LC-MS analysis of antimicrobial compound from bark extracts of *Poeciloneuron indicum* and *Suregada angustifolia*

Sandhya Rani, D\(^1\)*, Basavaraj GL\(^2\), Deepa R. Hebbar\(^3\), and Gopal GV\(^5\)

\(^1,3\)Associate Professor of Botany, Maharani’s Science College for Women, JLB Road, Mysuru, Karnataka, India
\(^2\)Associate professor of Botany, Government College for Women, Mandya, Karnataka, India
\(^5\)Professor, Department of Botany, Regional Institute of Education, Mysuru, Karnataka, India

Abstract

*Poeciloneuron indicum* Bedd. and *Suregada angustifolia* (Baill. ex Muell. Arg) Airy Shaw are endemic trees found in evergreen forest. Despite of high folkloric medicinal values of these plants, chemical profiling remains unexplored. This work is the first report dealing with identification of organic compounds present in the active fractions of these plants showing antimicrobial activity. The chloroform extract of *P. indicum* bark and ethanol extract of *S. angustifolia* bark were fractionated using silica column and the fractions showing activity against *Bacillus subtilis* 168 and *Pseudomonas aeruginosa* MTCC424 were subjected for LC-MS analysis. Around eight compounds were identified in the fraction F-2 of *P. indicum*. Besides, six compounds from F-1 fraction, five from F-4 and six compounds from F-6 fraction were confirmed in *S. angustifolia*. These compounds of superior biological activity support the therapeutic potential of selected taxa.

**Key words:** LC-MS, *Poeciloneuron indicum*, *Suregada angustifolia*, antimicrobial, fractionation

Introduction

In the last few decades, scientific world has witnessed the resurgence of plant derivatives in traditional medicine as safer and effective drugs in treatment of several diseases. Some of the chemical substances of medicinal plants play a definite physiological role in maintaining individual health. The unexplored possibilities of traditional medicines are sought for modern scientific world for newer remedies from novel compounds. In present scenario, analytical chemistry of medicinal plants or folk medicine is experiencing a huge expansion [1,2].
Rapid detection and characterization of natural metabolites play an important role in supporting the quality and efficiency of the metabolite as a drug. In this regard, chromatographic techniques are extensively used for separation of compounds and to achieve purity of the compound. The method allows authenticated fingerprints between unknown samples and standard, thereby facilitating removal of contaminations, quantitative measurement and precise identification.

HPLC provides efficient separation of non-volatile compounds [3]. HPLC can be used to detect and quantify a compound in the mixture by comparing the retention time with specific standard of known concentration. However for unknown samples, Liquid chromatography (LC) coupled with mass spectrometer (MS) and online database is used for appropriate detection and structural prediction. The selective response of individual compound, mode of ionization reflects the compound identity. LC-MS has been extensively used for analysis of complex plant extracts [4]. It is highly sophisticated and a powerful analytical technique applied for quantitative identification and mass analysis of non-volatile and semi-volatile inorganic as well as organic compounds. Kharyuk et al [5] have fantastically presented the application of LCMS for analysis of 74 species of medicinal plant extract and validating the species identification algorithms. Flavonol such as rutin, quercetin and their derivatives have been reported to be present in many plant extracts and juices by using LC-MS technique [6].

Poeciloneuron indicum Bedd. (Clusiaceae) and Suregada angustifolia (Baill. ex Muell. Arg) Airy Shaw (Euphorbiaceae) are endemic trees mostly found in the evergreen forests of Western Ghats, India. They are used in folk medicine and some herbal formulations to treat certain diseases. Bark of P. indicum is used in the treatment of dysentery, cholera and diarrhea [7]. Some of the tribals in Tamil nadu, India use the stem bark of Suregada angustifolia in mouth wash and also for treating skin infections. A number of species of these genera are well studied and various reports have been published in scientific journals regarding their possible application in treating various diseases. However, the data on the selected species is limited. In our previous study, we evaluated the antimicrobial and antioxidant property of various solvent extracts from P. indicum and S. angustifolia leaf and bark [8,9]. The chloroform extract of P. indicum bark and ethanol extract of S. angustifolia bark was found to show significant antimicrobial activity. This present investigation, for the first time, reveals the chemical composition of active fractions from these samples.

