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Original Article

Spirulina platensis as a Natural Antileishmanial Candidate: Effective Inhibition of LRV2+ and LRV2− *Leishmania major* Isolates In Vitro

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Abstract

Background: Drug resistance and treatment failure in *Leishmania* infections are major concerns. *Leishmania* RNA virus 2 (LRV2) enhances host inflammation, indirectly favoring the parasite. Thus, alternative treatments are needed. *Spirulina platensis* has shown antimicrobial potential.

Methods: The alcoholic extract of *S. platensis* was tested against *L. major* with or without LRV2. Anti-promastigote activity was evaluated directly on parasites, cytotoxicity on J774.A1 macrophages, and anti-amastigote effects using the MTT assay.

Results: The extract showed significant, dose-dependent antileishmanial activity against both LRV2+ and LRV2− promastigotes (IC_{50} = 62.5 µg/mL). J774.A1 cells remained viable at 62.5–2000 µg/mL (P = 0.0005). Amastigote growth was inhibited at 1000 and 2000 µg/mL in both strains.

Conclusion: *S. platensis* extract exhibits strong anti-leishmanial activity and low cytotoxicity, suggesting its potential as a natural therapeutic candidate against *L. major*, irrespective of LRV2 status. Further in vivo studies are warranted.

Introduction

Leishmaniasis is a neglected tropical disease caused by *Leishmania* spp., transmitted through bites of infected female phlebotomine sandflies (1). It remains endemic in 99 countries, presenting mainly as visceral and cutaneous forms (2). In Iran, cutaneous

leishmaniasis occurs as anthroponotic (*L. a tropica*) and zoonotic (*L. major*) types (3) the latter often self-healing but leaving disfiguring lesions (4). Despite the availability of first-line treatments like meglumine antimoniate (Glucantime®), sodium stibogluconate (Pentostam®), and Amphotericin B (AmB), treatment of CL is hampered by toxicity, limited efficacy, and increasing reports of treatment failure, especially among immunocompromised patients (5, 6). Thus, safer, affordable plant-based therapies with antimicrobial and immunomodulatory potential are gaining attention (7, 8). Previous studies on Iranian medicinal plants such as *Myrtus communis* and *Artemisia* species have demonstrated notable antileishmanial activities, further supporting the potential of natural products as complementary or alternative therapies (3, 9).

S. platensis (or *Arthrospira platensis*) is a blue-green microalga rich in protein, essential fatty acids, vitamins, and bioactive compounds such as phycocyanin, known for its antioxidant, anti-inflammatory, and immunomodulatory properties. These features distinguish *S. platensis* from other natural products and suggest mechanisms of antileishmanial activity beyond general antimicrobial effects (10). *S. platensis* not only acts directly on the parasite but also promotes macrophage activation, IL-12 and IFN-γ production, and Th1 immune responses. Unlike other algae mainly studied for antioxidant effects, *S. platensis* can restore immune balance, broadening its therapeutic potential (11, 12). While some antiparasitic properties have been documented, including activity against *Schistosoma mansoni* (13), evidence on its efficacy against *Leishmania* spp. remains limited. *Leishmania* RNA Virus 2 (LRV2), an endosymbiotic virus found in Old World *Leishmania* species, contributes to disease pathogenesis and treatment resistance by modulating host immune responses, potentially altering parasite survival and drug susceptibility (14, 15). LRV2 may modulate host immune responses, potentially altering parasite survival and drug susceptibility.

The prevalence of LRV2 in *L. major* isolates in Iran, combined with the high burden of cutaneous leishmaniasis in other endemic regions such as parts of the Middle East and South America, underscores the urgent and global need for novel, broadly effective therapies (16).

We aimed to evaluate the in vitro antileishmanial activity of *S. platensis* ethanolic extract against both LRV2-positive and LRV2-negative strains of *L. major*, with a focus on promastigote, amastigote, and cytotoxic responses in macrophage models.

Materials and Methods

Materials

Amphotericin B was purchased from Xgen Pharmaceuticals (Big Flatts, NY). Penicillin and streptomycin were obtained from Tehran Pharmacy, Iran, and were stored at room temperature (25°C) until testing. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] powder was purchased from Biobasic, Ontario, Canada. Fetal Bovine serum (FBS), RPMI-1640 medium with L-glutamine, and Tween 20 were prepared from Bioideaco (Tehran, Iran). All other chemicals and solvents were prepared from commercially available materials with the highest purity.

