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Antifungal Activity of *Poncirus trifoliata* Roots against Colletotrichum Species

Kumudini M. Meepagala*, Tamang Prabin

USDA-ARS, Natural Products Utilization Research Unit, University, MS, USA
Email: *kumudini.meepagala@usda

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Abstract

Fruits and vegetables are an essential part of a healthy diet, providing humans with vitamins, phytonutrients, and minerals. They are significantly vulnerable, however, to post-harvest diseases caused by numerous fungal and bacterial pathogens. These pathogens can cause significant quantitative and qualitative losses from harvest to consumption during the handling and storage processes. Chemical fungicides are commonly used but are likely to leave residues on the produce, rendering short shelf-life produce, such as berries, unsuitable for human consumption. Identifying eco-friendly methods to control post-harvest disease is, therefore, of utmost importance. The presence of antifungal constituents in the roots of *Poncirus trifoliata* extracts was detected by thin layer chromatography-based bioautography. The active constituents were isolated and identified by bioautography assay-guided fractionation using flash chromatography followed by spectroscopic techniques. In this study, xanthoxyletin, demethylsuberosin, dentatin, nordentatin, ponfolin, and clausarin were isolated from the root extracts. The antifungal activity of these compounds was moderate to weak compared to the commercial fungicide captan. This study reports the isolation and identification of natural compounds from *Poncirus trifoliata* that exhibited antifungal activity against *Colletotrichum fragariae* and *Botrytis cinerea*, two major post-harvest pathogens.

Keywords

Poncirus trifoliata, Bioautography, *Colletotrichum fragariae,* Fungicide

1. Introduction

Fresh fruits and vegetables are perishable commodities that require proper storage and transportation throughout the supply chain, from farmers to consumers. They are the major sources of vitamins, phytonutrients, and minerals for

humans. In 2019, the FAO (Food and Agriculture Organization) estimated that about 32% (by weight) of produce is wasted [1] during post-harvest handling and storage. These losses are attributed to post-harvest phytopathogenic fungal and bacterial pathogens. These pathogens can either directly spoil the produce (e.g., rotting or damage to physical appearance) or produce mycotoxins, making them unfit for human consumption.

Strawberry fruits are delicate, have a short shelf life, and are vulnerable to post-harvest fungal pathogens. Anthracnose caused by *Colletotrichum spp.* and gray mold caused by *Botrytis cinerea* are the two major post-harvest diseases of strawberries. Both fungal pathogens can cause significant economic losses in a wide range of hosts, including fruits, vegetables, legumes, and ornamentals in the field, during transportation, during storage, and in the market [2] [3] [4]. Globally, the major post-harvest loss of strawberries is attributable to anthracnose disease caused by *C. fragariae* [5]. *Colletotrichum gleosporides* and *C. acutatum* are the other two species of *Colletotrichum* that contribute to post-harvest loss of fruits and vegetables. *Colletotrichum fragariae* was first isolated and named by Brooks in 1931 when identifying anthracnose disease in strawberry plants [5] [6] [7].

There are various post-harvest treatments to maintain the quality and prevent spoilage of fruits and vegetables. These techniques include physical treatments, such as storage in a controlled-atmosphere (temperature and humidity) environment, radiation with low energy γ-rays, hot air and water treatment, controlling ethylene production, CO_2 treatment, use of biocontrol agents, and use of chemical fungicides [2] [5]. Fungicide application is the most widely used practice in the pre- and post-harvest prevention of fruit and vegetable loss due to fungal infections [2] [3]. Because of the emergence of resistance to currently used fungicides and the spread of fungal infections to new regions and hosts, there is a need to develop fungicides with novel modes of action and with different target sites from those currently in use. Natural products offer structurally diverse and environmentally friendly compounds that generally have fewer harmful effects and shorter half-lives than their synthetic counterparts. Plants and microbes develop defense compounds in their ecological niches as part of natural defense mechanisms and, therefore, such compounds have antifungal, phytotoxic, and insecticidal activities.

In this study, we report the isolation and identification of antifungal constituents from *Poncirus trifoliata* that exhibited antifungal activity against *Colletotrichum fragariae* and *Botrytis cinerea*, major post-harvest pathogens.

