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Proximate composition, nutritional values and phytochemical screening of *Piper retrofractum* vahl. fruits

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ABSTRACT

Objective: To investigate the proximate and mineral composition of the *Piper retrofractum* (*P. retrofractum*) vahl. Fruit and to evaluate its total alkaloids, phenol and flavonoid. **Methods:** The proximate composition of *P. retrofractum* fruit was ananlyzed using standard protocols according to Indonesian Standard and Association of Official Analytical Chemist. Meanwhile, mineral composition of the fruit was analyzed using inductively coupled plasma-mass spectrometry. Phytochemical screening and quantification were performed using standard protocols according to Harborn and spectrophotometric methods. **Results:** The results showed that *P. retrofractum* fruit contained carbohydrate (63.4%), crude protein (11.4%), total ash (4.29%), dietary fiber (28.8%) and total fat (2.97%). The fruit also contained calcium, copper, iron, magnesium, phosphor, potassium, sodium and zinc in different concentrations. Additionally, quinone, sterol, glycosides and alkaloid were detected in both *n*-hexane and ethyl acetate extracts. Moreover, tannin was presented also in ethylacetate and methanol extracts. Meanwhile, methanol extract contained sterol, glycosides, flavones, tannin and alkaloid. The results also revealed that methanol extract of the fruit contained highest phenol compared to other extract. Finally, small quantity of flavonoid (0.060 0%±0.000 2%) was observed. **Conclusions:** The overall results show that *P. retrofractum* contains potential nutritional and phytochemicals values, which support their function for pharmaceutical purposes.

1. Introduction

Tropical regions have been known as home of many fascinating medicinal plant species. This includes medicinal plants from the genus *Piper* that covers around one thousand species[1]. In terms of morphological characteristics, all species in this genus possess three plant forms including creeping, climbing and branching stems. In addition, all plants have distinct leaves color and shape. Apart from its role in pharmaceutical domains, plants from the genus *Piper* also used by traditional communities as supporting material for decorative arts, traditional ceremonies as well as food and beverages[2]. Among their various species, *Piper retrofractum* (*P. retrofractum*) vahl. is one of Piper species that are distributed mainly in the tropical region, including Thailand, Vietnam, Philipina and Indonesia.

Some Indonesian vernacular name of *P. retrofractum* have been reported including 'Cabe Puyang', 'Cabe Jawa', 'Cabe Jamu', and

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'Cabai Jamu' All these local names reflect its medicinal use. It is not surprising since this species contains various kind of phytochemical contents such as alkaloids/amides[3], which include more than 300 amide compounds that have been reported and thus become the prominent compounds found in *P. retrofractum*[4,5]. Furthermore, some terpenoid compounds have also been identified[6]. Recently, the presence of phenylpropanoids and alkylglycosides compound have been also reported and therefore the medicinal properties of this species were strengthened[7]. Many reports have demonstrated its biological and pharmaceutical functions, including antioxidant activity[8], potential bioresource for neurodegenerative diseases treatment[9], mast cell stabilization[10], mucolytic and expectorant agents[6] and potential regulation on human lipid metabolism[11], anti dengue[12] and larvacidal activity[13].

Based on the traditional use of *P. retrofractum* as potential component for herbal medicine development, providing its pharmaceutical analysis is necessarily needed. Therefore, this present study aims to determine the proximate composition, nutritional values and to conduct preliminary study on phytochemical content of *P. retrofractum* vahl. fruits.

2. Materials and methods

2.1. Collection and preparation of plant materials

P. retrofractum fruits were fresh-collected from the traditional market in Sumenep Region, Indonesia and were identified and authenticated by the Laboratory of Plant Biosciences and Technology, Department of Biology, Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia. The voucher specimen was deposited in the form of herbarium in the laboratory for further references. The fresh-collected materials were then subjected to three times serial washing using tap water for eliminating residual soil particles and other unnecesary debris. Subsequently, the fruits were subjected to open-air and non-directed sunlight drying.

