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Development of an extraction procedure to control rutin content from *Azadirachta indica* leaf extract for diabetes treatment

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Abstract. *Azadirachta indica*, a widely planted plant in southern Vietnam, is used in traditional medicine for its anti-cancer, antibacterial, and diabetes-fighting properties. However, there are few studies on using rutin as a marker in *A. indica*. This study aimed to evaluate the alpha-glucosidase enzyme inhibitory activity of the extract for diabetes treatment. The extract was optimized using ultrasound-assisted extraction and quantified using HPLC/PDA. The HPLC/DAD method was developed to determine rutin in *A. indica* leaf using a Phenomenex C_{18} column. The extraction efficiency was evaluated by the peak area of rutin in the extract sample. The methanol extract was selected as the extraction solvent due to its strong alpha-glucosidase enzyme inhibitory activity. The optimal extraction process involved methanol extraction with a volume of 30 mL, an ultrasound time of 30 minutes, an extraction temperature of 50 °C, and extraction three times. The rutin content in the extract was 5.53 mg/g.

Keywords: *alpha-glucosidase, diabetes, rutin, traditional medicine, and ultrasound-assisted extraction.*

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1. Introduction

Azadirachta indica A. Juss originates from Myanmar, India, and with diverse uses in treating diseases, it is also called the "Village medicinal plant" by Indian people (Sujarwo et al., 2016). Today, *A. indica* trees are still widely found in Bangladesh, Sri Lanka, Thailand, Malaysia, Mauritius, Fiji, South Africa, East Africa, and America (Khare, 2007; Kokate et al., 2017). In Vietnam, *A. indica* was introduced and experimentally planted in Ninh Thuan. With favorable climatic conditions as well as easy-to-grow characteristics that do not require much care, after a few years, the tree was widely planted in many southern provinces, such as An Giang and Kien Giang (Bich et al., 2006).

According to the US Environmental Protection Agency (EPA), A. indica is considered harmless to humans, birds, beneficial insects, and earthworms and is approved for use as a plant food (EPA, 2012). Different parts of A. indica, including leaves, bark, seeds, flowers, fruits, and roots, all have pharmacological effects with a variety of compounds found and extracted, such as azadirachtin, nimbolide, gedunin, azadirone, and salannin. Therefore, A. indica is widely used in traditional medicine systems (Patel et al., 2016). The pharmacological activities of *A. indica* are mentioned as anti-cancer, antibacterial, antifungal, antiviral, anti-epileptic, anthelmintic, anti-malarial, antipyretic, analgesic, anti-inflammatory, antidiabetic, anti-allergy, neuroprotection, cardiovascular protection, and insecticidal activities (Gupta et al., 2017; Saleem et al., 2018).

The nutritional value and medicinal uses of *A. indica* are due to the presence of phytochemicals. Compounds such as alkaloids, glycosides, carbohydrates, phenols, flavonoids, steroids, proteins, and amino acids found in the extract of *A. indica*'s leaves show the potential for the development of pharmacological and therapeutic activities of the plant in the field of medicine from medicinal herbs. Outstanding activities of the phenolic group, such as anti-oxidation, anti-inflammatory, antibacterial, and anti-cancer, in the extract of *A. indica* have also been reported in many studies (Sithisarn et al., 2006; Dhakal et al., 2016; Malar et al., 2020; Altayb et al., 2022). To our knowledge, there are currently very few studies on using rutin as a marker in *A. indica* (Figure 1).

Figure 1. Leaves of *Azadirachta indica* A. Juss.

The demand for using herbal medicines in the traditional way or from compounds of natural origin is increasing. In addition, herbal preparations, although they contain only one medicinal ingredient, contain many ingredients and many different compounds. Therefore, the identification and quantification of the main phenolic compounds accumulated in *A. indica* is necessary to improve the reliability, uniformity, and quality of the raw material, facilitating its use in the pharmaceutical industry. The high-performance liquid chromatography method uses preferred standards with outstanding advantages such as high sensitivity, good quantification, and high accuracy. The aim of the study was to evaluate the diabetes treatment of *A. indica* extract by alpha-glucosidase enzyme inhibiting activity. After that, the extract was optimized for ultrasound-assisted extraction and quantified for rutin as a marker of *A. indica* by HPLC/PDA.