2. Materials and Methods

2.1. Extraction of Plant Material

Air-dried, ground roots (450 g) of *Poncirus trifoliata* (voucher specimen CON140700-1-A) were obtained from the Medicinal Plant Garden at the Uni-

versity of Mississippi. Sequential solvent extraction was carried out on the root sample (400 g) with 2 L of hexane, ethyl acetate, and methanol, three times with each solvent in a 4-L beaker for 2 h at room temperature using a sonicator bath. The extracts for each solvent were filtered, and the combined extracts were evaporated to afford 13.5 g, 10.8 g, and 13.8 g of hexane, ethyl acetate, and methanol extracts, respectively.

2.2. General Chemical Methods

The solvents used in this study were reagent grade and were used without further purification. Plant extracts and column chromatography fractions were tested on 250-micron silica gel GF TLC plates (Analtech, Newark, DE). Iodine vapor, anisaldehyde spray reagent, and UV light (254 and 365 nm) were used to detect and visualize the constituents. Flash chromatography was carried out using a Biotage™ flash chromatography system (Biotage, Charlotte, NC). NMR spectra were recorded on a Bruker (400 MHz for ^{1}H and 101 MHz for ^{13}C) spectrometer (Billerica, MA). HR-ESI MS data were recorded on a JEOL ACCU TOFJMST 1000 mass spectrometer. Melting points were recorded on a MPA 100 OptiMelt™ (Sunnyvale, CA) automated melting point apparatus.

2.3. Direct Bioautography Assay

The direct bioautography technique was conducted as described by previously published methods [8] [9]. A total volume of 80 µL (10 mg·mL^{-1}) crude extract and each fraction from column chromatography (10 mg·mL^{-1}) were spotted onto a silica gel plate (Analtech Silica Gel GF UV 254). The inoculum was prepared as described previously [8] [9]. Briefly, the fresh conidia were harvested from a 7 - 10 days old *C. fragariae* culture by adding 10 mL distilled sterile water and filtering through a sterile double Mira cloth. The spores were counted in Countess 3 (Invitrogen). The spore suspension was centrifuged at 4000 rpm for 10 min to collect all the spores at the bottom of the tube. The supernatant was discarded, and the spore concentration was adjusted to 3×10^5 spores/mL by adding the required volume of PDB-TLC media (12.5 g PDB, 0.5 g agar, and 0.5 mL Tween 80 in 500 ml water). The inoculum was sprayed uniformly onto the extract-spotted silica gel plates with a glass TLC sprayer. The plates were incubated in a moisture chamber with 100% humidity at 26˚C for 3 - 4 days, and the antifungal activity of the crude extracts and fractions was assessed by the presence of clear zones (no fungal growth) on the TLC plate. For the control plate, the technical grade fungicides captan and azoxystrobin were used as positive controls with a 1 µL volume of 2 mM concentration in ethanol.

2.4. Fractionation of Root Extracts

The bioautography of the hexane extract showed the highest antifungal activity; thus, the hexane extract (7 g) was fractionated by Biotage™ flash column chromatography using silica columns (Biotage SNAP cartridge KP-SIL 50 g) with a

solvent gradient (0% - 100%) of ethyl acetate in hexane followed by a 10% methanol in ethyl acetate column wash. Fractions of 53 mL were collected, and fractions with similar TLC profiles were combined to obtain 24 fractions for the hexane extract.

2.5. Isolation and Identification of Antifungal Constituents against *Colletotrichum fragariae*

Fractions of the hexane extract from column chromatography showed antifungal activity in the TLC bioautography (Figure 1).

