Isolation, peroxisome proliferator-activated receptor-gamma transcription, glucose uptake, and molecular docking of tetramethoxyflavonoids from the leaves of *Rourea mimosoides* (Vahl) Planch

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**ABSTRACT**

*Rourea mimosoides* (Vahl) Planch. is used traditionally to treat various diseases. The ethanolic leaves extract was found to increase the transcription of peroxisome proliferator-activated receptor-gamma (PPARγ). Therefore, the present study is warranted to identify the active compounds from the dichloromethane and ethanolic leaves extract and examine their PPARγ binding and glucose uptake activity. The ground dried leaves were macerated successively in dichloromethane and ethanol and dried and fractionated using vacuum liquid chromatography. Exhaustive chromatographic procedures yielded to a new tetramethoxyflavonoid, quercetin tetramethyl ether-3-O-β-L-rhamnoside (1), along with a known quercetin tetramethyl ether (2) and a steroid (3). Compound 2 showed the highest PPARγ binding and glucose uptake activities at 0.313 and 2.5 µg/ml, respectively. The leaves extract and compounds 1–3 showed no cytotoxicity when evaluated using HepG2 cells. Molecular docking result showed that compound 2 is able to interact with important residues within the hydrophobic pocket of the ligand-binding domain, thus acting as an agonist of PPARγ.

**INTRODUCTION**

*Rourea mimosoides* (Vahl) Planch. belongs to the Connaraceae family. It is commonly known as “sembelit merah” (Grosvenor *et al.*, 1995). The decoction of the root is traditionally used to treat bloody diarrhea, bloody cough, dysentery, and leprosy and as diuretics (Grosvenor *et al.*, 1995), while the whole plant is used to treat colds in children and given to women after childbirth (Tan *et al.*, 2013). To the best of authors’ knowledge, no chemical compound is reported from *R. mimosoides* to date. Flavonoids, flavonoid glycosides, triterpenes, and phenolic acids were previously reported in *Rourea* sp. (He *et al.*, 2006; Kalegari *et al.*, 2011; Laikowski *et al.*, 2017; Oliveira *et al.*, 2012; Zhang *et al.*, 2008).

During our preliminary study, the ethanolic leaves extract of *R. mimosoides* was found to increase the transcription of the peroxisome proliferator-activated receptor-gamma (PPARγ) gene. PPARγ agonists such as rosiglitazone and pioglitazone activate the transcription of target genes, which improve glucose homeostasis by systemic insulin sensitization or direct action PPAR-γ on the transcription of genes involved in the glucose disposal (Rangwala and Lazar, 2004). PPARγ ligands improve insulin sensitivity, enhance insulin secretion, and improve glucose tolerance in diabetic patients (Janani and Ranjitha Kumari, 2015).

Molecular docking investigations on PPARγ transcription by natural products usually provide a detailed understanding of the ability of compounds to induce conformational changes on the cavity of the receptor upon binding (Pan *et al.*, 2017). Some of the

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residues that play a vital role in the transcription activity include Arg288, Ser289, Glu343, and Tyr473, as indicated by the site-directed mutagenesis approach (Mitro et al., 2013). Other binding interactions that are observed for transcription of PPARγ include the formation of the salt bridge to stabilize the PPARγ AF-2 helix, van der Waals interactions with residues I268, A272, N275, L309, I310, and F313 within the hydrophobic pocket, and electrostatic interactions with the side chain of residue R316 and backbone amide of A327 (Gampe et al., 2000).

Thus, the present study focuses on the identification of the active compounds from the leaves of R. mimosaoides and the examination of their PPARγ binding and glucose uptake activity. The docking study of the active compound with PPARγ ligand is also illustrated.

