 72, and 96-hour intervals, respectively. The 0.1mg/ml concentration of the root extract caused growth inhibition by 30%, 47%, 64%, and 73% after 24, 48, 72, and 96-hour intervals, respectively. The leaf extract inhibited cell growth of MDA-MB-231 cells in the same manner as bulb extract. This is also true for flower extract. The low concentrations (0.001 and 0.0001 mg/ml) of the different plant parts extracts had no inhibitory effects; they actually had some growth stimulating effects (P=0.28 and 0.20 for bulb extract at 0.001 and 0.0001 mg/ml, respectively). The IC50 after treatment with the highest concentration (0.1 mg/ml) were 0.039, 0.035, and 0.026 mg/ml after 48, 72, and 96 hours consecutively for bulb extract, 0.051 and 0.03 mg/ml after 72 and 96 hours consecutively for root extract and 0.048 mg/ml after 96 hours for flower extract.

The data are expressed as mean±SD for three independent experiments.

Effect of Extract on the Cell Cycle

To investigate the anti-proliferative action of P. maritimum bulb ethanol extracts further, we tested its biological effects on the cell cycle in MDA-MB231 cancer cells. As shown in figures 3 and 4, cells were significantly blocked in S phase and G2/M phase after 48-96 hours of treatment with various concentrations. It should be noted that the 0.1 mg/ml concentration significantly arrested cells at the S phase of cell cycle.

Effect of Extract on Bcl-2, Cyclin B1 and Ki67 Expression

P. maritimum extracts affected the expression of Bcl-2, cyclin B1 and Ki67 in MDA-MB-231 cells in a time- and concentration-dependent manner. The 0.1 mg/ml concentration decreased Bcl-2 expression significantly after 24 hours. However, Bcl-2 expression increased but remained below the normal levels by two-fold. While cyclin B1 expression decreased progressively with time, Ki67 expression pattern was different. It decreased and then returned to previous levels after 96-hour interval (figure 5).

Apoptosis Induction

P. maritimum bulb extracts showed minor effects on apoptosis induction, as evidenced by...
Annexin-V binding assay using flow cytometry (figure 6).

**Effect of Extract on Normal Lymphocytes**

The effects of high concentrations (0.1 and 0.02 mg/ml) of *P. maritimum* bulb extracts were also tested on stimulated and non-stimulated normal human lymphocytes in vitro. Our results did not indicate significant effects of *P. maritimum* bulb extracts on the distribution of stimulated lymphocytes on the various phases of cell cycle (figure 7). *P. maritimum* bulb extracts induced apoptosis in stimulated lymphocytes in a time- and dose-dependent manner. This effect was significant only after 72 hours of treatment with 0.1 mg/ml concentration (P=0.02). Interestingly, *P. maritimum* bulb extracts did not significantly increase apoptosis in non-stimulated lymphocytes (P=0.07).

**Discussion**

Carcinogenesis is known to involve many signaling pathways such as modulation of transcription factors, apoptotic proteins, protein kinases, cell cycle proteins (e.g. cyclins and cyclin-dependent kinases), cell adhesion molecules, enzymes, and growth factor signaling pathways.21

Many plants, including bulbous species contain many biologically active compounds that can be used as anticancer drugs.22,23 Many of these compounds are under investigation by various research groups, firms, and pharmaceutical companies. It is estimated that only 15% of this repertoire has been explored.24

These natural phytochemicals have potent and selective anti-cancer actions through mechanisms involving destabilizing microtubules formation in dividing cancer cells,25 inhibiting oncogenes expression, disrupting signal transduction pathways, preventing cell adhesion, and inhibiting enzymes. These actions also involve induction of cell differentiation, apoptosis, and tumor suppressor gene expression; in addition to induction of detoxification molecules and enzymes such as phase II enzymes, glutathione, peroxidase, catalase, and superoxide dismutase. Natural
phytochemicals are also known for their enhancement of immune response, preventing DNA adducts formation or DNA intercalation, blocking angiogenesis, and regulating hormones metabolism. Alkaloids in particular may interact with many cellular molecules and interfere with the growth and progression of the tumors. \textsuperscript{27} \textit{P. maritimum} contains up to 16 alkaloids, including lycorine, maritidine, lycoramine, galanthamine