2. Materials and Methods

2.1. Plant materials

A. indica A. Juss. specimens were gathered in the An Giang provinces, located in the Mekong Delta region of Vietnam. The plants were taxonomically classified at the Department of Biology, Can Tho University, with the scientific name *Azadirachta indica* A. Juss. Fresh leaf samples were gathered, air-dried, and crushed. All of the samples were housed in opaque glass containers and maintained at ambient temperature.

2.2. Chemicals, solvents and standard solutions

Sigma-Aldrich (Steinheim, Germany) provided the HPLC-quality methanol, acetonitrile (≥99.9%, with amylenes as stabilizers). The National Institute of Drug Quality Control (Vietnam) provided the rutin (98%) that was purchased. The α-glucosidase enzyme was taken from *Saccharomyces cerevisiae* and has an activity of 23 U (Sigma-Aldrich). Para-Nitrophenyl a-D-glucopyranoside was also taken from Sigma-Aldrich.

2.3. Alpha-glucosidase enzyme inhibiting activity

The alpha-glucosidase enzyme inhibiting activity was performed according to a method described by Shai et al. (2011) and with adjustments. Methanol extract was diluted in DMSO to obtain a final concentration of 1 mg/mL, and then centrifuged for 10 minutes to get the clear supernatant. This solution was added as the substrate in phosphate buffer (pH 7.0) to obtain concentration levels of 25, 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 (µg/mL). To start the reaction, 100 µL of the alpha-glucosidase enzyme (0.2 U/mL) was added to 50 εL of the reaction mixture. The mixture was then left to sit at 37 °C for 10

minutes. Then, 50 µL of 4 mM pNPG as a substrate for the reaction was added to the mixture and incubated at 37 °C for 20 minutes. The reaction was terminated by adding 1000 µL of 0.2 M Na_2CO_3. We measured the color intensity at 405 nm. Acarbose was used as standard drug control. The absorbance was measured at 405 nm. The alpha-glucosidase enzyme inhibiting activity was expressed as IC_{50}, and the inhibition percentage was calculated as given in the equation:

% inhibition = (Absorbance of control - Absorbance of sample)/ (Absorbance of control) × 100

The surveyed solvent (methanol, petroleum ether, or ethyl acetate) with the highest pharmacological activity will be selected as the extraction solvent for the next extraction experiment.

2.4. Optimization of sample preparation

Accurately weigh approximately 0.5 g of *A. indica*'s leaf sample and add it into a 15-mL centrifuge tube. The sample was extracted by the ultrasound-assisted extraction method at C (°C) temperature for B minutes with A mL of solvent; repeat the extraction D times. The efficiency of the extraction process was evaluated by the peak area of rutin in the extract. Four factors were optimized in ultrasound-assisted extraction, including solvent volume (A), extraction time (B), temperature (C), and times of extraction (D).

2.5. Chromatographic conditions

Method development, quantification, and validation were performed using an HPLC Hitachi Chromaster detector PDA. The type of HPLC C_{18} column used was the Phenomenex (250 mm × 4.6 mm i.d.; 5 µm), with isocratic elution of methanol: 0.1% formic acid/water (30:70). All chromatographic separations were carried out at a flow rate of 1.0 mL/min with an injection volume of 10 µL. Analytical identification was carried out using a 254 nm detection wavelength.

2.6. Method validation

The method was tested for selectivity, linearity, limit of detection, limit of quantification, precision, and accuracy in accordance with AOAC and ICH guidelines (AOAC, 2013).

2.7. Statistical analysis

The statistical analyses were conducted using Microsoft Excel 2016, with all measurements taken in triplicates, and all figures were generated using GraphPad Prism 8.

3. Results and Discussion

3.1. Alpha-glucosidase enzyme inhibiting activity

pNPG was used as a substrate to investigate the effect of *A. indica* extract on the alpha-glucosidase enzyme activity. Acarbose was a substance capable of inhibiting the enzyme alpha-glucosidase and was used as a positive control drug. Table 1 shows that the alpha-glucosidase enzyme inhibition effect of acarbose and methanol extracts of *A. indica* leaves got stronger as the concentration went up.

Table 1. Results of investigating the ability to inhibit α-glucosidase enzyme.