Extract preparation

The alcoholic extract of *S. platensis* (Ghazaye Sabze Khalij Company, Iran) was prepared by maceration in absolute ethanol, followed by rotary evaporation under reduced pressure. Fifty grams of powder were macerated with 500 mL ethanol at 25 ± 2 °C for three consecutive 48 h cycles with continuous stirring (150 rpm), filtering and remacerating with fresh ethanol after each cycle. The combined filtrates were concentrated under reduced pressure at 40 °C using a rotary evaporator and stored at 4 °C for further use. Ethanol was chosen for its safety, stability, and high efficiency in extracting lipophilic antiparasitic compounds such as salicylic acid. (17, 18). Subsequently, the ethanolic extract was vacuum-concentrated and refrigerated (19). Chemical characterization of the ethanolic

extract was not conducted; thus, the composition and levels of bioactive compounds like phycocyanin and salicylic acid remain unconfirmed. This limitation should be considered when interpreting the antileishmanial findings. The dried extract was dissolved in ≤1.6% DMSO (Sigma, USA) using 5 min sonication to prepare a stock solution, stored at −20 °C. Serial 2-fold dilutions (2000–62.5 µg/mL) were prepared in RPMI-1640 and sterilized through a 0.22 µm filter for anti-*Leishmania* assays.

Leishmania isolates

Two different strains of *L. major*, LRV2− isolate (MRHO/IR/75/ER) and LRV2+ isolate (Acc. No.: OR493488), available in the Leishmaniasis Laboratory, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, were included in this study. The presence of LRV2 was confirmed using a nested-PCR (20).

Parasite culture

Promastigotes of *L. major* isolates were prepared from the *Leishmania* Bank of the Leishmaniasis Laboratory, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. Promastigotes LRV2+ and LRV2− isolates were sub-cultured every 4-6 days in RPMI-1640 supplemented by 10% FBS and 1% Pen/Strep and incubated at 25 °C.

Anti-promastigote assay

Briefly, 2.5 × 10^6 LRV2+ and 1.75 × 10^6 LRV2- promastigotes in the stationary phase were added to 1 mL/well RPMI-1640 with 10% FBS in 24-well plates containing various extract concentrations. After 48 h at 25°C, viable parasites were counted using a Neubauer chamber and light microscopy (400×) after trypan blue staining (21). One hundred microliters of each suspension were transferred to 96-well plates, 10 µL of 5 mg/mL MTT was added, incubated 4 h at 25 °C, formazan dissolved in 100 µL DMSO, and absorbance measured at 570 nm. Amphotericin B (0.5 mg/mL) served as positive control; negative control: medium without parasites. Untreated parasites and 1.6% DMSO controls showed no significant difference. All assays were performed in duplicate and repeated. IC_{50} was calculated to determine extract efficacy.

J774.A1 culture and cytotoxicity assays

Murine macrophage cells (J774A.1; TIB-67) from the Pasteur Institute of Iran were cultured in RPMI-1640 with 15% FBS and 1% Pen/Strep at 37 °C, 5% CO_2, with medium changed every 2–3 days. To assess *S. platensis* cytotoxicity, 4×10^3 cells/well were seeded in 96-well plates and treated with extract (62.5–2000 µg/mL) for 48 h (22). Cell viability was measured via MTT assay: after incubation, 20 µL of 5 mg/mL MTT was added for 3 h at 25 °C, supernatant removed, 100 µL DMSO added, and absorbance recorded at 570 nm using an ELISA reader (23, 24). CC50 was calculated based on the proportion of viable cells (25, 26).

Anti-intracellular amastigotes activity

J774.A1 mouse macrophages were cultured in RPMI-1640 with 10% FBS on 8-well chamber slides (Nunc, Denmark) at a density of 4 × 10^5 cells/well. After 24 h, cells were infected with LRV2+ and LRV2− promastigotes in the log phase (7 days post subculture) at a multiplicity of infection (MOI) of 5 and incubated overnight at 37 °C with 5% CO_2 without antibiotics. Unattached parasites were removed by washing with RPMI-1640, after which cells were treated with 2000 and 1000 µg/mL extract (500 µL/well) for 48 h under the same conditions.