**MATERIALS AND METHODS**

**General instrumentation**

Thin-layer chromatography was performed on the thin layer chromatography (TLC) silica gel 60 F₂₅₄ aluminum sheets (1.05554, Merck, Germany) and visualized under ultraviolet light at 254 and 365 nm. Preparative TLC was carried out using TLC silica gel 60 F₂₅₄ glass plates (1.00390, Merck Germany). Column chromatography (CC) was performed using silica gel 60 (1.09385, 230–400 mesh, Merck, Germany). Vacuum liquid chromatography (VLC) was carried out using silica gel 60 PF₂₅₄ (1.07745, Merck, Germany). All the solvents used for extraction and isolation were of analytical grade. ¹H- and ¹³C-NMR spectra were acquired on a Bruker Ascend 600 (Switzerland) at 600 and 150 MHz, respectively, using CDCl₃ or CD₃OD as solvent. Chemical shifts were reported in ppm and δ scale with the coupling constants given in Hz. The mass spectra were recorded using LCMS/MS QTOF Agilent Technologies 6520 (Agilent, Santa Clara, CA). The UV absorbance for the in vitro analysis was obtained by Spectrostar Nanospectrometer (BMG Labtech, Ortenberg, Germany), while the 96-well plates were shaken on Scilogex SCI-M Microplate Mixer (Scilogex, US).

**Plant material**

*Rourea mimosaoides* (Vahl) Planch. was collected in lowland and hill forest at elevation up to 750 m with latitude and longitude of N 05°33.654’, E 101°20.293’ of Royal Belum Rainforest in 2017. The plant was identified by Dr. Shamsul Khamis, a botanist from Universiti Kebangsaan Malaysia. The voucher specimen (IPH-423) was deposited at the Malaysian Institute of Pharmaceuticals and Nutraceuticals.

**Extraction and isolation**

The leaves (200 g) were air-dried, ground, macerated successively in CH₂Cl₂ (1 l) and EtOH (1 l) for 48 hours, filtered, and dried under reduced pressure using Heidolph Rotavapor (Germany). The maceration was repeated two times for each solvent to give a total of 5 and 3 g of dichloromethane and ethanol extracts, respectively. CH₂Cl₂ extract (5 g) was subjected to fractionation using VLC eluted with n-hex:EtOAc-MeOH (n-hex:EtOAc, 9:1 → 0:10; EtOAc:MeOH, 9:1 and 8:2) as elution system to give 63 fractions. Fractions 33–44 (500 mg) were further purified using CC eluted with CH₂Cl₂:EtOAc of increasing polarity (CH₂Cl₂:EtOAc, 10:0 → 0:10) to give 45 subfractions. Subfractions 4–15 (20 mg) were pooled and subjected to preparative thin layer chromatography (PTLC), which yielded compound 2 (1 mg). Fraction 9 (435 mg) was further fractionated using CC eluted with n-hex:CH₂Cl₂ (n-hex:CH₂Cl₂, 9:1 → 1:9) to give 15 subfractions, of which purification of subfraction 5 (13.4 mg) using PTLC (n-hex:CH₂Cl₂, 1:9) afforded compound 3 (5.1 mg). The fractionation of ethanolic extract (3 g) using CC eluted with n-hex:EtOAc-MeOH of increasing polarity (n-hex:EtOAc, 9:1 → 1:9; EtOAc:MeOH, 9:1 and 8:2) afforded 73 fractions. Fraction 59 (50 mg) was subjected to PTLC developed in CH₂Cl₂:MeOH (95:5) to give compound 1 (2 mg).

**PPAR gamma assay**

PPAR gamma assay was carried out based on a previously reported study with slight modifications (Christensen et al., 2010). HepG2 (ATCC HB-8055) cell lines were obtained from the Department of Pharmaceutical Pharmacology & Chemistry, Faculty of Pharmacy, Universiti Teknologi MARA. The cells were seeded in a 96-well plate (clear bottom) at a cell density of 8 × 10⁴ cell/ml and incubated in an incubator supplemented with 5% (v/v) CO₂. Cells were then transfected using FuGENE® HD transfection reagent (Promega Corporation, Carlsbad) and later added to the plasmid composition of pSV-Sport PPARγ, pACOX peroxisome proliferator response element (PPRE) ×3, pSV-Sport retinoid X-receptor (RXR) α, and pRL-CMV. Transfecting cells were incubated for another 24 hours and treated with the compounds, concentration ranging from 20 to 0.039 µg/ml. These concentrations were used to evaluate the best binding activity of PPARγ exhibited by the compounds and compared to the positive control, rosiglitazone, which gave its best binding activity at 1 µM (Christensen et al., 2010).