Figure 4: Flow cytometric dot plots showing the effects of \textit{P. maritimum} extract (0.1 mg/ml) on the cell cycle/proliferation of MDA-MB-231 cells analyzed by BrdU incorporation assay: (A) control, (B) after 24h, (C) after 48h, (D) after 72h, and (E) after 96h of incubation. Cyan cluster represents apoptotic cells in the sub G0/G1 phase. Red cluster represents cells at the G0/G1 phase. Green cluster represents cells at the S phase. Pink cluster represents cells at the G2/M phase.
narciclasine, lycoricidine, and pancratistatin. Some of these alkaloids and their congeners have demonstrated potent cytotoxicity against cancer cell lines in vitro and potent antitumor activity in vivo.\textsuperscript{5,11,12} Pancratamine B and N-methyl-8, 9-methylenedioxyphenanthridine extracted from \textit{P. maritimum} have antiproliferative and antimigratory activity against the highly metastatic human prostate cancer cell line PC-3 cells without cytotoxicity.\textsuperscript{11}

In this study, we investigated the antiproliferative effects of \textit{P. maritimum} extract on the MDA-MB-231 cell line in vitro. This cell line is an invasive adenocarcinoma cell line that is ESR-, PR-, HER2-, and has non-functional p53. Our results showed that \textit{P. maritimum} extracts inhibited the growth and the proliferation of MDA-MB-231 breast cancer cell line. This inhibition was dose- and time-dependent. As evident from IC\textsubscript{50} values, the inhibition effect
of bulb extract on MDA-MB-231 cell growth was superior to that of the root, flower, and leaf extracts sequentially. However, lower concentrations of different parts extracts did not inhibit cell growth. The increased effect of bulb extract can be explained by differences in the content of active substances among different parts of the plant.

The low concentrations of *P. maritimum* extracts, apparently, did not interfere with...
cell viability. This is consistent with the results obtained by Ryu\textsuperscript{28} and Jeong\textsuperscript{29} on Orostachys japonicas extracts.

\textit{P. maritimum} extracts arrested cycling MDA-MB-321 cells at S and G\textsubscript{2}/M phases. This selective blocking of cancerous cells in specific cell cycle phase is a favorable characteristic of anti-cancer drugs.\textsuperscript{30} The mechanism involved in this arrest might be through interfering with DNA synthesis pathways\textsuperscript{31} or blocking the formation of mitosis spindle.\textsuperscript{32} Many studies have shown that alkaloids induce depolymerization of microtubules in cancer cells.\textsuperscript{33,34} Recent studies also suggest that, at nanomolar concentrations, narciclasine and alkaloid found in \textit{P. maritimum} extracts suppress the growth and proliferation of glioblastoma multiforme cancer cells by inhibiting protein synthesis pathways.\textsuperscript{35} Our results showed that \textit{P. maritimum} extracts down-regulated expression of cyclin B1 (proliferation regulating molecule) and Ki67 (nuclear antigen expressed by cancerous proliferating cells).

The cyclin B1 and Cdc2 kinase regulate the entry and progression of the mitotic phase in eukaryotic cells. Cyclin B1 and Cdc2 kinase activity is known to be involved in the G2/M phase transition of the cell cycle. This G2/M transition requires the accumulation of cyclin B1 and Cdc2.\textsuperscript{36,37} Here, we demonstrated that treatment of MDA MB-321 cells cancer cells...
with \(P. maritimum\) extracts resulted in the decrease of cyclin B1 levels as evidenced by the flow cytometric expression profile of cyclin B1. In particular, down-regulation of cyclin B1 expression is the supporting evidence of the inhibiting effects of \(P. maritimum\) extracts on MDA-MB-321 cells proliferation.

