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**Antitumor activity, cell cycle arrest
and apoptosis induction in human
colon cancer cell line by *Primula
macrophylla* extracts**

Antitumor activity, cell cycle arrest and apoptosis induction in human colon cancer cell line by *Primula macrophylla* extracts

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Abstract

The primary objective of the current work was to investigate the antitumor potential of *Primula macrophylla* extracts in human colon cancer cell line (Colo-205) along with evaluating the effects on apoptosis induction, cell cycle arrest and mitochondrial membrane potential. Cell viability was assessed by tetrazolium-based MTT assay. Flow cytometry measurement was carried out to assess the effect of the extract on cell cycle phase distribution and mitochondrial transmembrane potential. Results showed that *P. macrophylla* methanol extract was effective and exhibited highest cell growth inhibition (IC₅₀ value, 26.17 µg/mL). Methanol extract significantly increased the side-scattering profile of Colo-205 cells in concentration-dependent pattern. Exposure of Colo-205 cells with different concentrations of the methanol extract (0-80 µg/mL) caused dose-dependent G₀/G₁ cell cycle arrest along with inducing apoptotic cascade by increasing the population of cells at G₀/G₁ phase. Furthermore, methanol extract treatment caused an increase in mitochondrial membrane depolarization in Colo-205 cells.

Introduction

Among all types of cancers, colon cancer stands as the third most commonly diagnosed and second most cause of cancer-related deaths in the United States (Rebecca et al., 2014). Intake of fruits and vegetables is known to impart a reduction in the incidence of different types of cancers including colon cancer and ischemic heart diseases (Mathew et al., 2004; Fleischauer et al., 2003). Emphasis has now been shifted towards the screening of apoptotic inducers from natural sources particularly from plants in the form of extracts or as isolated compounds that specifically increase apoptotic cell death in cancerous cells.

Primula macrophylla D. Don. (Primulaceae) is an important member of genus *Primula*, which represent more than 400 species (Richards, 1993). *P. macrophylla* is a Himalayan species which is widely distributed from

Afghanistan to China. The species flourishes best in moist rocky slopes near melting glaciers and wet meadows, at elevations ranging from 3,500-5,000 meters above sea level. *P. macrophylla* is a perennial clump-forming herb with compact heads of many flowers. The aqueous extract of *P. macrophylla* in the traditional system of medicine is used for the treatment of asthma. The plant is reported to have antispasmodic property and strong anti-proliferative potential (Tsarong, 1994). The present study reports the anti-proliferative and apoptotic effects of different extracts of *P. macrophylla* in human colon cancer cells (Colo-205).

Materials and Methods

Materials

Methanol, ethyl acetate (HPLC grade) and dimethyl



sulfoxide (DMSO) were purchased from Merck, Germany). RPMI 1640 medium, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), RNase, trypsin/EDTA, phosphate buffer saline (PBS), triton X-100, rhodamine-123, fetal bovine serum (FBS), propidium iodide, streptomycin, penicillin and 5-fluorouracil were obtained from Sigma-Aldrich (USA). The other chemical reagents used were of analytical grade.

Plant collection and extract preparation

P. macrophylla was collected at the peak stage of flowering in July 2015 from the Hangzhou City, Zhejiang Province, China. Fresh aerial parts of *P. macrophylla* were cleaned with distilled water, dried under shade for several days. After drying, the plant material was cut into small pieces and made into fine powder. The plant powder was packed in Soxhlet apparatus and extracted first with ethyl acetate and then with methanol at desirable temperatures. The extracts were then filtered through Whatman filter paper. The pellets were discarded and the supernatant was collected and concentrated under reduced pressure at required temperature using rotavapor. The extracts were dried, labeled as ethyl acetate extract and methanol extract and stored at 4°C until used for experimental analysis.

Cell line and cell culture conditions

The Colo-205 cells were obtained from the American type culture collection (ATCC). Cells were cultured in RPMI-1640 medium complemented with 10% fetal calf serum, 100 µg/mL streptomycin and 100 U/mL penicillin. The Colo-205 cells were then grown in a CO₂ incubator at 37°C in 5% CO₂ with 98% relative humidity atmosphere.