**Glucose uptake assay**

Glucose uptake assay was performed as described previously (Tedong et al., 2010), with few modifications. Myoblast cells (C2C12 and ATCC CRL-1772) were obtained from the Malaysian Institute of Pharmaceutical & Nutraceuticals (IPharm). The cells were plated in 96-well plates (black and clear bottom) at a cell density of 8 × 10⁴ cell/ml and incubated in an incubator supplemented with 5% (v/v) CO₂ at 37°C for overnight to allow cell attached. The next day, cells were differentiated with differentiation medium including Dulbecco’s Modified Eagle’s Medium (Sigma-Aldrich, Germany), supplemented with 2%
(v/v) of Horse Serum (Sigma-Aldrich, Germany) and 1% (v/v) of 1,000 µg/ml of penicillin/streptomycin (Nacalai Tesque), and followed by incubation for 5–8 days. During this process, cells were differentiated at least 85% from myoblasts to myotubes. Differentiated myoblast was then treated with compounds, with concentrations ranging from 20 to 0.039 µg/ml and incubated for 24 hours. These concentrations were used to determine the efficacy of the compounds in the stimulation of glucose uptake and compared to the positive control, insulin with slight modification on maximal concentration, 200 µM (Anandharajan et al., 2006). Cells were then washed and lysed using a buffer. Cell lysates were transferred into a 96-well plate (clear bottom) and analyzed for relative fluorescence units using a microplate reader at 465/540 nm (Tecan, Morrisville, NC). The fold increase in glucose uptake activity was calculated.

**Cytotoxicity assay**

Cytotoxicity assay was performed on both HepG2 and C2C12 differentiated cells based on the previously reported protocol (Alwi et al., 2019). Briefly, 150,000 cells/ml were plated into the 96-well plates (clear bottom) and incubated in an incubator supplemented with 5% (v/v) CO₂ at 37°C overnight to allow cells to be attached. The next day, cells exposed to compounds with concentration ranged from 100 to 1.5625 µg/ml and incubated for 24 hours. The ranges of concentrations were chosen to access the highest test concentrations that caused dose-dependent cytotoxic effects on cells (Swamy and Tan, 2000). After 24 hours of incubation, the test plates were inspected under an inverted microscope. 3-(4,5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (Sigma) solution of 1 mg/ml was added to each well of test plates and incubated for an additional 3–4 hours. The assay plate was analyzed by a microplate reader (Tecan, Morrisville, NC) at 570 nm. The concentration required for a 50% inhibition of viability inhibitory concentration (IC₅₀) was determined.

**Molecular docking**

Molecular graphics laboratory tools and AutoDock4.2 were used to perform molecular docking. Discovery studio visualizer 4.2 was used to visualize and analyze the molecular docking result. Molecular docking was performed using the Lamarckian genetic algorithm for ligand conformational searching which is used for the docking. A three-dimensional docking grid with the size of 50 × 50 × 50 Å and 0.375 Å spacing was used. This grid was centered on the native crystal ligand with the x, y, and z coordinates of 16.986, −21.062, and 11.751, respectively. Docking was performed using PPARY complexed with rosiglitazone (PDB:1FM6). Validation was performed using native crystal ligand and rosiglitazone to ensure the validity of molecular docking results.