\(P. maritimum\) extracts also affected the expression pattern of Bcl2 (anti-apoptosis regulating molecule). Many studies on the extracts of other plant species\(^{28,38}\) also reported changes in the expression patterns of Bcl2 and other molecules involved in the apoptosis pathways in treated cells. Alkaloids have been shown to induce cell growth arrest, phosphorylation of Bcl-2, increased Bax protein levels, and finally cell death.\(^{39,40}\) Bcl-2 is known to play an important role in the intrinsic apoptosis pathway and protects the microtubule integrity.\(^{18}\) Pancratistatin, a major alkaloid in \(P. maritimum\) extracts, may also contribute to cell death by indirect activation of pro-apoptotic Bcl-2 proteins and/or interfere with antioxidant response mechanisms.\(^{41}\) Pancratistatin also induces an autophagic response in p53-mutant prostate cancer cells that might be due in part to possible down-regulation of Bcl-2 proteins by pancratistatin.\(^{42}\) Lycorine, another alkaloid found in \(P. maritimum\) extracts, has been implicated in down-regulation of anti-apoptotic Bcl-2 family member Mcl-1.\(^{43}\) Recent studies have indicated that lycorine also activates caspase-3, -8, -9 and modulates the expression of anti-apoptotic Bcl-2 family member, Mcl-1, leading to apoptosis in leukemia cells.\(^{43,44}\) It is therefore possible that \(P. maritimum\) extract may be modulating the anti-apoptotic effect of Bcl-2 proteins to contribute to cell death. However, in this study, \(P. maritimum\) extracts showed minor effects on apoptosis induction. This indicates that the inhibition of MDA-MB-231 cells growth is not through triggering apoptosis induction pathways or that the cell line is resistant to apoptosis induction. This is in agreement with the findings of Ryu.\(^{28}\) In general, our results are in accord with the findings of studies carried out on different plant species extracts and on different cancerous cell lines.\(^{45,46}\)

The effects of \(P. maritimum\) extract on normal lymphocytes proliferation was minor and nonspecific. This is consistent with Griffen findings that pancratistatin, a major alkaloid in \(P. maritimum\) extract, is selective in inducing apoptosis specifically in Jurkat cells (a human lymphoma cells) and in clinical leukemia samples with minimal effect on non-cancerous peripheral blood mononuclear cells.\(^{42}\) Studies have indicated that pancratistatin induces apoptosis in several different types of cancer including colon, prostate and breast carcinomas, leukemia, melanoma and glioblastoma, with minimal toxicity to non-cancerous counterpart cells.\(^{47,48}\) Pancratistatin selectively targets cancer cell mitochondria and induces apoptosis independent of p53, a frequently mutated tumor suppressor in human carcinomas.\(^{49}\)

Our results showed that \(P. maritimum\) extract has no capacity to enhance the proliferation of stimulated human lymphocytes. However, stimulated lymphocytes were more susceptible to apoptosis; the reason for the extract being more effective on stimulated lymphocytes.

### Conclusion

The results show that \(P. maritimum\) extract at 0.1 mg/ml concentration has a profound and significant antiproliferative effect on MDA-MB-231 cancerous cell line in vitro. This is the first report regarding the antitumor effects of \(P. maritimum\) extracts on MDA-MB-231 cell line. The antiproliferative effect implies the involvement of mechanisms that leads to cell proliferation inhibition, arresting cells at S and G\(_2\)/M phases, and cyclin B1, Bcl-2, Ki67 expression down-regulation. In addition to testing the effects of those fractions in vivo, we intend to conduct future studies on fractionated compounds of bulb extract and test their effects on different neoplastic cell lines to identify the most effective fraction. The mechanism of observed effects also requires further detailed investigation.

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### Conflict of Interest: None declared.

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