Isolation of xanthoxyletin (**1**)

Fraction 11 (92 mg) was concentrated and crystallized from CH_2Cl_2 and hexane to afford white crystals (68 mg, m.p. 132˚C - 133˚C). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.85 (d, J = 9.7 Hz, 1H), 6.56 (d, J = 10.25 Hz, 1H), 6.51 (s, 1H), 6.2 (d, J = 10.0 Hz, 1H), 5.69 (d, J = 10.25, 1H), 3.86 (s, 3H), 1.47 (s, 6H). ^{13}C

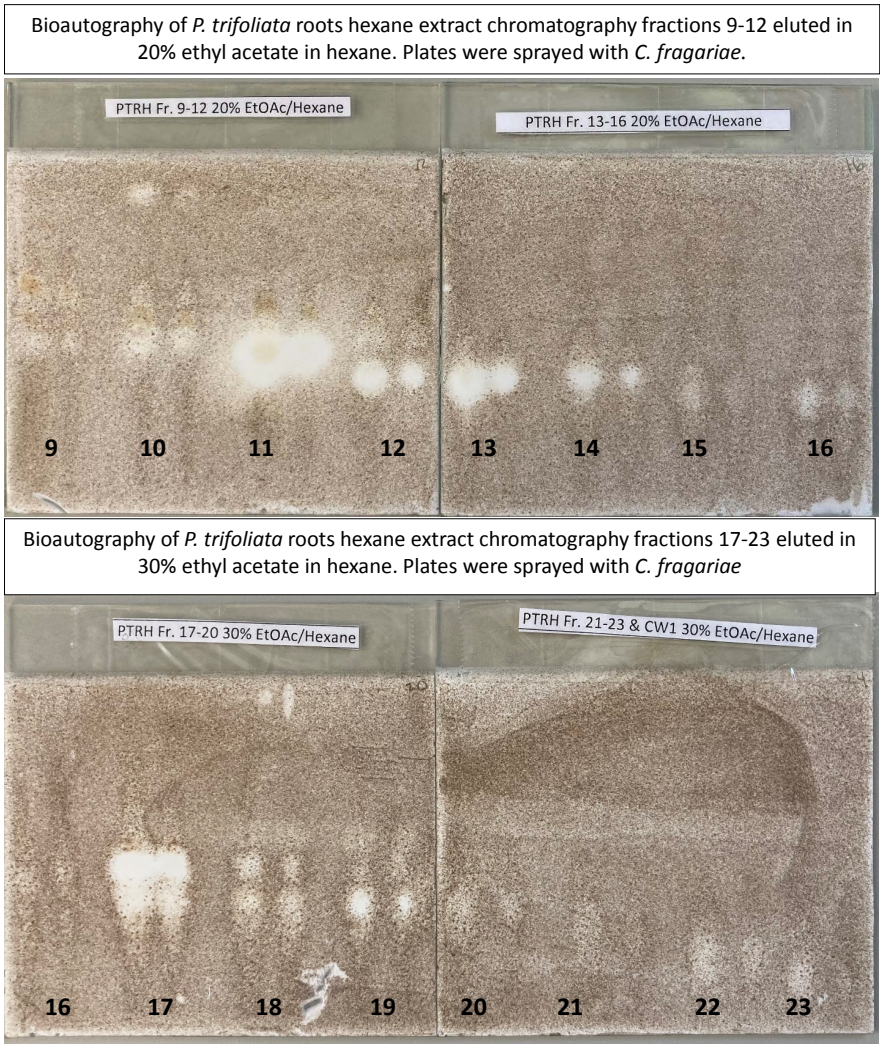


Figure 1. TLC bioautography plates of column fractions of *P. trifoliata* hexane extract. The plates are sprayed with *C. fragariae* spores.

NMR (101 MHz, $CDCl_3$) δ 161.1, 157.6, 155.6, 152.8, 138.5, 130.60, 115.80, 112.3, 111.3, 107.4, 100.8, 77.53, 63.6, 28.1. The experimental data are consistent with the data reported in the literature [10].