Based on prior cytotoxicity assessments on J774.A1 macrophages, concentrations of 1000 and 2000 µg/mL were selected, as doses below 1000 µg/mL showed minimal antiparasitic activity, while concentrations above 2000 µg/mL approached the cytotoxic threshold for host cells. Anti-amastigote activity was evaluated on methanol-fixed, Giemsa-stained chamber slides following standard procedures. Amastigotes were counted in 100 macrophages, and the survival index was calculated as (% infected macrophages × mean amastigotes per infected macrophage ÷ total macrophages). Inhibition percentage and IC_{50} values were determined relative to

controls, as previously described for CC_{50} (24, 26).

Statistical analysis

All analyses were performed using GraphPad Prism v9.0.0 (GraphPad Software, San Diego, CA, USA). One-way ANOVA with Tukey's post-hoc test compared extract concentrations in anti-promastigote and anti-amastigote assays. The Wilcoxon–Mann–Whitney test was applied for non-parametric comparisons between LRV2+ and LRV2− strains. One-sample t-test assessed differences between treated and control groups. Data normality and variance homogeneity were verified before analysis.

Results

In vitro antileishmanial effects of S. platensis extract

Anti-promastigote activity

S. platensis ethanolic extract showed strong, dose-dependent antileishmanial activity against both LRV2+ ($P < 0.0001$) and LRV2− ($P = 0.0001$) L. major promastigotes after 48 h. The IC_{50} value was 62.5 µg/mL for both isolates. No significant association was found between LRV2 presence and resistance to the extract ($P > 0.05$). Mann–Whitney U analysis confirmed no significant difference in inhibition rates between LRV2+ and LRV2− isolates. In LRV2+ *L. major*, the highest and lowest inhibition occurred at 2000 µg/mL (93.53 ± 2.08) and 125 µg/mL (61.35 ± 2.81), respectively, while in LRV2− isolates these were 95.23 ± 0.21 and 58.5 ± 6.58, respectively (Fig. 1, Table 1). The relatively wide 95% confidence intervals observed at lower extract concentrations (e.g., 250 and 125 µg/mL) reflect natural experimental variability due to small sample sizes and inherent differences in parasite susceptibility. These intervals were constrained within the biologically plausible range of 0–100% to ensure interpretability.

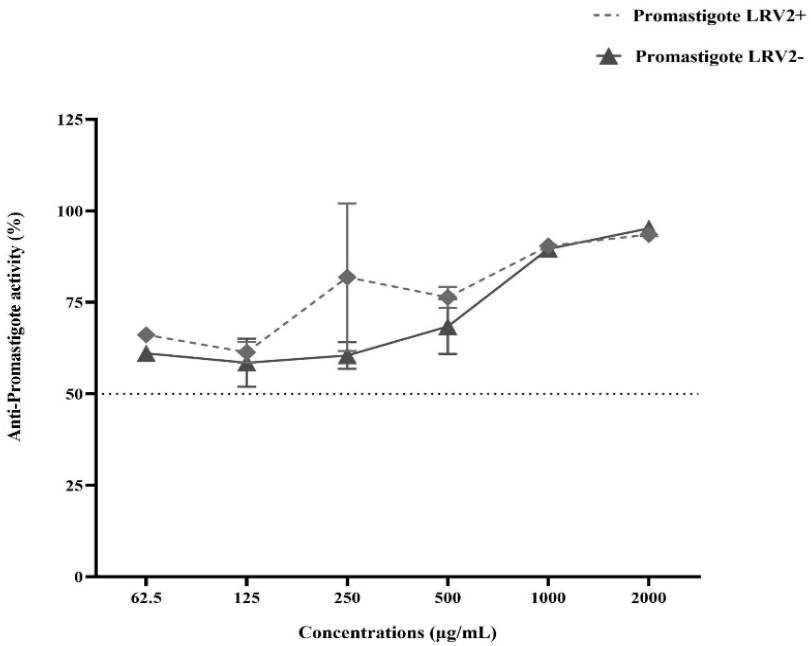

Fig. 1: Dose-dependent anti-promastigote activity of *S. platensis* ethanolic extract against LRV2+ and LRV2− *L. major* after 48 h. X-axis: extract concentrations (µg/mL); Y-axis: % viable promastigotes vs. control. Significant reduction observed at all concentrations; $IC_{50} < 62.5$ µg/mL. Data: mean ± SD of three independent experiments in triplicate. Statistical analysis: one-way ANOVA with Tukey's post hoc ($P < 0.05$)