**RESULTS AND DISCUSSION**

**Phytochemical results**

A new tetramethoxyflavonoid (1), a known tetramethoxyflavonoid (2), and a known steroid (3) were isolated from the leaves extracts of *R. mimosoides* (Fig. 1). The structure of stigmast-4-one-3-ene (3) was identified by spectral data analysis and comparison with literature data (Gaspar and das Neves, 1993). Quercetin tetramethyl ether (2) was reported from *Sterculia foetida* (Anjaneyulu and Murty, 1981); however, the spectral data were not available for comparison. Its spectral data were compared to its synthetic counterpart (Wang et al., 2014). The structure of quercetin tetramethyl ether-3-O-ß-L-rhamnoside (1) was established from the analysis of 1D and 2D and MS spectral data. There is no recent report on the natural occurrence of compound 2. Hence, this paper discusses the characterization of compound 1, including full spectroscopic data compound 2 for future references. All these structures are presented in Figure 1.

**Compound 1** was isolated as colorless crystals. The molecular formula of C₆₁H₇₁O₳₄ was generated from LC-MS QTOF with its [M+H]⁺ at m/z 505.2082 (cald 504.4760 for C₆₁H₇₁O₳₄). The 1H NMR displayed a 5,7-disubstituted pattern of ring A with the presence of a pair of meta-coupled signals at δ₇.643 (1H, d, J₆-7 = 2.4 Hz, H-6) and δ₇.63 (1H, d, J₆-7 = 2.4 Hz, H-8) in addition to two methoxyl signals at δ₄.394 (3H, s, 7-OCH₃) and δ₃.95 (3H, s, 5-OCH₃). The ABX-system of aromatic signals at δ₇.73 (1H, d, J₆-7 = 1.8 Hz, H-6) and δ₈.6 (1H, d, J₆-7 = 8.4 Hz, H-5) and δ₇.73 (1H, d, J₆-7 = 1.8 Hz, H-2) together with methoxy proton signals at δ₄.93 (3H, s, 5-OCH₃) and δ₄.38 (3H, s, 4-OCH₃) indicated the presence of a 3,4′-disubstituted phenylpropanoid moiety at ring B. The spectral data of the flavanol moiety were in good agreement with spectral data published previously (Wang et al., 2014). A doublet signal at δ₇.27 integrated for three protons and several glucoside proton signals were observed in the up field region, suggesting that the glycoside is rhamnose. An anomeric proton was observed at δ₅.52 as a doublet with a J value of 1.2 Hz, suggesting that the sugar has ß configuration (Agrawal, 1992). The APT-NMR spectrum showed 24 carbons, including five methine carbons, 10 quaternary carbons, including the characteristic carbonyl of flavones at δ₇.164, and five glycosidic carbons. The anomeric carbon was observed at δ₁.399, suggesting that the sugar moiety is linked to aglycone via α O-glycosidic linkage. The presence of one primary carbon at δ₁.84 confirms the rhamnose’s nature of the sugar. The assignment of the methoxy groups at C-5, C-7, C-3′, and C-4′ was made based on the heteronuclear multiple bond correlation (HMBC) correlations observed between H-6 (δ₆.63) and C-5 and C-7 and H-2 (δ₇.73) and H-6 (δ₁.68) with C-3′, respectively. Sugar moiety is assigned at C-3 based on HMBC cross-peaks observed between H-5′ (δ₇.28) and H-6′ (δ₁.68).
with C-3. Analysis of the spectra data and information found in the literature allowed us to characterize compound 1 as quercetin tetramethyl ether-3-O-ß-L-rhamnoside derivative (Table 1).