Isolation of dentatin (**2**)

Fractions 12 and 13 (168 mg) were dissolved in ethyl acetate and were concentrated. Needle-like colorless crystals formed upon adding hexane (1:1) to the concentrated solution. The crystals were filtered and washed with hexane and then recrystallized with ethyl acetate and hexane (approx. 1:1 V/V) to obtain pure dentatin (69 mg, m.p. 92˚C - 93˚C). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.87 (d, J = 9.6 Hz, 1H), 6.57 (d, J = 9.9 Hz, 1H), 6.31 (dd, J = 17.4, 10.6 Hz, 1H), 5.70 (d, J = 10.0 Hz, 1H), 4.94 (dd, J = 17.4, 1.1 Hz, 1H), 4.88 (dd, J = 10.6, 1.1 Hz, 1H), 3.83 (s, 3H), 1.67 (s, 6H), 1.45 (s, 6H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 160.8, 156.0, 151.2, 149.8, 138.9, 130.4, 119.3, 116.4, 111.7, 108.2, 107.5, 77.5, 63.4, 41.2, 29.4, 27.6. The experimental data are consistent with the data reported in the literature [10].

Isolation of demethylsuberosin (**3**)

Combined fractions 18-20 (309 mg) were further purified with a 25 g Biotage silica column using a 5-50% gradient of ethyl acetate in hexane to afford five subfractions. Subfraction 1 was crystallized with hexane in ethyl acetate to afford demethylsuberosin (**4**) as white crystals (87 mg).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.67 (d, J = 9.4 Hz, 1H), 7.19 (s, 1H), 7.05 (s, 1H), 6.22 (d, J = 9.4 Hz, 1H), 5.38 - 5.18 (m, 1H), 3.42 - 3.29 (m, 2H), 1.76 (dd, J = 18.9, 1.3 Hz, 6H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 162.9, 158.9, 154.0, 144.7, 134.5, 128.2, 126.3, 121.2, 112.1, 111.8, 102.9, 28.16, 25.83, 17.86.

The structure was confirmed via NMR and MS data by comparison with those reported in the literature [11] [12].

Isolation of nordentatin (**4**)

Subfraction 3, obtained from fractions 18 - 20 (described above), was concentrated, and upon addition of hexane, nordentatin was obtained as colorless crystals (159 mg). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.05 (d, J = 9.6 Hz, 1H), 6.54 (d, J = 9.9 Hz, 1H), 6.30 (dd, J = 17.4, 10.6 Hz, 1H), 6.17 (d, J = 9.6 Hz, 1H), 5.99 (s, 1H), 5.72 (d, J = 9.9 Hz, 1H), 4.94 (dd, J = 17.4, 1.2 Hz, 1H), 4.88 (dd, J = 10.6, 1.2 Hz, 1H), 1.64 (s, 1H), 1.46 (s, 6H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 161.58, 155.96, 154.14, 150.06, 146.61, 139.32, 130.00, 116.16, 114.96, 110.24, 108.08, 106.08, 103.90, 77.11, 41.03, 29.57, 27.32. The experimental data are consistent with those reported in the literature [10].

Isolation of clausarin (**5**)

Subfraction 5 (68 mg) was crystallized twice in ethyl acetate in hexane to obtain colorless crystals. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.93 (s, 1H), 6.59 (d, J = 9.9 Hz, 1H), 6.39 (s, 1H), 6.22 (ddd, J = 41.4, 17.4, 10.6 Hz, 2H), 5.65 (d, J = 9.9 Hz, 1H), 5.11 - 5.03 (m, 2H), 4.95 - 4.81 (m, 2H), 1.62 (s, 6H), 1.47 (s, 6H), 1.42 (s, 6H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 160.48, 154.97, 153.23, 150.11, 146.69, 145.61, 133.75, 129.53, 128.74, 115.54, 115.30, 111.96, 107.94,

106.14, 104.22, 77.34, 40.90, 40.22, 29.46, 27.23, 26.14. The identity of the sample was confirmed by comparison of NMR data with those in the literature [10]. Structures of the isolated compounds are shown in **Figure 2**.