Concentration (µg/mL)	Percent inhibition of alpha-glucosidase (%)			
	Acarbose	Methanol	Petroleum ether	Ethyl acetate
25	15.98^{k}±0.13	15.54^{k}±0.34	7.76^{j}±0.16	8.49^{j}±0.18
50	22.15^{j}±0.02	23.57^{j}±0.20	14.18^{i}±0.15	16.25^{i}±0.34
100	31.24^{i}±0.21	30.63^{i}±0.16	19.93^{h}±0.26	30.41^{h}±0.18
150	37.97^{h}±0.12	41.50^{h}±0.09	32.06^{g}±0.18	38.52^{g}±0.26
200	47.53^{g}±0.19	55.46^{g}0.24	50.08^{f}±0.25	45.15^{f}±0.29
250	55.88^{f}±0.04	63.63^{f}±0.23	55.48^{e}±0.09	57.14^{e}±0.27
300	66.36^{e}±0.17	70.71^{e}±0.16	64.07^{d}±0.43	55.65^{d}±0.16
350	77.35^{d}±0.07	80.65^{d}±0.24	70.58^{c}±0.17	77.68^{c}±0.11
400	79.22^{c}±0.17	87.81^{c}±0.13	77.75^{b}±0.24	81.29^{b}±0.24
450	84.94^{b}±0.13	93.86^{a}±0.19	77.4^{b}±0.28	84.76^{a}±0.24

On the basis of IC_{50}, acarbose showed an inhibitory effect with an IC_{50} of 220.6±0.18 µg/mL. They both had weak inhibitory effects on the alpha-glucosidase enzyme, with IC_{50}s of 237.2±0.69 µg/mL for the petroleum ether extract and 228.4±0.56 µg/mL for the ethyl acetate extract of *A. indica* leaves. However, methanol extract showed the strongest inhibitory effect with an IC_{50} of 19.2±0.14 µg/mL, as shown in Figure 2.

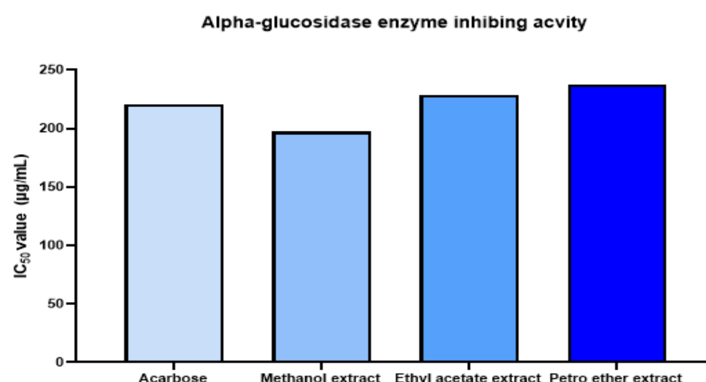

Figure 2. Alpha-glucosidase enzyme inhibiting activity (IC_{50}(µg/mL) of methanol extract, petroleum ether extract, ethyl acetate extract of *A. indica* and standard (acarbose).

The higher the IC_{50}, the lower the inhibition effect, and vice versa. The alpha-glucosidase enzyme inhibitory activity of petroleum ether extract and ethyl acetate extract of *A. indica* is not as strong as that of acarbose. Meanwhile, methanol extract has stronger alpha-glucosidase enzyme inhibitory activity than acarbose. This proves that many of the compounds in *A. indica* extract that can stop the alpha-glucosidase enzyme from working are made up of substances with a medium polarity. *A. indica* extracts are extracted with many solvents of different polarities. Each extract's composition, amount of inhibitors, and ability to act on the alpha-glucosidase enzyme are not the same, so there are differences in the percentage of enzyme inhibition in the research. Based on IC_{50}, the ethyl acetate extract of *A. indica* leaves was 228.4 g/mL higher than the Saini et al. (2015) study, which had an IC_{50} result of 208.92 g/mL. It was found that the methanol extract of Dagar et al. (2022) had an IC_{50} of 50 mg/mL, which is higher than the IC_{50} of 197.2 µg/mL for the methanol extract of *A. indica*. Graphic conditions, sample collection time, extraction method, and chemicals may be the causes that affect the active ingredients in the raw materials, leading to these differences.