**Biological results**

PPARγ binding activity assay was conducted to access the natural PPARγ ligand present in the compound isolated from the natural resources and binds to PPAR, which then dimerizes with RXR to form a heterodimer which in turn, binds to PPRE in the promoter region, and consequently triggers the transcription of the luciferase gene (Straus and Glass, 2001). The signal produced by the reporter gene was measured by a luminometer. HepG2 cell line was used in this assay because the suppression of PPARγ and RXRα was found at low levels in the cells to differentiate the transcriptional activation activity of other PPAR isoforms. Thus, expression vectors of PPARγ and RXRα are transfected in this assay system (Han et al., 2002). Compound 2 demonstrated the highest binding of PPARγ ligand activity at 0.313 µg/ml with a 2.188 ± 0.195-fold increase (Fig. 2). The result of the PPARγ transactivation assay demonstrated that compound 2 induced PPARγ activation when it produced a ratio of luciferase activity higher than rosiglitazone. Polyphenols were known to increase PPARγ expression when administered as part of a high-fat diet in animal models, particularly in the liver and adipose tissue except for quercetin and catechins (Beekmann et al., 2015). However, compound 2, the methyl ether of quercetin, showed high binding energy with PPARγ in the present study. Compounds 1 and 3 exhibited similar dose-dependent manner with the best binding

<table>
<thead>
<tr>
<th>Position</th>
<th>Compound 1</th>
<th>Compound 2</th>
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<tbody>
<tr>
<td></td>
<td>δ_1H</td>
<td>δ_13C</td>
</tr>
<tr>
<td>2</td>
<td>149.2</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>154.9</td>
<td>12.86, 1H, s</td>
</tr>
<tr>
<td>4</td>
<td>176.4</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>162.2</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>6.43, 1H, d, J = 2.4 Hz</td>
<td>97.2 C-5, C-7, C-8, C-10</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>166.4</td>
</tr>
<tr>
<td>8</td>
<td>6.63, 1H, d, J = 2.4 Hz</td>
<td>93.8 C-6, C-7, C-9-C10</td>
</tr>
<tr>
<td>9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>110.1</td>
</tr>
<tr>
<td>1'</td>
<td>–</td>
<td>126.3</td>
</tr>
<tr>
<td>2'</td>
<td>7.73, 1H, d, J = 1.8 Hz</td>
<td>113.6 C-2, C-3′, C-6′</td>
</tr>
<tr>
<td>3'</td>
<td>–</td>
<td>151.2</td>
</tr>
<tr>
<td>4'</td>
<td>–</td>
<td>142.5</td>
</tr>
<tr>
<td>5'</td>
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<td>118.0 C-3, C-1′, C-3′</td>
</tr>
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<td>6'</td>
<td>7.68, 1H, dd, J = 8.4 Hz, J = 1.8 Hz</td>
<td>122.9 C-2′, C-2, C-3</td>
</tr>
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<td>1''</td>
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<td>100.4 C-2′, C-5′</td>
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<tr>
<td>2''</td>
<td>4.13, 1H, dd</td>
<td>71.9 C-4′, C-3′</td>
</tr>
<tr>
<td>3''</td>
<td>3.49, 1H, t</td>
<td>73.9 C-4′, C-5′</td>
</tr>
<tr>
<td>4''</td>
<td>3.94, 1H, m</td>
<td>72.4 C-3′, C-5′</td>
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<tr>
<td>5''</td>
<td>3.76, 2H, dd</td>
<td>71.0 C-3′, C-4′</td>
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<tr>
<td>6''</td>
<td>1.27, 3H, d</td>
<td>18.4 C-5′, C-3′</td>
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<tr>
<td>5-OCH_3</td>
<td>3.95, 3H, s</td>
<td>57.2 C-5</td>
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<td>7-OCH_3</td>
<td>3.94, 3H, s</td>
<td>166.4 C-7</td>
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<tr>
<td>3′-OCH_3</td>
<td>3.93, 3H, s</td>
<td>56.9 C-3′</td>
</tr>
<tr>
<td>4′-OCH_3</td>
<td>3.82, 3H, s</td>
<td>56.6 C-4′</td>
</tr>
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of PPARγ activity at 0.556 ± 0.085-fold increase at 1.25 µg/ml and 0.735 ± 0.073-fold increase at 0.625 µg/ml, respectively. Compound 1, which has a similar aglycone as compound 2 except for additional glycoside, showed weaker activity than compound 2.