MATERIALS AND METHOD

Media Chemicals, reagents and solvents

Solvents such as Methanol, Chloroform, Trifluoro acetic acid were purchased from SRL, India.
BHI (Brain heart infusion) media was procured from Hi Media, India. Sephadex G-75 was procured from Sigma, India. Column chromatography and LCMS facilities were obtained from Institute of Excellence (IOE), University of Mysore, Mysore, Karnataka, India.
Plant material and extracts

Poeciloneuron indicum Bedd. was collected from Sirimane falls, Kigga, Sringeri (Tq), Chikmagaluru (Dist) and Suregada angustifolia (Baill. ex Muell. Arg) Airy Shaw was collected from Western Ghats near Hosnagar, Shimoga district in Karnataka, India. The botanical identity of the collected specimens was authenticated by taxonomists from University of Agricultural Sciences, Bangalore, India and voucher specimens were deposited (UASB-5278 and UASB-5279 respectively). The bark of each plant was excised separately, powdered and extracted using soxhlet apparatus. The extraction solvent chloroform was used for P. indicum bark extraction, whereas ethanol was used for S. angustifolia bark. The extracts were dried under reduced pressure and stored in desiccator until further use.

Fractionation of P. indicum and S. angustifolia bark extracts

The chloroform extract from P. indicum bark and ethanol extract of S. angustifolia bark were subjected to a column chromatography using silica 100 and eluted with methanol. Each fraction obtained was analyzed for total phenol content by Folin-Ciocalteau Reagent method as described by Volluri et al., [10].

Antimicrobial activity of the extracts

The fractions obtained from column chromatography were checked for antimicrobial activity against Pseudomonas aeruginosa MTCC424 and Bacillus subtilis 168 by disc diffusion assay. Briefly, freshly grown bacterial cultures were spread on BHI agar plates using sterile swabs. Sterile filter paper disc of 4 mm in diameter was placed on the media at equidistance. Then, 10 μl of filter sterilized extract was spotted on the disc carefully and allowed to absorb. Later, plates were incubated at 37°C for 24-48 h. After incubation, the zone of inhibition was measured in mm and recorded. Antibiotic chloramphenicol (1mg/ml) was used as positive control.

Liquid Chromatography- Mass Spectrometry (LCMS) Analysis

The chemical constituents of the active fraction (F2) from chloroform extract of P. indicum bark and fractions (F1, F4 and F6) from ethanol extract of S. angustifolia were determined by using LC-MS. Waters UPLC model Acquity UPLC, USA equipped with degasser, binary pump, an autosampler, a column heater and a diode array detector was used. The chromatographic separation was performed using an BEH C18 (Ethylene bridge hybrid) analytical column (2.1 mm x 50 mm x 1.7 μm particle size). The mobile phase consisted of Solvent A (water with 1%formic Acid) and solvent B (methanol). The flow rate was kept at 0.4 ml/min. The gradient elution started with 98% A/2% B 0-4 min, 2%A/98%B 4-6 min, 98% A/2% B 6-7 min, 98% A/2% B 7-8 min. Photodiode array detector was set at 324 nm for acquiring chromatograms. The injection volume was 2 μl and peaks were monitored at 254 nm.
Mass Spectrometric Analysis

Mass spectrometric analysis was performed using a Xeno G2 Xs-QToF mass spectrometer. Mass spectral data was recorded on an ionization mode for a mass range of m/z 50-1500. Mass spectrometer conditions were as follows: capillary voltage 2KV, source temperature 120°C, nebulizing gas pressure: 40 psi; desolvation gas flow: 750L/h; drying gas temperature: 450 °C; nebulizing gas flow: 1.5 L/min. The specific negative and positive ionization modes were used to analyze the compounds.

RESULTS

Goal of the present investigation was to perform comprehensive characterization of bioactive compounds present in the chloroform extract of *P. indicum* bark and ethanol extract of *S. angustifolia* bark.