Research has been conducted on the inhibitory activity of plant extracts on α-glucosidase enzymes. In vitro and in silico evaluations showed that *Ocimum basilicum* methanol extract inhibited alpha-glucosidase and α-amylase activity at concentrations of 500 µg/mL. Mahdi et al. (2020) tested hydro-methanol decoction extract, ethyl acetate fractions, and *n*-butanol fractions for their ability to stop alpha-glucosidase from working in a lab setting. According to Kidane et al. (2018), the methanolic extract of *Psiadia punctulata* had the most inhibitory activity at 17.29±9% mg/dL. The chloroform extract of Meriandra *bengalensis* also had the most activity at 30±5% mg/dL. According to Dhakal et al. (2016), the methanol extract of *A. indica* was a much better antioxidant than the chloroform extract. The IC_{50} for the methanol extract was 80.28 µg/mL, while it was 439.60 µg/mL for the chloroform extract. The methanol extract with the highest pharmacological activity will be selected as the extraction solvent for the next extraction experiment.

3.2. Optimization of extraction procedure

Ultrasound-assisted extraction (UAE) is a novel extraction technique with advantages over traditional methods like less time, high yield, and easy control. The optimal conditions for rutin assay in *A. indica* extracts were analyzed using parameters like solvent volume, extraction time, number of extractions, and temperature.

As the solvent volume increases, the ability to dissolve substances also increases, resulting in higher extraction efficiency. Because efficiency is limited, the amount of solvent used must be optimized to ensure economy. The rutin peak area resulted in a nearly equal distribution between 30 mL and 40 mL. To limit the amount of organic solvent, 30 mL of methanol was used. The

extraction times, including 10, 20, 30, and 40 minutes, were investigated. The result of the rutin peak area was almost equal between 30 and 40 min. Therefore, the extraction process took longer than 30 minutes. Prioritizing the appropriate temperature selection is crucial for achieving high performance, with the investigated temperatures being 30 °C, 40 °C, 50 °C, and 60 °C, respectively. The highest peak area of rutin was extracted at 50 °C. The solubility of active ingredients in medicinal herbs will increase as the temperature increases; however, too high a temperature can decompose some active ingredients that are less stable with heat. Furthermore, we evaluated the extraction efficiency four times. The result shows that the remaining rutin peak area in the 4th extraction was only <1% (about 0.42%) compared to the total rutin peak area after extraction four times (Figure 3). Therefore, the optimal number of extractions was chosen for the process to be three to save time.

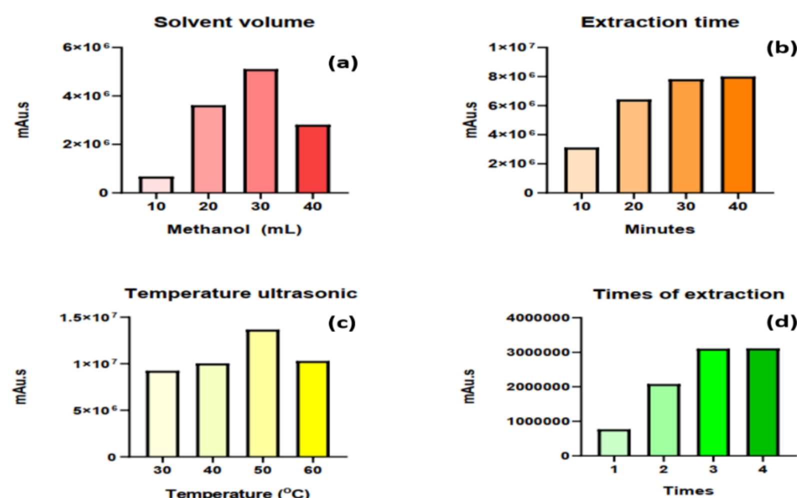

Figure 3. The result of optimization of extraction procedure: (a) solvent volume; (b) extraction time; (c) temperature ultrasonic and (d) times of extraction.