2.2. Determination of water content of P. retrofractum vahl. fruit

Water content was measured according to the Indonesian National Standard (SNI No. 01-2891-1992)[14]. Briefly, 2 g of fresh *P. retrofractum* fruit was weighted (W_a) carefully and then subjected to an oven at 105 ℃ until a constant weight was reached (W_b). Finally, the percentage of water content was calculated using the following formula:

Water content (%)=(W_a-W_b)/W_b×100

2.3. Determination of total ash content of P. retrofractum vahl. fruit

Total ash content was quantified according to the Indonesian National Standard (SNI No. 01-2891-1992)[14]. In this process, all volatile materials were vaporized and the organic matters would be perfectly burned, thanks to the presence of oxygen (O_2) into carbondioxide (CO_2), but not the organic materials. Firstly, an empty evaporating dish was pre-treated by heating at 600 ℃ in a muffle furnace and then directly cooled and weighed (W_1). Subsequently, 2 g of sample (W) was placed into the evaporating dish and then was heated at 550 ℃ using muffle furnace until it was perfectly turned into grayish-white ash (W_2). The later represents the total amount of minerals within the *P. retrofractum* fruits. The total ash content was then determined using the following formula:

Total ash content (%)=(W_2-W_1)/W×100

2.4. Crude protein analysis

Crude protein of the *P. retrofractum* vahl. was represented by measuring the nitrogen (N) content. This determination was done based on the Indonesian National Standard (SNI No. 01-2891-1992[14]. Briefly, 0.51 g of sample was placed in the 100 mL kjeldahl flask. Subsequently, 2 g of selenium mixture (2.5 g of SeO_2; 100 g of K_2SO_4 and 30 g of $CuSO_{45}H_2O$) and 25 mL of concentrated H_2SO_4 were added into the kjeldahl flask. All the components were mixed thoroughly and heated until the mixture became clear and greenish. Distilled water was then added until the mixture reached 100 mL. Next, 5 mL of the mixture was pipetted and placed into distillation system and subsequently was added by 5 mL of 30% NaOH solution and few drops of mixed indicator solution (10 mL of 0.1 mL bromocresol green and 2 mL of methyl red). Distillation was done within 10 min and then the produced NH_3 was collected (in the form of NH_4OH) in the conical flask supplemented with 10 mL of 2% boric acid solution and few drops of mixed indicator solution. Then, the distillate was subjected to titration against 0.01 N HCl. Finally, crude protein of *P. retrofractum* vahl. fruit was calculated as:

Crude protein content (%)=6.25×[(V_1-V_2)×N ×0.014×f/W] ×100

Where V_1, V_2, N, f and W are the sample titration reading, blank titration reading, HCl normality, sample dilution and sample weight, respectively. The 0.014 constant is the mili equivalent of nitrogen.

2.5. Analysis of total lipid content

Analysis of total lipid content within the *P. retrofractum* vahl. fruit was conducted using soxhlet extractor and based on the Indonesian National Standard (SNI No. 01-2891-1992)[14]. Briefly, 2 g of fruit sample was wrapped and dried using oven at 80 ℃ and then was placed into the lipid flask within the soxhlet extractor. The sample was then extracted using hexane solvent. The extract obtained was then distilled and subsequently dried using oven at 105 ℃. The later step was repeated until a constant weight was achieved. Total lipid content was calculated as follow:

Lipid content (%)=(Weight of *n*-hexane extract)/(Weight of

sample)×100

2.6. Determination of carbohydrate content and caloric value of P. retrofractum vahl. fruit

Carbohydrate content of the *P. retrofractum* fruit was determined by involving the addition of crude protein, lipid, water content, total ash content), as the equation below :

Carbohydrate (%)=100%-(Water content+Total ash+Total lipid +Crude protein)

Meanwhile, the caloric value of the *P. retrofractum* fruit was calculated as the sum of crude protein, carbohydrate and total lipid content (each was multiplied by a factor of 4.0, 3.5 and 9.0 kcal/100 g, respectively[15].