2.6. Microdilution Broth Assay

An in vitro 96-well micro broth susceptibility assay was used to assess the antifungal activity of the isolated fraction against the fungal pathogens *C. fragariae and B. cinerea*. The inoculum was harvested and prepared as described above, and the spore concentration was adjusted to 1×10^4 spores/mL in RPMI media and Alamar blue. Technical grade commercial captan was used as a positive fungicide standard. A two-fold dilution series of each compound, starting with 10 mg·mL^{-1} (10,000 ppm), was prepared (10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, and 0.019 mg·mL^{-1}) in dimethyl sulfoxide (DMSO). These compounds were further diluted in RPMI media (1:5 dilution) and followed by another 1:20 dilution in RPMI media (containing fungal inoculum) to achieve the final corresponding concentrations of 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, and 0.19 μg·mL^{-1}. Captan was also similarly diluted to achieve the final starting concentration of 25 μg·mL^{-1}, as it was effective at a low dosage. The positive control

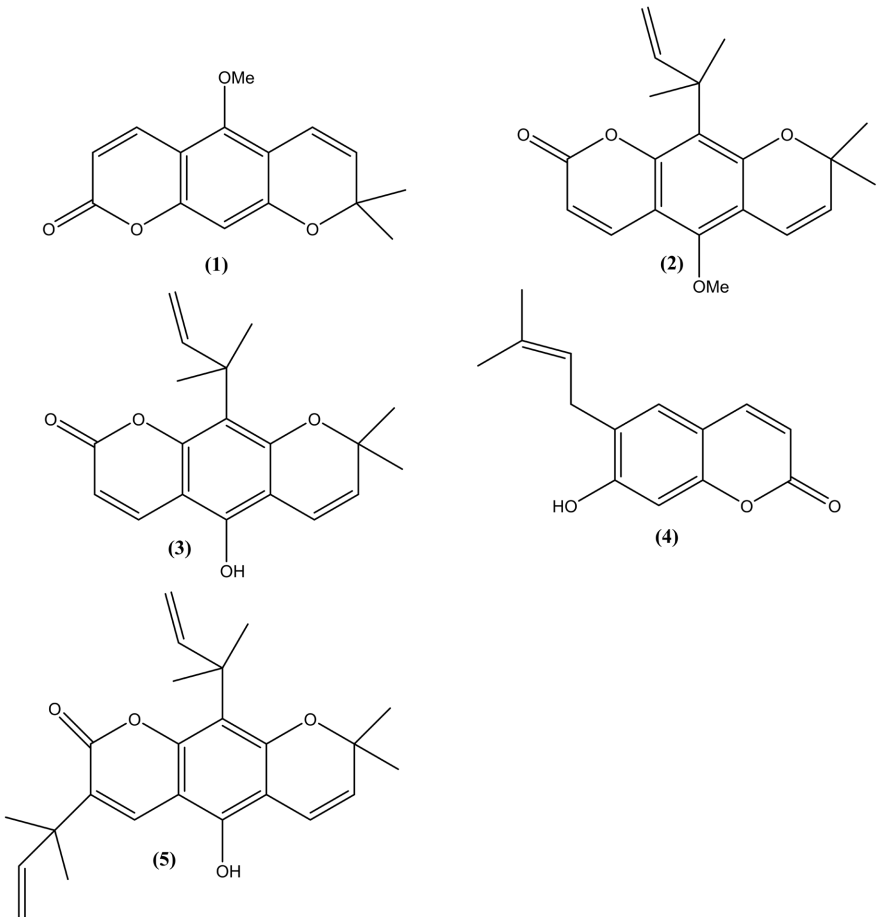


Figure 2. Structures of the isolated compounds.

(DMSO with inoculum) and the negative control (without inoculum and DMSO) were also included in the 96-well plate assay. All wells received a final volume of 200 µL, comprising test compounds, inoculum in RPMI, and Alamar blue. The final concentrations of DMSO and Alamar blue were 1% and 5%, respectively. Each compound was evaluated in duplicate, and the experiment was repeated twice. The plates were incubated at 27˚C - 28˚C in the dark in a growth chamber. The fungal growth was assessed by measuring the fluorescence readings of each culture solution at 544 nm excitation and 590 nm emission on a microplate reader (BioTek Synergy H1 Multimode Reader) 48 hours after incubation. The fluorescence readings were corrected using the value of the blank well without inoculum. The lethal concentration for 50% growth reduction (LC_{50}) was calculated from the mean of fungal growth (2 replications) by using the "nonlinear regression (curve fit)" function in P_{RISM} 8 software.