Cytotoxicity assessment by tetrazolium-based assay (MTT assay)

To assess the effect of *P. macrophylla* leaf extract on Colo-205 cells, (3-(4, 5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) protocol was used as previously reported (Mosmann, 1983). Colo-205 cells (2 × 10⁵ cells/mL) were grown in 96-well culture plates and incubated for 24 hours at 37°C and 5% CO₂ level. After 24 hours of incubation, the supernatant was removed from the adhered cells and washed. Different concentrations (0-80 µg/mL) of plant extracts were added to respective labeled wells and incubated further for 48 hours. MTT solution (10 µL) was added to the cell culture and incubated for 2 hours. To dissolve the formazan crystals so formed, 100 µL of DMSO was used. Optical density was recorded on microplate reader (Bio-Rad Laboratories) at 570 nm. Production of formazan is proportional the number of viable cells. The percentage cell growth inhibition was calculated by the equation:

$$\% \text{Cell growth inhibition} = 100 - [\text{OD of treated cells} / \text{OD of control cells}] \times 100$$

Dot-blot light scattering analysis

Colo-205 cells at a density of 2 × 10⁵ cells/mL were plated in 96-well culture plates and then treated with different concentrations (0, 10, 20, 40 and 80 µg/mL) of methanol extract and incubated for 24 hours at 37°C and 5% CO₂ level. FACScan flow cytometer (Becton Dickinson, USA) was used to analyze dot-blot light scattering profiles (Schrantz et al., 2001).

Cell cycle analysis and DNA content

For the analysis of cell cycle phase distribution and apoptotic populations, flow cytometry was used (Fraker et al., 1995). Colo-205 were plated in 6-well culture plates at a cell density of 2 × 10⁵ cells/mL and incubated for 24 hours at 37°C and 5% CO₂ level. Cells were then treated with methanol extract of *P. macrophylla* at different concentrations (0, 10, 20, 40 and 80 µg/mL) in cell medium supplemented with 10% (v/v) FBS for 48 hours. After treatment, cells were harvested by centrifugation at 1,000 rpm for 5 min, washed with PBS, and then fixed with 70% (v/v) ethanol (ice-cold) for 1 hour and then staining was done using cell cycle reagent (20 µg/mL propidium iodide, 0.1 mM EDTA, 10 µg/mL RNase (DNase free) and 1% triton X-100 prepared in PBS) for 30 min in dark. The contents were then analyzed by flow cytometry using FACScan flow cytometer (Becton Dickinson, USA) using Cell Quest software to measure the DNA content and cell cycle distribution phase. For each experiment, at least 10,000 cells were analyzed. The population of apoptotic cells with sub-G1 DNA content were calculated as a percentage of total number of cells.

Analysis of mitochondrial transmembrane potential

In this experiment, the effects of *P. macrophylla* on mitochondrial transmembrane potential ($\Delta\Psi_m$) was assessed by employing Rh-123 as dye (Cao et al., 2007). Briefly, Colo-205 cells at a density of 2 × 10⁵ cells/mL were seeded in 6-well culture plates and incubated with different concentrations (0, 10, 20, 40 and 80 µg/mL) of methanol extract of *P. macrophylla* for 48 hours. One hour before termination of the experiment, Rh-123 dye (10 µg/mL) was added and again incubated for 30 min at 37°C followed by washing with PBS. Upon centrifugation at 1,000 rpm for 5 min, pellets were collected, resuspended in PBS and immediately analyzed using FACScan flow cytometer (Becton Dickinson, USA) at an exciting wavelength of 488 nm. Cells without plant extract served as control. Population of apoptotic cells with low mitochondrial membrane potential ($\Delta\Psi_m$) were counted as a percentage of total number of cells.

Statistical analysis

Results from replicated experiments were depicted as mean ± standard deviation (SD). One-way ANOVA followed by Dunnett's test was used and p value <0.05

was accepted as statistically significant. The concentration of the extract at which 50% of cell growth inhibition (IC_{50} value) was calculated from concentration inhibition curve.