Antimicrobial activity of fractions obtained by Column Chromatography

The extracts from *P. indicum* and *S. angustifolia* were subjected to column chromatography on silica 100. Three fractions (F1–F3) were obtained from the extract of *P. indicum* and seven (F1-F7) were obtained from *S. angustifolia* extract. Fraction F2 of *P. indicum* showed good inhibitory activity against the tested pathogens with 12 mm zone of inhibition (Figure 1a and 1 b). On the other hand, fractions F1, F4 and F6 of *S. angustifolia* bark displayed inhibitory activity with 10-12 mm zone of inhibition (Figure 1c and 1d).
Figure 1: Antimicrobial activity of active fractions from *P. indicum* (F1, F2 and F3) against (a) *Bacillus subtilis*; (b) *Pseudomonas aeruginosa*; Active fractions of *S. angustifolia* (F1-F7) against (c) *Bacillus subtilis*; (d) *Pseudomonas aeruginosa*.

Identification of bioactive compound by LCMS

The chemical composition of active fractions showing antimicrobial activity was determined using the LC-MS technique. In the present study, to obtain satisfactory separation, the extract was analyzed using gradient mobile phase consisting of 0.1% formic acid in water. Flow rate and MS conditions were optimized. Base peak chromatogram of *P. indicum* and *S. angustifolia* active fractions are presented in figure 2. Retention time (RT), molecular formula, and observed m/z of identified compound is presented in table 1 and 2.
Figure 2: LC-MS chromatogram of (a) F-2 from *P. indicum* chloroform bark extract; (b) F-1; (c) F-4; (d) F-4 from *S. angustifolia* ethanol bark extract

Table 1: Details of bioactive compounds identified by LC-MS in the active fraction of *P. indicum* chloroform bark extract

<table>
<thead>
<tr>
<th>Compound identified</th>
<th>Retention time (RT)</th>
<th>Molecular formula</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyclovir</td>
<td>0.338</td>
<td>C₈H₁₁N₅O₃</td>
<td>225</td>
</tr>
<tr>
<td>Methocarbamol</td>
<td>0.338</td>
<td>C₁₁H₁₅NO₅</td>
<td>241</td>
</tr>
<tr>
<td>Gliclazide</td>
<td>0.338</td>
<td>C₁₅H₂₁N₃O₃S</td>
<td>323</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.440</td>
<td>C₁₁H₁₂N₂O₂</td>
<td>203</td>
</tr>
<tr>
<td>Vanilly alcohol</td>
<td>1.15</td>
<td>C₈H₁₀O₃</td>
<td>153</td>
</tr>
<tr>
<td>Demexiptiline</td>
<td>3.315</td>
<td>C₁₈H₁₈N₂O</td>
<td>279</td>
</tr>
<tr>
<td>Procyanidin</td>
<td>3.315</td>
<td>C₃₀H₂₆O₁₃</td>
<td>579</td>
</tr>
<tr>
<td>Quercetin derivative</td>
<td>3.99</td>
<td>C₁₅H₁₀O₇</td>
<td>301</td>
</tr>
</tbody>
</table>
Table 2: Details of bioactive compounds identified by LCMS in the active fractions of *S. angustifolia* ethanol bark extract