2.7. Determination of dietary fiber of P. retrofractum vahl. fruit

The analysis of dietary fiber was done by combining both enzymatic and gravimetric methods[16]. Briefly, samples were digested with sequential enzymatic reaction involving α-amylase, protease and amyloglucosidase followed by incubation at 60 ℃ for 30 min with constant agitation. Hereafter, 225 mL of 95 % ethanol was added to each digest product and then pre-heated to 60 ℃. The solution was precipitated at room temperature and then filtered. The residues were then washed twice using 15 mL of 78% ethanol, 95% ethanol and acetone. The washed residues were subsequently dried in oven at 105 ℃ until the constant weight was achieved. The residues were then cooled using dessicator and then weighed (W). All these analysis were done in duplicate, where the first sample was used to measure the sample protein residue (Pr) (using kjeldahl method) and the second sample was used to determine total ash residues (As). Dietary fiber was finaly determined using the following equation :

Dietary fiber (%)=(W-Pr-As-blank)/(Weight of sample)×100

Where the value of blank was measured using:

Blank=Blank residue-Blank protein residue-Blank ash residue

2.8. Determination of mineral content of P. retrofractum vahl. fruit

Mineral contents were analyzed using inductively coupled plasma-mass spectrometry. These minerals include calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphor (P), potassium (K), sodium (Na) and zinc (Zn). In brief, a 0.3 g of sample was used for each tested mineral. In addition, all samples used were also spiked to the internal standard. Then, 6 mL of concentrated nitric acid (HNO_3) was carefully added. Pre-digestion process was conducted around 10-15 min prior the digestion process. Hereafter, the sample was cooled and then diluted with deionized water. Finally, diluted samples were analyzed using inductively coupled plasma-mass spectrometry. The value of each minerals were represented as mg/100 g DW (dry weight).

2.9. Phytochemical screening of P. retrofractum vahl. fruit

Prior conducting phytochemical screening, the samples were prepared for extraction. A maceration procedures using three different solvents including methanol, ethyl acetate and *n*-hexane (Brataco Chemical) were performed by procedure described previously[8]. Phytochemical screening of *P. retrofractum* fruit was done firstly by qualitative analysis to test the presence of alkaloid, tannin and flavones, flavonoid, glycosides, sterols and quinone. These analysis were conducted using standard procedures described by the Indonesian Herb Pharmacopoeia and Harborne[17,18].

Quantitative analysis of total alkaloid of *P. retrofractum* was also conducted using Folin-Ciocalteu[19]. Briefly, 2.5 g of powdered samples were weighed and extracted using maceration with 5% of acetic acid for 4 h. Then, the mixture was filtered to remove cellular debris until the volume reached one quarter using water bath at 70 ℃. A concentrated ammonium hydroxide (NH_4OH) was applied dropwise until pH reached 10 or the precipitate was complete. The solution was then centrifuged and the precipitate was washed using 1% of ammonium hydroxide and was de novo centrifuged. Finally, the obtained residu, which represented the total alkaloid, was weighed after previously being dried. The total alkaloid was represented as percentage using the following equation:

Total alkaloid (%)=Residue/(Weight of sample)×100

Total phenol content of methanol, ethyl acetate and *n*-hexane extraxts were also determined using Folin-Ciocalteu method. Briefly, 1 mL of the extracts were mixed with 0.5 mL of Folin-Ciocalteu reagent. The mixture was left for 5 min. Subsequently, 2 mL of 10% (w/v) sodium carbonate. The mixture was then allowed to stand for 10 min in the dark and then the absorbance was measured using spectrophotometer at 770 nm. Total phenolic content was expressed as mg of gallic acid equivalent per g dry weight of the extract using a calibration curve generated with gallic acid.

Total flavonoid content was also measured. Firstly, 1 g of powdered samples was weighed and placed in a boiling flask round bottom. Then, 1 mL of 0.5% (w/v) hexamethylenetetramine, 20 mL of acetone and 2 mL of 25% (w/v) HCl were added. The mixture was then filtered and placed into a volumetric flask. Subsequently, 100 mL of acetone was then added and homogenized. A total of 20 mL of filtrate obtained was added by 20 mL of distilled water. Hereafter, 15 mL of ethylactetate and then mixed thoroughly. The ethylacetate phase was separated and subjected to three additional 10 mL ethylacetate extraction. All the ethylacetate fractions were mixed together and then washed two times using 50 mL distilled water followed by filtration using cotton. Finally, filtrate was diluted using ethylacetate to 50 mL. Quantification of flavonoids was conducted based on Christ-Muller's method. Briefly, 10 mL of ethylacetate fraction was mixed with $AlCl_3$ in a methanol-acetic acid. The absorbance was measured at 425 nm using spectrophotometer. The percentage of flavonoids were determined using the following

formula :

Total flavonoids (%)=Absorbance of sample×1.25/(Weight of sample)×100

2.10. Statistical analysis

Statistical analysis were performed using Minitab 17 Statistical Software for Windows. Data were expressed as mean±standard deviation. Determination of mineral composition and phytochemical quantification were carried out in triplicate and duplicate, respectively. Comparison between extraction solvent used in total phenol quantification was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD *post hoc* test.