Table 1: Percentage inhibition of *L. major* promastigotes (LRV2+ and LRV2−) at various concentrations of *S. platensis* extract

Concentrations (µg/mL)	Anti-Promastigote activity for LRV2+	95% CI	*P*-value	Anti-Promastigote activity for LRV2−	95% CI	*P*-value
2000	93.53 ± 2.07	89.40 to 97.66	<0.0001	95.23 ± 0.20	94.63 to 95.83	0.0001
1000	90.40 ± 0.10	90.20 to 90.60	-	89.58 ± 1.13	87.32 to 91.84	-
500	76.37 ± 2.88	70.61 to 82.13	-	68.38 ± 7.50	54.18 to 82.58	-
250	81.87 ± 20.16	61.63 to 100.00	-	60.49 ± 3.66	54.83 to 66.15	-
125	61.35 ± 2.80	55.91 to 66.79	-	58.50 ± 6.58	50.77 to 66.23	-
62.5	66.14 ± 2.08	61.26 to 71.02	-	61.05 ± 1.49	58.56 to 63.54	-

Note: One-sample *t*-test was employed to evaluate the statistical correlations among samples

Cytotoxic effects

Cytotoxicity analysis showed that J774.A1 cells remained viable at all *S. platensis* extract concentrations, with no significant toxicity (Fig. 2). Effects on cell viability were dose-dependent (P = 0.0005), with highest toxicity at 2000 µg/mL (87.9 ± 0.466; 83.71–92.1) and lowest at 62.5 µg/mL (206.43 ± 1.46; 193.31–219.55) (Table 2).

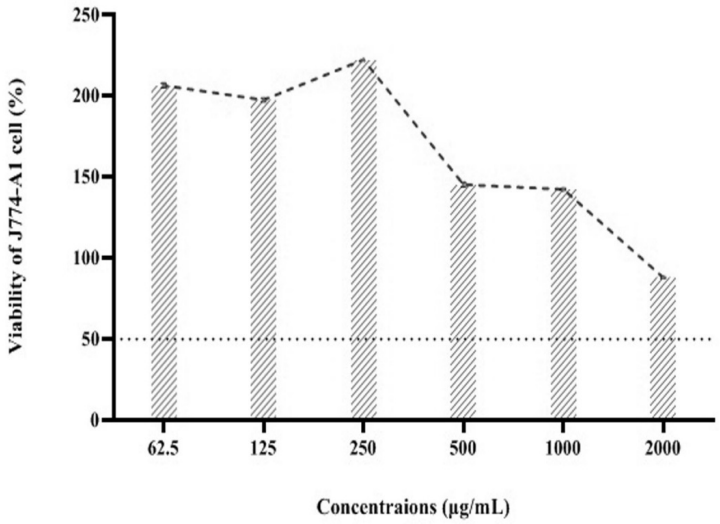

Fig. 2: Cytotoxicity of *S. platensis* ethanolic extract on J774.A1 macrophages after 48 h. X-axis: extract concentrations (µg/mL); Y-axis: cell viability (%) by MTT assay. No significant cytotoxicity observed up to 2000 µg/mL. CC_{50} > 2 mg/mL (p = 0.0005). Data: mean ± SD of three independent experiments in triplicate. Macrophages were cultured in RPMI-1640 + 10% FBS at 37 °C, 5% CO_2

Table 2: Viability (%) of J774.A1 macrophages after 48-hour exposure to different concentrations of *S. platensis* extract. Values above 100% indicate potential stimulatory effects on cell proliferation or metabolic activity

Concentrations (µg /mL)	Cytotoxicity assay		*P*-value
	Mean ± SD (%)	95% CI	
2000	87.90 ± 0.46	83.70 to 92.09	0.0005
1000	142.31 ± 0.68	136.15 to 148.47	
500	145.20 ± 1.42	132.35 to158.04	
250	222.32 ± 0.10	221.40 to 223.23	
125	197.49 ± 1.33	185.48 to 209.50	
62.5	206.43 ± 1.46	193.31 to 219.55	