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Compound identified</th>
<th>Retention time (RT)</th>
<th>Molecular formula</th>
<th>m/z</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>Methyl 2,2-difluoro-2-(fluorosulfonyl) acetate</td>
<td>3.992</td>
<td>C$_3$H$_3$F$_2$O$_2$S</td>
<td>192.12</td>
</tr>
<tr>
<td></td>
<td>1,2-O-isopropylidene D-glucofuranose</td>
<td>4.398</td>
<td>C$_9$H$_6$O$_6$</td>
<td>220.22</td>
</tr>
<tr>
<td></td>
<td>γ-glutamyl-cysteinyl-glycine</td>
<td>4.838</td>
<td>C$_{10}$H$_7$N$_2$O$_4$S</td>
<td>307.33</td>
</tr>
<tr>
<td></td>
<td>3-acetyl-6-bromo coumarine</td>
<td>5.142</td>
<td>C$_{11}$H$_7$BrO$_3$</td>
<td>267.07</td>
</tr>
<tr>
<td></td>
<td>2,6,7-tri hydroxy-1,4-naphthoquinone</td>
<td>5.142</td>
<td>C$_{10}$H$_6$O$_5$</td>
<td>206.15</td>
</tr>
<tr>
<td></td>
<td>1,8-dihydroxy-4,5-dinitroantraquinone</td>
<td>5.751</td>
<td>C$_{14}$H$_6$N$_2$O$_8$</td>
<td>330.21</td>
</tr>
<tr>
<td>4</td>
<td>Tetratriacontane</td>
<td>4.36</td>
<td>C$_{34}$H$_0$</td>
<td>478.9</td>
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<tr>
<td></td>
<td>p-(2,4,6-trinitroanilino) phenyl acetate</td>
<td>4.84</td>
<td>C$<em>{14}$H$</em>{10}$N$_4$O$_8$</td>
<td>362.3</td>
</tr>
<tr>
<td></td>
<td>5-(p-amino phenyl)-4-(m-bromophenyl)-2-thiazolamine</td>
<td>5.14</td>
<td>C$<em>{15}$H$</em>{12}$BrN$_3$S</td>
<td>346.2</td>
</tr>
<tr>
<td></td>
<td>N-acetyl-phytosphingosine</td>
<td>5.48</td>
<td>C$<em>{20}$H$</em>{41}$NO$_4$</td>
<td>359.54</td>
</tr>
<tr>
<td></td>
<td>Cholesteryl heptadecanoate</td>
<td>5.75</td>
<td>C$<em>{44}$H$</em>{78}$O$_2$</td>
<td>639.1</td>
</tr>
<tr>
<td>6</td>
<td>Xanthosine-5’-monophosphate</td>
<td>1.15</td>
<td>C$<em>{10}$H$</em>{13}$N$_4$O$_5$P</td>
<td>364.04</td>
</tr>
<tr>
<td></td>
<td>1,2,3,4-tetrachloronaphthalene-bis(hexachlorocyclopentadiene)</td>
<td>4.36</td>
<td>C$_{20}$H$<em>4$Cl$</em>{16}$</td>
<td>811.4</td>
</tr>
<tr>
<td></td>
<td>L-alanyl-L-prolylglycine</td>
<td>4.84</td>
<td>C$<em>{10}$H$</em>{17}$N$_3$O$_4$</td>
<td>243.26</td>
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<tr>
<td></td>
<td>6-bromo-2-(2-nitrostyryl)-1H-imidazo (4,5-b) pyridine</td>
<td>5.18</td>
<td>C$_{14}$H$_6$BrN$_4$O$_2$</td>
<td>345.2</td>
</tr>
<tr>
<td></td>
<td>N-acetyl-phytosphingosine</td>
<td>5.48</td>
<td>C$<em>{20}$H$</em>{41}$NO$_4$</td>
<td>359.54</td>
</tr>
<tr>
<td></td>
<td>Bis(2,2,2-trifluoroethyl) phthalate</td>
<td>5.75</td>
<td>C$_{12}$H$_8$F$_6$O$_4$</td>
<td>330.18</td>
</tr>
</tbody>
</table>
DISCUSSION

The study was focused to identify the bioactive molecule showing antimicrobial activity in the chloroform bark extract of *P. indicum* and ethanol bark extract of *S. angustifolia*. For this purpose, column chromatography followed by LC-MS has been used to fully characterize the compounds present in the extract. The highly selective MS detection allowed the specific and rapid analysis of components. Further, Electrospray Ionization (ESI) was applied to identify the active compounds. The compounds were identified based on systematic search for molecular ions obtained by mass chromatograms and comparing the obtained data with previous literature [11-13].

**Compounds identified in the Fraction F-2 of *P. indicum* chloroform bark extract**

The extracts of *P. indicum* bark contained metabolites with molecular masses predominantly in the range \([M + H] 150–1200\). The LC-MS chromatogram displayed eight peaks at different retention times. Each peak was fragmented, and the LC-MS spectrums interpretation was performed using a spectrum database for organic compounds in Spectral Database for Organic Compounds (SDBS) application.

Peak 1 (RT 0.338) exhibited a base peak \([M_H]\) at \(m/z\) 225 and 241. The compound was identified as Acyclovir and methocarbamol (guaiacol glycerol ether carbamate) [14,15]. Earlier report support for the biological activity of acyclovir, especially anti-herpes activity [16]. Methocarbamol has been known for the treatment of muscle spasticity. In the same peak, a fragment at \(m/z\) 323 was observed which was matched with gliclazide similar to sample reported by Al-Qaim *et al* [17]. Gliclazide is a drug used to treat type 2 diabetes [18].