3. Results

3.1. Proximate composition of P. retrofractum vahl. fruits

Water content of *P. retrofractum* fruit was found to be 18%. High water content in this fruit support its function as supplemental herbal beverages. Some traditional and modern beverages derived from the plants also showed similar value in their water content[15,20]. Meanwhile, total ash and fat of this fruit were 4.29% and 2.97%, respectively. Dietary fiber of the fruit was found to be 28.8%. This fiber represents polysaccharides as well as lignin and other plant fiber that are not easily digested by human's digestive system. In addition, dietary fiber can also be an alternative source of energy. Nevertheless, we found high amount of carbohydrate in this fruit (63.4%). The crude protein was measured using Kjeldahl method by determining total nitrogen content within the fruit. Therefore, it includes nitrogen from the proteins and other nitrogen containing compounds. The value of crude protein reached 11.4%. Finally, the calorie was found to be 326 kcal/100 g. This means that *P. retrofractum* fruits possesses an important value of energy and it could support the traditional uses of this fruit as supplement and medicinal beverages.

3.2. Mineral composition of P. retrofractum vahl. fruits

Determining the mineral content of the fruit or other source of food is pivotal for human health reasons. We observed high quantity of potassium (K), calcium (Ca), phosphor (P), and magnesium (Mg) which were (1 361.03±24.00); (414.97±5.85); (203.30±0.26) and (166.4±0.8) mg/100 g DW, respectively. Meanwhile, among the minerals observed in this study, the amount of manganese found in this fruit was found to be the lowest [(0.72±0.06) mg/100 g DW]. The ratio level between zinc (Zn) and copper (Cu) was found in relatively balanced condition, where the concentration of Zn and Cu were (0.93±0.08) and (0.91±0.04) mg/100 g DW. Other trace elements such as iron (Fe) and sodium were found to be (5.12±0.06) and (9.41±0.32) mg/100 g DW, respectively.

3.3. Phytochemical screening of P. retrofractum vahl. fruit

The phytochemical screening of *P. retrofractum* fruit was preliminary conducted using three different extracts, including methanol, ethylacetate and *n*-hexane extracts. Phytochemical parameters used in this study comprise quinone, sterols, glycosides, flavones, tanin and alkaloids. The results showed that methanol extract was positive for almost all parameters, except quinone. Meanwhile, ethylacetate extract of *P. retrofractum* fruit was positive for quinone, sterols, glycosides, tanin and alkaloids but negative for flavones. Finally, we observed the presence of quinone, sterols, glycosides and alkaloids in the *n*-hexane extract of the fruit. Our observation also showed that *P. retrofractum* fruit contains low quantity of flavonoids and alkaloids, which were 0.060 0%±0.000 2% and 0.265%±0.008% (w/w), respectively. Furthermore, we did three extractions using different solvents including methanol, ethylacetate and n-hexane. Each type of extracts exhibited significant different amount of total phenols ($P<0.05$). Methanol extract showed the highest total phenol content [(16.31±0.05) ppm] followed by ethylacetate and n-hexane [(5.78±0.02) and (5.31±0.01) ppm respectively]. These indicate that the selection of solvent for an efficient extraction is important.