Based on the aforementioned findings, we selected methanol as the ideal extraction solvent, utilizing a volume of 30 mL, a 30-minute ultrasound duration, and an extraction temperature of 50 °C. According to research by Biney et al. (2021), the methanol extract of *A. indica* contained more phytochemical compounds at a concentration of 0.1 mg/mL than the acetone/water extract. Besides that, according to Benisheikh et al. (2019), solvents used in research were chloroform, hexane, methanol, and ethyl acetate. The results also showed that chloroform and methanol extracts had the highest and moderate activity against the tested microbial pathogens, respectively, while other extracts had the lowest activity.

3.3. Optimization of chromatographic conditions

In order to examine phenolic acids and flavonoids, it is common practice to include acidic modifiers in the mobile phase. This helps to reduce the ionization of polyphenolic compounds and silanols. The mobile phase employed for the separation of phenolic acids and flavonoids by RP-HPLC, as documented in several prior research (Snyder et al., 2009), consisted of a mixture of water and methanol, water and acetonitrile, or a combination of water, methanol, and acetonitrile. In addition, the mobile phase was typically altered by including phosphoric acid, formic acid, or tetrahydrofuran. Several variables, like as the characteristics of the substance being analyzed, the properties of the stationary and mobile phases, pH, flow rate, and temperature, have an impact on the separation of compounds in chromatography. Consequently, rutin possesses a structure with moderate polarity. Therefore, the mobile phase components selected for analysis were methanol (MeOH) and water (H_2O).

Under the condition of a mobile phase MeOH:H_2O ratio of 25:75 (v/v), the chirality coefficient and capacity coefficient are both met, and the separation between rutile and impurities is complete, but the run time is too long (more than 40 minutes). All the chromatographic conditions are met when the mobile phase is MeOH:H_2O (v/v) at ratios of 35:65, 40:60, and 50:50 (v/v). However, the main peak and impurity peaks of the test sample are not completely separated. At the mobile phase ratio of 30:70, the separation between rutin and impurities is complete, and the retention time is about 20-21 minutes. However, under this

condition, the rutin signal is unstable in the mobile phase; this is overcome when adding 0.1% formic acid to H_2O, and the rutin signal is more stable, so choosing the ratio MeOH:H_2O (0.1% formic acid) 30:70 (v/v) is the optimal condition.

The pH of the buffer solution was investigated at 2.5, 3.0, and 4.0 with the mobile phase ratio selected. According to the survey results, the peak area of rutin was nearly the same at pH 2.5 and pH 3.0. At pH 4.0, the peak area decreased a little but not too much, possibly because at pH 4.0, the stability of rutin began to be affected due to the interaction of OH-phenols with the solution. NaOH is used to standardize the pH of the buffer solution.

3.4. Method validation

System stability. To check how stable the system was, a mixed standard solution with a concentration of 10 µg/mL was injected six times. Different parameters were then measured, such as the theoretical plate number (N), resolution (Rs), symmetry factor (As), and repeatability (shown by the relative standard deviation (RSD) of retention time (RT) and area) of the analytes. The relative standard deviation (RSD) values of the peak area and retention time (RT) for all analytes were below 2.0%. Hence, the suggested approach fulfilled this criterion (Figure 4).

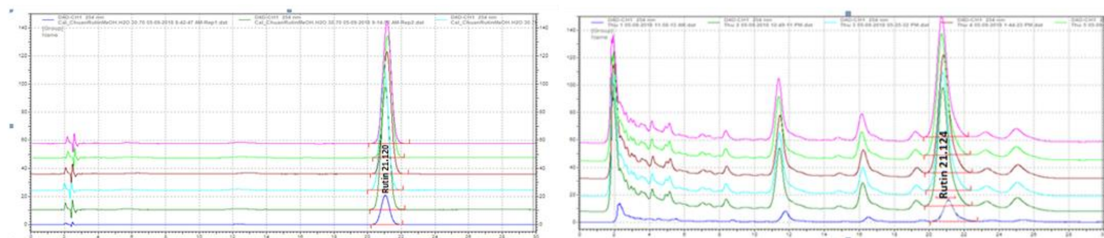

Figure 4. System stability chromatograms on rutin standard and test samples.

Specificity. The selectivity was assessed by utilizing the High Performance Liquid Chromatography (HPLC) technique to evaluate the leaf extracts of *A. indica*. The evaluation was conducted by comparing the retention time (RT) and ultraviolet (UV) absorption spectra of each component in standard solutions with the peaks obtained by analyzing the extracts (Figure 5 and Table 2).