The 2-deoxyglucose uptake assay was designed to evaluate the insulin-like and insulin-sensitizing activity for isolated compounds from *R. mimosoides*. C2C12 myoblast cells were used since skeletal muscle is one of the insulin-responsive tissues responsible for glucose uptake for the maintenance of whole-body glucose homeostasis (Choi *et al.*, 2009). Insulin-induced glucose uptake in adipocytes by binding to insulin receptor proteins within the cell leading to the translocation of glucose transporters 4 to the cell surface (Rathi *et al.*, 2002). Compound 2 exhibited the highest uptake of glucose activity at 2.5 µg/ml with a 7.022-fold increase (Fig. 3), while compounds 1 and 3 presented a similar dose-dependent manner and significant difference with minor glucose uptake activities.

The cytotoxicity was conducted to evaluate any toxicity on the cell lines used in this study which were HepG2 cell line and C2C12 myoblast cell. Initially, the cells were tested with the concentration of compounds starting from 1.56 to 100 µg/ml. The ethanolic leaves extract of *R. mimosoides* and compounds 1–3 showed no toxicity on the HepG2 cell line with the IC₅₀ values of more than 100 µg/ml.

**Molecular docking**

Molecular docking was carried out to identify the plausible binding position of compound 2. Before performing molecular docking of quercetin tetramethyl ether (2) on the target receptor, a validation docking run was done to ensure the reliability and accuracy of the results obtained. Results obtained from the validation run of rosiglitazone suggest that the method developed can reproduce binding position with an acceptable root mean square deviation of 0.54 Å (Fig. 4). The result for plausible binding position of compound 2 showed that compound 2 is capable of interacting with PPARγ primarily through carbon-hydrogen bonds involving ring B.

The chromone moiety can fit in the hydrophobic pocket and interact with various residues within the ligand-binding domain (Fig. 5). Hydroxyl on chromone ring was observed to form a conventional hydrogen bond with the main-chain carbonyl oxygen atom of residue Leu340 at a distance of 2.29 Å. The oxygen atom...
of the methoxy on ring A of the chromone ring forms a carbon-hydrogen bond with the backbone (Ha) of Gly284 at a distance of 2.34 Å. The oxygen atom of the methoxy at the para position of ring B forms a carbon-hydrogen bond with the backbone (Ha) of important residue Glu343 at a distance of 2.83 Å, while the oxygen atom on the other methoxy at the meta-position of ring B forms a carbon-hydrogen bond with the backbone (Ha) of Ser342 at a distance of 2.45 Å. The hydrogen atoms of the methoxy at the para position of ring B can form carbon-hydrogen bonds with the backbone (Oe2) of Glu343 and backbone (Oe1) of Glu291 at a distance of 2.43 and 2.68 Å, respectively. On the other hand, the hydrogen atom of the methoxy at the meta-position of ring B formed a carbon-hydrogen bond with the backbone (Oe2) of Glu291 at a distance of 2.62 Å (Fig. 6). The molecular docking result supports the fact that interaction with Arg288 and Glu343 is vital for stable binding between compound 2 and PPARγ (Li et al., 2008).

CONCLUSION

Purification of the extract afforded two tetramethoxyflavonoids (1–2) and a triterpene (3). Compound 2 showed the highest PPARγ binding activity and glucose uptake activity. *Rourea mimosoides* extract and compounds 1–3 showed no cytotoxicity when evaluated using HepG2 cell line. Molecular docking revealed that compound 2 is capable of occupying the hydrophobic ligand-binding domain and interacting with crucial residues Arg288 and Glu343 through hydrogen-bonding interactions and thus may have resulted in compound 2 acting as an agonist for PPARγ. The bioactive compounds identified in this study can be further used as biomarkers for the development of *R. mimosoides* as a botanical drug.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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