Peak 2 (RT 0.44) exhibited a base peak \([M_H]\) at \(m/z\) 203 a strong fragment related to tryptophan [19].

Peak 3 (RT 1.15) exhibited a base peak \([M_H]\) at \(m/z\) 153 suggesting as vanillyl alcohol [20]. In Peak 4 (RT 2.3), none of the fragmentation matched with any reported compound, hence it is reported as unknown compound.

Peak 5 (RT 3.315) exhibited a base peak \([M_H]\) at \(m/z\) 279 and 579 suggesting as demexiptiline and procyanidin B-2 [21,22]. Demexiptiline is known to have antidepressant effect [23] and procyanidin are polyphenol compounds with potential antioxidant properties [21].

Peak 6 (RT 3.99) exhibited a base peak \([M-H]\) at \(m/z\) 463 and a strong fragment related to an aglycon ion at \(m/z\) 301. The loss of 162 amu from the pseudomolecule is related to the sugar glucose, thereby suggesting the compound as quercetin 3-O-glycoside [24]. Similarly, Hajji *et al.* [25] identified eleven phenolic components by HPLC chromatogram in aqueous extract of *Mirabilis jalapa* tubers. They report negative molecular ion with \([MS−H]+\) at an \(m/z\) of 447 and a negative fragment ion \([MS2−H]+\) of 301. The loss of 162 amu from the intermediate ion has been indicated as loss of sugar rhamnose. Thereby, the corresponding compound was predicted as quercetin-3-O-rhamnoside. Similarly, Kumar *et al.* [26] have shown characteristic fragment ion at \(m/z\) 301 \([Y]\)- and reported as quercetin derivatives. Peak 7 and 8 didn’t show any similarity with the reported compounds, hence are considered unknown.
Compounds identified in the fractions (F1, F4 and F6) of *S. angustifolia* ethanol bark extract

**Fraction 1:**

The fraction F-1 in ethanolic extracts of *S. angustifolia* bark contained metabolites with molecular masses predominantly in the range [M + H] 150–1100. From the LCMS chromatogram, six peaks were identified at different retention times. Peak 1 (RT 3.992) exhibited a base peak [M_H] at m/z 191.18 and 192.13. The compound was identified as Methyl 2,2-difluoro-2-(fluorosulfonyl) acetate. Although the compound has not been documented in any plants, but it has been used as one of the ingredient in trifluromethylation of pyridone 14 to give novel pyridone 8. Pyridone 8 stimulates ghrelin hormone receptor and mediates calcium mobilization [27]. Peak 2 (RT 4.398) exhibited a base peak [M_H] at m/z 220.22 with strong fragment corresponding to 1,2-O-isopropylidene D-glucofuranose. It is a hydrolyzed derivative of D-Glucose and was evaluated for antibacterial functionality against six human pathogens. The study revealed that the compound was effective against Gram positive bacteria than Gram negative bacteria [28]. Peak 3 (RT 4.838) exhibited a base peak [M_H] at m/z 307.26 suggesting as γ-glutamyl-cysteinyl-glycine or glutathione. It is a tripeptide thiol molecule found in several plants and plays a significant role in giving tolerance to environmental stress. It chelates toxic metal ions and protects proteins from oxidative denaturation [29]. Peak 4 (RT 5.142) with m/z 267.08 was matched as 3-acetyl-6-bromo coumarine, a coumarin derivative. Coumarine has been considered to be promising antibacterial agents [30]. Peak 5 (RT 5.514) exhibited a base peak [M_H] at m/z 206.05 suggesting as 2,6,7-tri hydroxy-1,4-naphthoquinone. It is a common metabolite in plants and is known for diverse biological activity including antibacterial and antitumor activity [31]. Peak 6 (RT 5.751) exhibited a base peak [M_H] at m/z 330.15 suggesting as 1,8-dihydroxy-4,5-dinitroantraquinone. It is an anthroquinone derivative reported as secondary metabolite in several plants. It is acetate derived compound exhibiting diverse biological functions including antimicrobial, anticancer, immunostimulant and antiplasmodium activities [32].