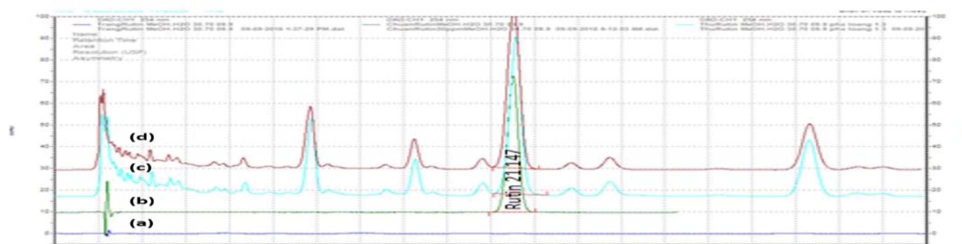

Figure 5. Chromatography of specificity: (a) mobile phase solvent; (b) rutin standard sample; (c) *Azadirachta indica* leaf sample; (d) rutin spiked *Azadirachta indica* leaf sample.

Table 2. Purity on standard and test samples of rutin.

	Time saved (minute)	Peak area (mAUs)	Purity (%)
Standard	21.113	3961036	99.45
Test samples	21.207	3110924	97.78

Linearity, limit of detection, and limit of quantification. Stock solutions were mixed with rutin at different concentrations, and calibration curves were obtained. The linearity was evaluated using HPLC, with LOD and LOQ values of 0.125 ppm and 0.412 ppm, respectively.

Table 3. Precision, recovery, calibration parameters, LOD and LOQ.

	Calibration curve		Precision		Recovery (%)		
Substance	Regression equation	R^2	Intra-day RSD (%) (n=6)	Inter-day RSD (%) (n=18)	Low-level	Mid-level	High-level
Rutin	y = 78661x	1	2.91	2.44	3.72	2.29	4.82

Precision. The method's accuracy was confirmed by comparing the intraday and interday precisions. As an accuracy indicator, the relative standard deviation (%RSD) was used. The precision of intra-day was performed on 6 test samples, and the precision of inter-day was conducted over 3 consecutive days with a total of 18 specimens. The precision results presented in Table 3 indicate that the total intra- and inter-day fluctuations (%RSD) were less than 6%, which aligns with the AOAC recommendations.

Accuracy. Looking at the method's accuracy by recovery experiments. The blank samples were treated with three quantities of reference compounds: low (80%), medium (100%), and high (120%). The spiked samples were then included and measured using the aforementioned techniques. The findings showed that the developed method had a good level of accuracy, with a total recovery falling between 94.6% and 103.7%. The method was thought to be accurate in light of the recovery test's outcomes.

Application. All leaf samples detected rutin, and the quantitative results show that the average rutin content is 5.53 mg in 1 g of extract (Table 4).

Table 4. Quantitative results of 18 samples of *A. indica* leaves.

Sample	Rutin content (mg/g)	Sample	Rutin content (mg/g)
1	5.1721	10	5.6034
2	5.3398	11	5.6828
3	5.2474	12	5.3868
4	5.5046	13	5.7816
5	5.3899	14	5.6636
6	5.3749	15	5.8228
7	5.4746	16	5.8270
8	5.6011	17	5.6093
9	5.4812	18	5.5434

4. Conclusions

The methanol extract of *Azadirachta indica* had the most potent inhibitory action on the alpha-glucosidase enzyme, with an IC_{50} value of 197.2±0.14 µg/mL. A method combining ultrasound-assisted extraction and RP-HPLC with diode array detection was devised to determine the concentration of rutin in *A. indica* leaves. The optimal extraction process resulted in high efficiency and extraction yields. In addition, the HPLC methodology allowed for the qualitative separation of the chemicals and demonstrated high efficiency, precision, and accuracy. The quantitative analysis reveals that the mean rutin concentration is 5.53 mg per gram of extract. This study reveals that *A. indica* in Vietnam possesses numerous promising components that could potentially transform the plant into a valuable source of medicinal herbs for treating diabetes in the future.

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Conflicts of interest. The authors mentioned that none of them have a conflict of interest when it comes to this article.

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