Results

Effect on cell proliferation

Anti-proliferative effect of methanol and ethanol extracts of *P. macrophylla* at different concentrations (10, 20, 40, and 80 $\mu\text{g}/\text{mL}$) was evaluated against Colo-205 cells by tetrazolium-based MTT assay. The methanol extract was effective and exhibited the highest percentage of cell growth inhibition of 89.4 ± 1.2 at 80 $\mu\text{g}/\text{mL}$ concentration, with IC_{50} value of 26.1 $\mu\text{g}/\text{mL}$. In contrast, the proliferation of Colo-205 cells was minimally affected by ethanol extract with highest cell growth inhibition of 61.5 ± 2.1 and IC_{50} value of 51.0 $\mu\text{g}/\text{mL}$ (Table I). The lower IC_{50} value indicates the higher anti-proliferative potential of the plant extract. The findings of our study show that methanol extract was most effective against Colo-205 cells. Therefore, further experiments were carried out to study the apoptotic induction of methanol extract on Colo-205 cells.

Effect on dot-blot light scattering

One of the important characteristics of any anti-cancer drug is the inhibition of cell growth proliferation and apoptosis induction in cancer cells (Lowe and Lin, 2000). Methanol extract induced apoptosis in Colo-205 cells in concentration-dependent pattern (Figure 1). Treatment of Colo-205 cells with different concentrations of extract (0-80 $\mu\text{g}/\text{mL}$) significantly increased the side-scattering (SSC) profile due to increased cellular

Table I			
Anti-cancer activity of <i>P. macrophylla</i> against Colo-205 cancer cells			
Sample	Concentration	% Growth inhibition	IC_{50} value
Untreated control	0 $\mu\text{g}/\text{mL}$	1.3 ± 2.6	
Methanolic extract	10 $\mu\text{g}/\text{mL}$	26.1 ± 1.2	26.1
	20 $\mu\text{g}/\text{mL}$	47.3 ± 2.8	
	40 $\mu\text{g}/\text{mL}$	68.8 ± 1.7	
	80 $\mu\text{g}/\text{mL}$	89.4 ± 1.2	
Ethyl acetate extract	10 $\mu\text{g}/\text{mL}$	14.0 ± 2.0	51.0
	20 $\mu\text{g}/\text{mL}$	29.7 ± 1.8	
	40 $\mu\text{g}/\text{mL}$	46.2 ± 1.9	
	80 $\mu\text{g}/\text{mL}$	61.5 ± 2.1	
5-Fluorouracil	20 μM	63.9 ± 2.5	