**Fraction 4:**

Fraction F-4 from ethanol extract of *S. angustifolia* displayed 5 peaks. Peak 1 (RT 4.36) exhibited a base peak [M_H] at m/z 477.21. The compound was identified as tetratriacontane. Swamy *et al.* [33] have identified tetratriacontane as one of the major compound in the acetone extract of *Plectranthus amboinicus* leaves which exhibited potential antioxidant and antimicrobial properties. Peak 2 (RT 4.84) with base peak [M_H] at m/z 362.15 corresponds to p-(2,4,6-trinitroanilino) phenyl acetate. It is a nitrated amine derivative known for potent antitumor activity [34]. Peak 3 (RT 5.14) base peak [M_H] at m/z 346.15 was identified as 5-(p-amino phenhl)-4-(m-bromophenyl)-2-thiazolamine. However, up to our knowledge and literature search, biological activity has not been reported in this compound. Peak 4 (RT 5.48) with m/z 360.17 was matched as N-acetyl phytoshingosine. It is a lipid component found abundantly in plants. It is used in treating skin inflammation and is also known to exhibit antimicrobial and anti-inflammatory activity [35]. Peak 5 (RT 5.75) with base
peak [M_H] at m/z 637.37 is suggested as cholesteryl heptadecanoate. It is a fatty acid ester that has been studied for antibacterial activity against human nasal organisms [36].

**Fraction 6:**

Fraction F-6 from ethanol extract of *S. angustifolia* displayed 6 peaks. Peak 1 (RT 1.15) exhibited a base peak [M_H] at m/z 365.16. The identity of the compound was determined as xanthosine-5'-monophosphate. It is a nucleoside derived from xanthine, an alkaloid. It show several biological functions including antibacterial and antifungal activity [37]. Peak 2 (RT 4.36) with base peak [M_H] at m/z 811.39 is a strong fragment related to 1,2,3,4-tetrachloronaphthalene-bis(hexachlorocyclopentadiene). However the biological function of this compound has not been reported. Peak 3 (RT 4.84) displayed a base peak [M_H] at m/z 243.32 and it is suggested as L-alanyl-L-prolylglycine. Biological activity of this compound has not been reported till date. Peak 4 (RT 5.18) exhibited a base peak [M_H] at m/z 345.15, which corresponds to 6-bromo-2-(2-nitrostyryl)-1H-imidazo (4,5-b) pyridine. The compound is reported to have fungicidal activity [38]. Peak 5 (RT 5.48) exhibited a base peak [M-H] at m/z 360.17 suggesting as N-acetyl phytosphingosine. This compound was also detected in fraction 4 at same retention time (5.48). Peak 6 (RT 5.75) showed a base peak [M-H] at m/z 330.16 suggesting as Bis (2,2,2-trifluoroethyl) phthalate. It has been reported to have insecticidal properties [39].

LC-MS facilitates the identification and characterization of known and unknown compounds on the basis of their molecular formula, mass and fragmentations [26, 40]. It is a good choice for such metabolomic profiling studies as it generally detects a larger amount of compounds of varying polarities than the other commonly used techniques. However, this method is limited to studies of the mid-highly polar compounds and is not as useful for studies aimed at highly non-polar compounds. In recent advances in plant science research using LC-MS for qualitative estimation along with high throughput bio-analytical support for a new drug discovery is in need.

**CONCLUSION**

In the present study, an analytical method of column chromatography and LC-MS was applied for rapid characterization of phytochemicals in the chloroform extract of *P. indicum* bark and ethanol extract of *S. angustifolia* bark. Totally eight compounds were successfully characterized in *P. indicum* chloroform fraction by comparing retention time, mass and fragmentation data from the earlier literature and online software. On the other hand, six compounds from fraction-F-1, five compounds from fraction-F-4 and six compounds from fraction-F-6 were identified in the ethanol extract of *S. angustifolia*. Thus, the present work paves way for finding novel drugs. The work is significant and has lot of scope for unknown compounds and can be taken up for further studies for efficacy against various diseases.
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Conflict of Interest

The authors declare that they have no conflicts of interest to disclose.

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