Note: Viability values >100% may indicate stimulatory effects of *S. platensis* extract on macrophage proliferation/metabolic activity, consistent with its reported immunostimulatory properties

Anti-amastigote assay

The effects of *S. platensis* on the amastigote form of *L. major* were investigated through staining and counting viable *L. major* infected J774.A1 cells. *S. platensis* significantly inhibited amastigote development in 2000 and 1000 µg/mL in both LRV2+ (P = 0.025) and LRV2− (P = 0.032) isolates compared with untreated controls. No difference between LRV2+ and LRV2− strains indicates that inhibition was independent of LRV2 status. Amphotericin B (positive control) strongly suppressed amastigotes, while untreated cells showed normal proliferation. Moreover, *S. platensis* enhanced host cell viability in a dose-dependent manner (Fig. 3).

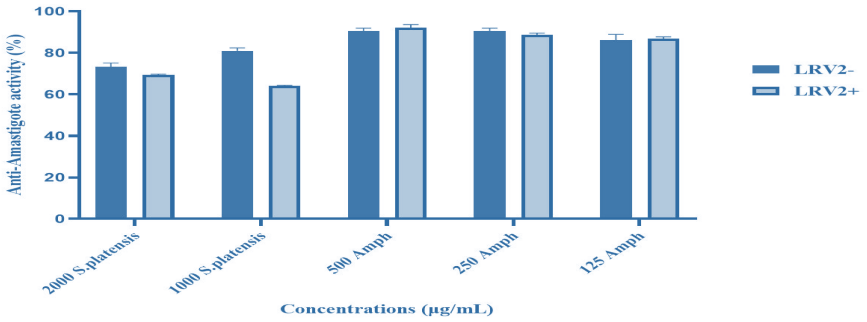

Fig. 3: Inhibitory effect of *S. platensis* ethanolic extract on intracellular amastigotes of LRV2+ and LRV2− *L. major* in J774.A1 macrophages. X-axis: treatment groups (control, 1000 µg/mL, 2000 µg/mL, AmB). Y-axis: amastigotes per 100 macrophages (Giemsa stain). After 48 h, both extract concentrations significantly reduced amastigote load. Data: mean ± SD of three independent experiments in triplicate. Statistical analysis: one-way ANOVA with Tukey's post hoc ($p < 0.05$). Macrophages were infected with promastigotes at 1:10 ratio and incubated in RPMI-1640 + 10% FBS at 37 °C, 5% CO_2

Discussion

The increasing resistance to conventional antileishmanial drugs such as meglumine antimoniate and AmB, along with their adverse side effects, has intensified the need for new, safer, and more effective therapies (27). In this context, herbal extracts with immunomodulatory and antimicrobial properties have gained attention as promising candidates (28).

In our study, *S. platensis* ethanolic extract demonstrated significant dose-dependent antileishmanial effects on both LRV2+ and LRV2− *L. major* isolates. The extract showed

an IC_{50} of 62.5 µg/mL against promastigotes. Although less potent than AmB (IC_{50}: 0.1–1 µg/mL in similar models), its low cytotoxicity (CC_{50} > 2 mg/mL) suggests a favorable safety profile. Unlike AmB, which causes nephrotoxicity and infusion-related side effects limiting its use (29). *S. platensis* extract combines antileishmanial efficacy with high biocompatibility, making it a promising option for long-term or adjunctive therapy (30).

The markedly lower IC_{50} of *S. platensis* extract against *L. major* compared with bacterial (*E. coli*, *S. aureus*) and fungal (*C. albicans*) pathogens indicates selective antiparasitic activity. This may stem from interactions with *Leishmania*-specific targets—such as surface glycoconjugates, the kinetoplast, or unique metabolic pathways—absent in other microbes, supporting its targeted and safe potential.