The results are expressed as mean \pm SD of three independent experiments

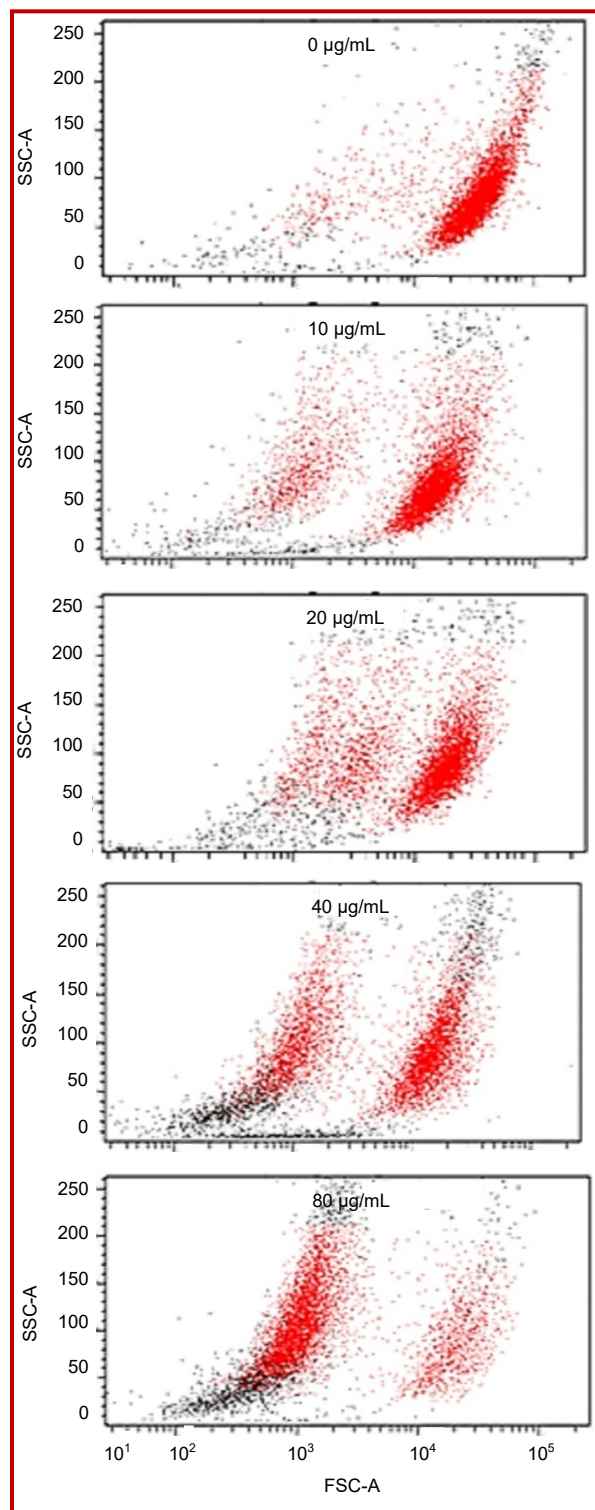


Figure 1: Effect of methanol extract of *P. macrophylla* on dot-blot light scattering profile of Colo-205 cancer cells. Treatment of Colo-205 cells with different concentration of extract (0-80 $\mu\text{g}/\text{mL}$) significantly increased the side-scattering (SSC) profile due to increased cellular granularity because of chromatin fragmentation and significant decrease in forward-scattering (FSC) profile due to shrinkage of cells