4. Discussion

Food and beverages industries apply a very strict regulation to ensure the quality of the product. The latter is necessarily required since this type of industry directly correlates with human health. Furthermore, medicinal based industry, especially natural-based drugs where some of the components are obtained from the living things, including plants, also possess similar regulation. Therefore, proximate analysis would give a preliminary data for determining the quality of the products as well as its nutritional value[21]. This dioceous climbers tree, *P. retrofractum* possess a cylindrical berry fruits, where the apex part of the fruit is rounded. The color of the fruit is green and become reddish to brown when they reach its maturity. In term of traditional uses, the *P. retrofractum* fruit is commonly used as flavours in the folk cuisine preparation (*i.e.* curries). In addition, the Indonesian local wisdom knowledge of grinding, mashing and pounding the plant material into a valuable medicinal beverages, commonly called as 'Jamu' has made *P. retrofractum* as an economically important plants. Some litteratures have cited this plant for its biological function as antioxidant[8], hepatoprotective[22], anticancer[23], gastroprotective[24], antiobesity[11], potential mast cell stabilizer[10], antileishmanial[25], and antilarvacidal[13].

The water content analysis revealed that *P. retrofractum* exhibited relatively high water content compared to other *Piper* species including *Piper trichostachyon* and *Piper nigrum* (*P. nigrum*) which

possessed 4.31% and 4.55%, respectively[26]. The water content analysis helps to determine the usable and safety time of the product. High water content might provoke the presence and enhance the bacteria and mould growth, in some extent, this condition might reduce the quality of the product[27]. More than 14% of food water content is susceptible for microbial contamination[28]. Hence, good storage condition also plays a crucial role to prevent spoilage. Nevertheless, high water content is the best characteristic for plant-based beverages. Some reports showed that plant parts that are used for beverage production have high water content[14]. We observed the presence of high carbohydrate content (63.4%) within the fruit. This is could be the major contributor of energy, since crude protein and total fat contribute only in small portion. These values are lower than that observed in other Piper species, such as *P. nigrum*. The crude protein content and total fat of *P. nigrum* had been reported to be 25.45% and 5.34%, respectively. However, carbohydrate content of *P. retrofractum* is higher than *P. nigrum*, which is only 37.36%[29]. The contribution of the three parameters (carbohydrate, crude protein and total fat) make *P. retrofractum* as a valuable high energy plant source. This could be interesting since high calorie diet increases weight gain in anorexia nervosa incidence[30]. Eventhough *P. retrofractum* possess high caloric value (326 kcal/100 g), this species contains piperidine alkaloids, which regulates lipid metabolism by activating the AMP-activated protein kinase. The biological function of the antiobesity constituent of *P. retrofractum* is indicated by an increase of protein expression, which is responsible for fat burning and a decrease of protein expression involved in fat storage mechanism[11].

Dietary fiber has been always associated with human health, especially to certain diseases including cardiovascular, type Ⅱ diabetes, obesity and cancer[31]. Dietary fibers have been defined as non enzymatically digestible. The dieatry fiber was found to be relatively higher than other spices plants such as *Ocimum gratissimym*, *Penrgularia extensa* and *Tetrapleura tetraptera* which are ranging from 17.00%-20.24%[32]. In addition, our observation also showed that the dietary fiber contained in *P. retrifractum* is also higher than *P. nigrum* (23.6%)[29]. These results also made *P. retrofractum* as a potential dietary fiber source. Hence, it also supports the medicinal function of this spice plant. The minerals content of this species is also comparable to other Piper species. Calcium, magnesium and potassium were found higher that those observed in *Piper guineense* (8.50-12.38; 1.14-1.21; and 0.72-1.2 mg/100 g DW, respectively)[33] and in *P. nigrum* (195; 52 and 663 mg/100 g DW, respectively)[29]. Calcium functions not only for mediating the vascular contraction and vasodilatation, neural transmission and muscular contraction[34], but also plays an important role in bone health, including lowering the risk of osteoporosis[35]. Regarding to magnesium, this intracellular cation is also associated with calcium metabolism in the human bones and in some extent might be associated with hypertension, diabetes and cardiac perturbation[36]. As an extracellular cation, potassium also plays a pivotal role in maintaining blood pressure. Therefore, high uptake of potassium could reduce the hypertension[37]. Our observation showed that *P. retrofractum* posses high potassium and low level of sodium. This low Na/K ratio might also contribute to better dietary source[15].