The antileishmanial effect was unaffected by LRV2 status, indicating activity independent of viral endosymbiosis. Moreover, *S. platensis* has been shown to enhance Th1 immune responses (IL-12, IFN-γ), and induce nitric oxide and ROS production, facilitating macrophage-mediated parasite clearance (31, 32). These mechanisms are crucial for controlling *Leishmania* infections, and *S. platensis* appears to exploit them to counter the parasite.

Conversely, *Leishmania* RNA virus 2 (LRV2) suppresses IL-12 and IFN-γ production, weakens Th1 responses, and alters macrophage signaling, reducing antileishmanial activity. Since *S. platensis* showed similar efficacy against LRV2-positive and -negative strains, its action seems independent of LRV2. This independence is therapeutically valuable, as LRV2 can diminish the efficacy of conventional drugs like meglumine antimoniate and worsen lesion severity. Thus, by bypassing LRV2-associated immune evasion, *S. platensis* may provide consistent efficacy across infections, particularly in regions with high LRV2 prevalence (33, 34).

Treatment with *S. platensis* at 1000 and 2000 µg/mL caused over 50% inhibition of intracellular amastigotes in both LRV2+ and LRV2− macrophages. Since amastigotes are the clinically relevant and drug-resistant form (38), this finding is notable. The extract showed low cytotoxicity on J774.A1 cells (CC_{50} > 2 mg/mL), indicating good biocompatibility, consistent with previous reports on *S. platensis* components such as phycocyanin and polysaccharides (35-37).

Although immunomodulatory mechanisms such as enhanced nitric oxide (NO) and reactive oxygen species (ROS) production were not directly measured in this study, these pathways are well-established mediators of parasite clearance. NO and ROS are produced by activated macrophages and exert cytotoxic effects by inducing oxidative and nitrosative stress, damaging parasite DNA, proteins, and membrane lipids, ultimately leading to apoptosis-like death of Leishmania spp (25). Studies with other natural compounds support this mechanism, demonstrating that increased NO and ROS enhance macrophage-mediated parasite killing (38, 39).

These functions are critical in the control of *Leishmania* infection, especially in the context of intracellular survival and immune evasion. LRV2, previously shown to interfere with treatment outcomes and exacerbate lesion severity in some clinical studies (40), did not alter the efficacy of *S. platensis* in this study. This suggests that the extract may act through mechanisms independent of viral modulation and could overcome one of the challenges associated with traditional therapies. The minimum inhibitory concentrations of *S. platensis* extract against *E. coli*, *S. aureus*, and *Candida albicans* (5–32 mg/mL) (41), are much higher than the effective doses against *L. major* observed here, indicating selective antiparasitic activity. This selectivity may result from interactions with *Leishmania*-specific features—such as surface glycoconjugates, the kinetoplast, or unique metabolic pathways—absent in bacteria and fungi.

Thus, *S. platensis* likely disrupts parasite-specific processes while sparing host cells and other microbes, supporting its potential as a safe, targeted antileishmanial agent. Overall, the findings support the notion that *S. platensis* extract, either as a monotherapy or in combination with existing antileishmanial drugs, could offer a safe, affordable, and effective strategy for the treatment of cutaneous leishmaniasis. However, further investigations using in vivo models and different *Leishmania* species are warranted to validate its clinical applicability and define its exact molecular mechanism of action.

Limitations

While this study demonstrates the promising in vitro antileishmanial activity of *S. platensis* extract against *L. major*, some limitations exist. The lack of in vivo validation limits translational relevance, and the long-term safety and pharmacokinetic profiles remain unassessed. The lack of detailed characterization of the extract (e.g., bioactive components and their concentrations) may impact reproducibility and limit mechanistic clarity. The study also did not assess the extract's impact on immune responses or signaling pathways in infected cells, which could clarify its mechanism of action. Future studies should include animal models, toxicity tests, extract characterization, and molecular immunological analyses to better define the therapeutic potential of *S. platensis*.

Conclusion

The ethanolic extract of *S. platensis* shows significant antileishmanial activity against both LRV2+ and LRV2− L. major promastigotes (IC_{50} = 62.5 µg/mL) and low cytotoxicity toward J774.A1 cells. Its LRV2-independent activity highlights potential as an alternative or adjunct therapy for cutaneous leishmaniasis. Further in vivo studies and mechanistic investigations are needed to confirm therapeutic applicability.

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Conflict of interest

There is no conflict of interest.

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