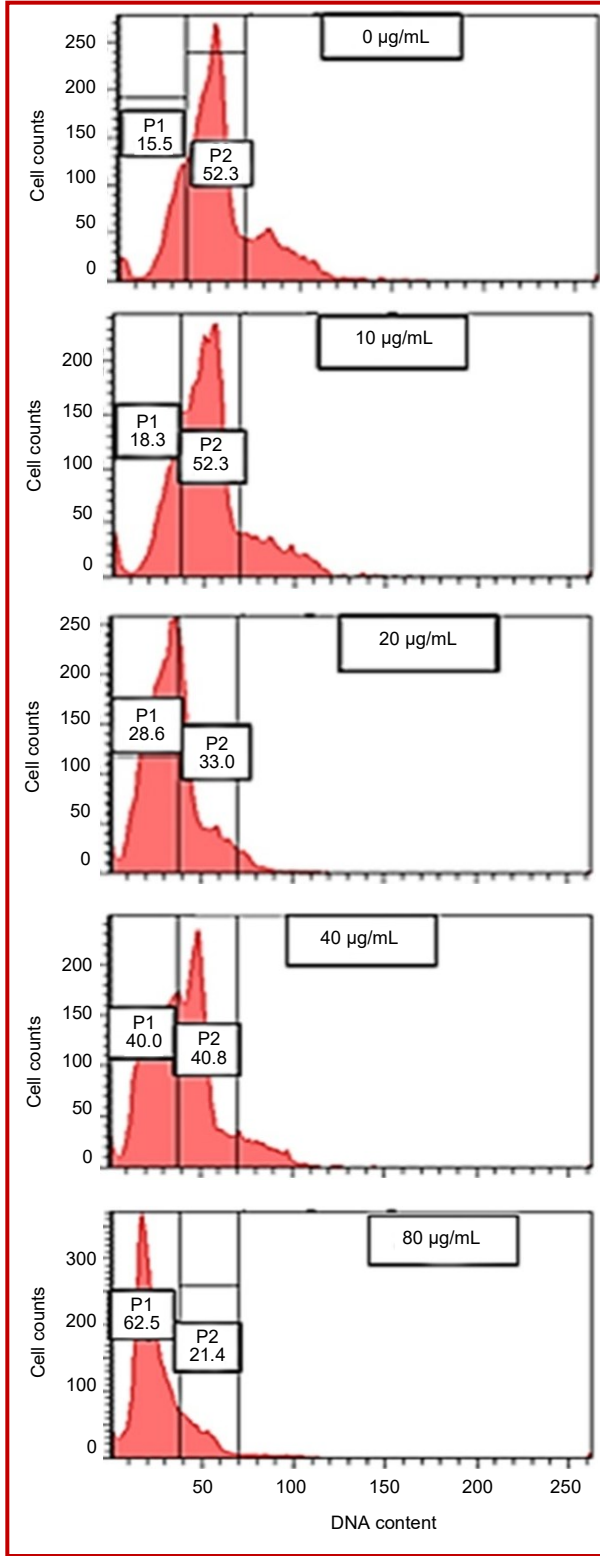


Figure 2: Methanol extract of *P. macrophylla* induced cell cycle arrest in COLO-205 cells at G₀/G₁ phase of cell cycle. Exposure of cells with different concentrations of extract caused dose dependent cell cycle arrest at G₀/G₁ phase and triggered apoptosis by increasing accumulation of cells at sub-G₀ phase

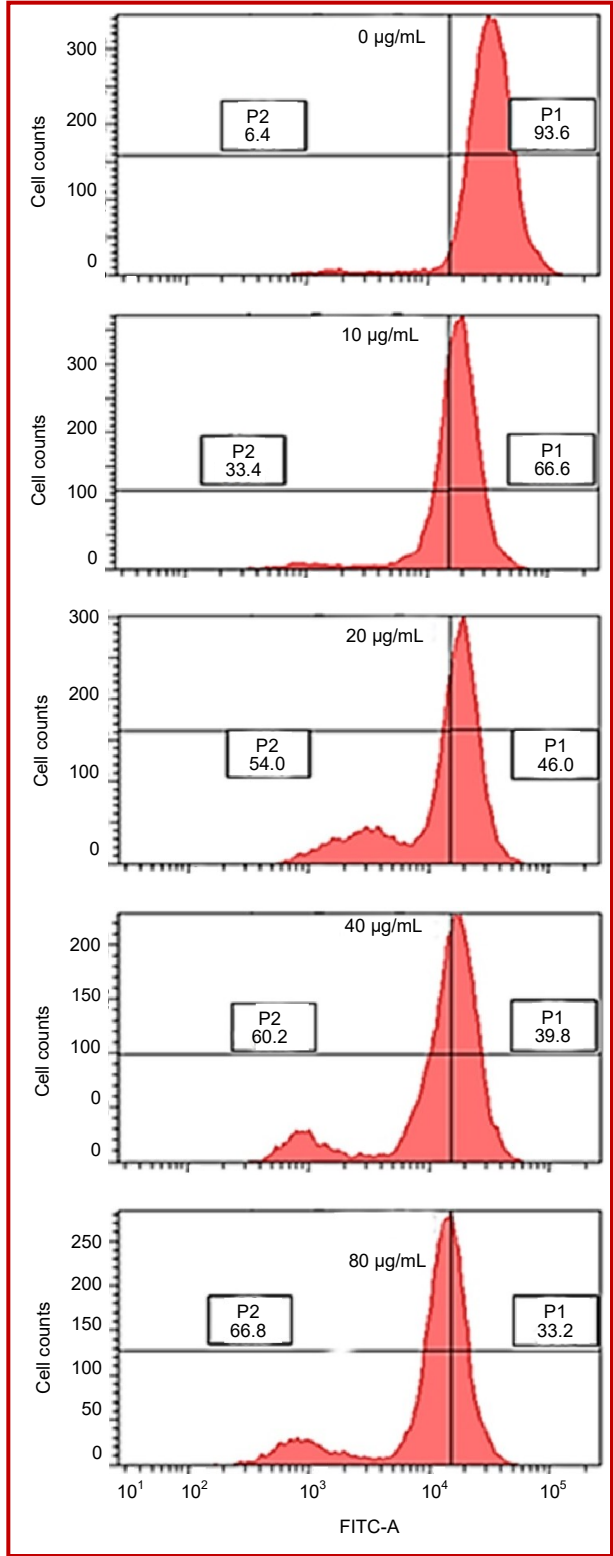


Figure 3: Effect of methanol extract of *P. macrophylla* on mitochondrial membrane potential ($\Delta\Psi_m$). Extract induced significant increase in mitochondrial membrane potential ($\Delta\Psi_m$) loss in concentration dependent manner

granularity because of chromatin fragmentation and significant decrease in forward-scattering (FSC) profile due to shrinkage of cells (Shan et al., 1998).