A non significantly difference between copper and zinc content was observed in this study. The balance between those two minerals is essential for reducing the risk of coronary diseases by modulating the concentration of cholesterol within the plasma[38]. Furthermore, zinc and copper have been known for its function as co-factor of metalloproteins, including superoxide dismutase. Recent data showed that these two elements function to ensure and accelerate the oxidative refolding of metalloprotein. In other word, these elements play a role in preventing protein misfolding which is responsible for many serious diseases[39]. Our observation showed that zinc content of *P. retrofractum* is higher than that of *Piper guineense* (0.09-0.18 mg/100 g DW)[33]. Meanwhile, copper content of *P. retrofractum* is lower than that observed in *P. nigrum* (1.3 mg/100 g DW)[29].

Compared to zinc, iron is abundantly found in earth and also plays an important role in human health. It involves in the formation of hemoglobin and ensures the oxygen transport through blood circulation. Low intake of iron can lead to the anemia disease and possibly can also cause neurodegenerative diseases[40]. Major source of iron is from meat, fishes and also from some vegetables. However, the absorption of iron from vegetables is reported to be lower than those absorbed from meat and fishes. This is might be due to other food components present in the vegetables including polyphenols, calcium and peptides[41]. In this study we found that iron is detected in comparable quantity compared to other *Piper* species. It is lower than that observed in *P. nigrum* and *Piper trichostachyon* (25 mg/100 g DW and 10.84 mg/100 g DW, respectively)[26] and higher than iron observed in *Piper guineense* (0.62-0.85 mg/100 g DW)[33]. Nonetheless, *P. retrofractum* could be potential and alternative source of iron in human diet.

High phosphorous and low manganese content are found in *P. retrofractum*. Together with calcium, phosphorous involves and plays an important role in ensuring human bone health[42]. However, other report showed that high consumption of phosphorous is often associated with cardiovascular diseases[43]. Similar to the function of phosphorous and calcium, manganese is also essential micronutrient needed in supporting human bone formation. Additionally, it plays an important role as co-factor of some important enzymes involved in the neurotransmitter metabolism[44]. Our results showed that the quantity of phosphorous and manganese found in *P. retrofractum* fruits were higher than *P. nigrum* (0.14 mg/100 g DW and 5.18 mg/100 g DW, respectively) and lower than *Piper trichostachyon* (0.16 mg/100 g DW and 7.54 mg/100 g DW, respectively)[26].

Phytochemical screening of *P. retrofractum* fruits extracted from *n*-hexane, ethylacetate and methanol result in different profile of natural compounds ranging from quinone, sterols, glycosides, flavones, tannin and alkaloids. This demonstrates that the choice of solvent for extraction process is necessarily required and determines

the type of phytochemicals. This is in accordance with the previous study which showed that different solvent mixture influenced the extracted compounds. Furthermore, the choice of solvent might also be species dependent[45]. Naturally occuring phytochemicals are also influenced by the genetic of the species, geographical factors, climate, ecological and plant part used for extraction methods[33,46]. Regarding to the effect of different solvents used for extraction, it is also reflected in the results of total phenol quantification, which indicated that the use of different solvents resulted in different quantities of total phenol. Methanol is the best solvent for total phenol quantification since it gave the highest quantity compared to others. Our results also showed that total phenol contained in the fruit of *P. retrofractum* through methanol extraction is silghtly lower than that obtained in the *P. nigrum* fruits (1.728 mg/g). Meanwhile, flavonoid content in the *P. retrofractum* is higher than that observed in *P. nigrum* (1.087 μg/g)[47]. Flavonoids have been known as a natural compounds that play an important role in some biological mechanisms of living thing. Previous reports showed that flavonoids obtained in *Piper aduncum* possess anti-inflamatory effect[48]. Moreover, flavonoid compounds found in *Piper delineatum* and *Piper sarmentosum* have been reported to act as inhibitor of quorum sensing inhibitor[49] and cytoprotective against oxidative stress[50], respectively. Alkaloids are also major constituents in plant belongs to the genus *Piperaceae*[1]. Among them, piperine constitutes the main alkaloids found in this genus. Many reports have demonstrated the biological function of alkaloids including hepatoprotective, antidepressant and anti cancer[51]. Our results showed that total alkaloids found in *P. retrofractum* was lower than alkaloid found in *P. nigrum* fruit (112.39 mg)[52]. Nonetheless, the overall results support the medicinal function of *P. retrofractum* fruits.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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