2.7. TLC-Based Antifungal Assay to Determine Minimum Inhibitory Concentration (MIC)

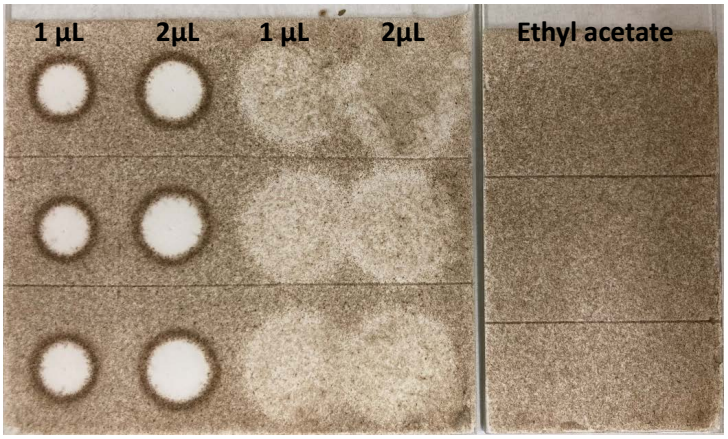
The antifungal activity and minimum inhibitory concentration (MIC) for each of the isolated compounds were assessed by the TLC bioautography procedure published in the literature [13]. The compounds were spotted in triplicate at varying concentrations (10, 5, 2.5 1.25, 0.675 mg·mL^{-1}) on TLC plates and sprayed with the spore suspension as described in the bioautography assay. Clear zones indicated inhibitory activity. The concentration at which the fungal growth is barely inhibited is estimated as the MIC (**Figure 3**). Two commercial fungicides, captan and azoxystrobin, served as positive controls, and ethyl acetate (solvent of the samples) served as the negative control.

3. Results and Discussion

No antifungal activity was detected against *C. fragariae* and *B. cinerea* for any of the five compounds tested in the micro broth susceptibility assay, even though the TLC bioautography showed antifungal activity. As a result, LC_{50} values were not determined for the tested compounds. The control fungicide captan showed antifungal activity with MIC values of 3.13 and 0.78 µg·mL^{-1} and LC_{50} values of 0.95 and 0.44 µg·mL^{-1}, against both pathogens, *C. fragariae and B. cinerea*, respectively. DMSO (1%) and Alamar Blue (5%) had no effect on the growth of the fungi. The test compounds precipitated at higher concentrations (greater than 1000 µg/mL) in the 96-well plates; therefore, the TLC bioautography-based method was used to estimate the MIC values (**Figure 3**). (All data are not shown.) From the TLC plate assay for determination of MIC values, it is evident that captan was extremely active, but azoxystrobin was not very effective against *C. fragariae*. Among the isolated compounds, xanthoxyletin (1) showed the highest activity, with an MIC value of 1.25 mg·mL^{-1} (**Figure 3**). The MIC value for demethylsuberosin (2) was estimated to be 1.25 - 2 mg·mL^{-1}. The MIC for dentatin was 5 mg·mL^{-1}, and the MIC for nordentatin was greater than 5 mg·mL^{-1}. Clausarin

Xanthoxyletin MIC =1.25 mg mL^{-1}

Demethylsuberosin MIC =2.5-1.25 mg mL^{-1}



Captan Azoxystrobin EtOAc negative control

Figure 3. TLC-based bioautography plates to estimate MIC values of isolated compounds.

showed negligible activity even at 10 $mg{\cdot}mL^{-1}$. These results suggest that the activity observed in **Figure 1** could be due to synergism with some minor constituents in the plant extract. Demethylsuberosin and its methylated analog have been reported to possess antifungal and insecticidal activity [12] [14]. According to **Figure 1**, our results indicate the presence of antifungal constituents in the

hexane root extract of *P. trifoliata.* Even though the individually measured antifungal activity is not particularly high, our data indicate that these compounds may be acting synergistically to produce fungitoxicity. Also, upon fungal infection of the plant tissues, the plant may produce higher levels of these compounds as part of a natural defense mechanism. This effect should be investigated to see if the metabolites produce a phytoalexin-type behavior and to further understand the ecological importance of the metabolites.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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