Induced cell cycle arrest

Effect of methanol extract of *P. macrophylla* on cell cycle progression in Colo-205 cells was evaluated by propidium iodide stained flow cytometry. Exposure of Colo-205 cells with different concentrations of methanol extract (0-80 µg/mL) caused dose-dependent cell cycle arrest at G₀/G₁ phase and triggered apoptosis by increasing accumulation of cells at sub-G₀ phase, confirms that methanol extract induced apoptosis in Colo-205. The percentage of cells undergoing apoptosis improved from 15.5% in untreated cells to 18.3, 28.6, 40.0 and 62.5% respectively at 10, 20, 40 and 80 µg/mL methanol extract (Figure 2).

Effect on mitochondrial membrane potential ($\Delta\Psi_m$)

The methanol extract of *P. macrophylla* induced significant increase in mitochondrial membrane potential ($\Delta\Psi_m$) loss in concentration-dependent manner. The loss of mmp ($\Delta\Psi_m$) increased from 6.4% in case of untreated control cells to 33.4, 54.0, 60.2 and 66.8% respectively at 10, 20, 40 and 80 µg/mL of the extract (Figure 3).

Discussion

In the current study, the main aim was to explore the antitumor potential of different extracts of *P. macrophylla* namely methanol extract and ethyl acetate extract against Colo-205 human colon cancer cells. MTT is a soluble tetrazolium dye which is reduced in the mitochondria of glycolytically active viable cells by NADPH-dependent dehydrogenases to insoluble purple formazon complex. The amount of formazon production is directly proportional to the number of viable cells (Mir et al., 2015). Treatment of Colo-205 with different concentrations of methanol and ethanol extract induced a significant decrease in cell proliferation in a concentration-dependent manner. *P. macrophylla* is known to have anti-cancer property (Quzi et al., 2009).

The cytotoxic activity of *P. macrophylla* is due to the presence of phenolic glycosides and saponins (Muller et al., 2006). A number of saponin compounds (macrophyllin, sistostrol, macrophyllin, etc.) have been isolated from *P. macrophylla*. The cell cycle is the major indicator of anti-proliferation effect in cancer cells. Mechanism of cell cycle regulation may be altered by anti-cancer drugs, which ultimately results in arresting of cells at different phases of cell cycle. This leads to inhibition of cell growth and proliferation of cancer cells (Shah and Schwartz, 2005).

Anti-cancer effects and induction of apoptosis in colo-205 cells have been shown by acetoxycyloleanone

exhibits selective through the mediation of NF- κ B and caspase-3 signalling pathways (Zhong et al., 2016).

Cell cycle phase distribution analysis indicates that the methanol extract caused significant increase in sub-G₀ population, possibly due to DNA fragmentation. Exposure of Colo-205 cells with different concentrations of methanol extract (0-80 µg/mL) caused dose-dependent cell cycle arrest at G₀/G₁ phase and triggered apoptosis by increasing accumulation of cells at sub-G₀ phase. Mitochondria plays key role in cell survival and cell death because the majority of cellular energy (ATP) is produced by mitochondria. ATP synthesis requires electric potential across inner mitochondrial membrane (mitochondrial transmembrane potential ($\Delta\Psi_m$)). The permeabilization of mitochondrial outer membrane due to the opening of membrane permeability transition pore induces depolarisation of mitochondrial membrane potential ($\Delta\Psi_m$), release of apoptogenic factors into the cytosol (Dave et al., 2008) and loss of oxidative phosphorylation. All these factors constitute critical events in apoptosis (Liu et al., 2015). Thus, for the assessment of apoptosis, evaluation of mitochondrial membrane potential ($\Delta\Psi_m$) is of critical importance. In addition, anticancer effects and apoptosis induction through a mitochondrial pathway involving DNA damage in Colo-205 cells have been shown by the ethanol extract of *Artemisia sieversiana* (Tang et al., 2015).

Conclusion

Methanol extract inhibits the growth of Colo-205 cancer cells. Treatment of cells with methanol extract results in dose-dependent cytotoxicity, cell cycle arrest at G₀/G₁ phase, increasing side-scattering profile due to nuclear fragmentation and induction of apoptosis via mitochondrial mediated pathways.

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Conflict of Interest

Authors declare no conflict of interest

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