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Optimization of extraction parameters for phenolic and flavonoid in *Artocarpus altilis* (breadfruit) leaf extract

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Abstract
Artocarpus altilis (*A. altilis*), commonly known as breadfruit, is widely exploited for food and non-food uses. In Southeast Asian regions, the leaves of *A. altilis* are consumed for their medicinal properties following traditional medicine. A growing body of literature points toward the extract of *A. altilis* leaves possessing anti-cancer, antioxidative, photoprotective, anti-inflammatory, hepatoprotective, renoprotective, and cardioprotective properties. Furthermore, experimental and computational methods have demonstrated that there is an association between the antioxidative property with the flavonoid and phenolic contents of *A. altilis* extract. In an attempt to identify the optimal extraction parameters for the Soxhlet extraction of *A. altilis* leaves, the extraction time and the sample-to-solvent ratio were optimized by one-factor-at-a-time methodology. The optimized extraction parameters were identified as: 6-hour Soxhlet extraction, sample-to-solvent ratio of 4:160 (g:mL), with ethanol:water (60:40, v/v) as solvent. The optimized *A. altilis* leaf extract exhibited total phenolic content and total flavonoid content values of 86.96±0.2130 mg GAE g^{-1} DW and 33.76±0.2991 mg QE g^{-1} DW, respectively. The optimized *A. altilis* leaf extract demonstrated potential for the development of nutraceutical and pharmaceutical products due to its strong antioxidant activity.

Keywords: Antioxidant, *Artocarpus altilis*, Breadfruit, One-factor-at-a-time method, Radical scavenging, Soxhlet extraction

Introduction
Artocarpus altilis, commonly known as breadfruit, is a member of the genus Moraceae which contains about 50 species [1]. *A. altilis* originated in the Western Pacific and spread throughout the tropical regions after the European explorers recognized the potential of the *A. altilis* fruit as a highly productive and cheap source of nutrition.[2, 3] The mature fruit of *A. altilis* is consumed as a stable food in many Pacific Islands countries due to its high productivity and similarity in taste to bread [2, 3].[3] Other parts of *A. altilis* are also consumed as construction materials (tree, timber), adhesives (latex), animal feed (fruit, leaves), and medicine (leaves, bark, latex).[3] Although *A. altilis* is widely exploited for food and non-food uses in the Pacific Island regions, it is a minor crop in Vietnam and other Southeast Asian regions [2, 4]. Despite this, the leaves of *A. altilis* are consumed for their medicinal effects in the Southeast Asian and South American regions [3-5].

A growing body of literature has investigated the therapeutic application of the leaves of *A. altilis* and they suggested the extract of *A. altilis* leaves to possess therapeutic actions and demonstrate anti-cancer, antioxidative, photoprotective, anti-malarial, anti-hypertension, anti-inflammatory, hepatoprotective, renoprotective, and cardioprotective properties. *A. altilis* leaf extract showed potential in inhibiting the proliferation of cervical cancer cell [6], human lung cancer cell, human liver cancer cell *in vitro* [7]. *A. altilis* extract suppressed the structural alterations in skin induced by ultraviolet radiation [8]. In the same vein, the methanolic extract of *A. altilis* leaf exhibited significant tyrosinase and α-glucosidase inhibitory activities [9]. The aqueous ethanol extract of *A. altilis* leaf was shown to inhibit the growth of malarial parasite [10]. The aqueous extract of *A. altilis* leaf also demonstrated anti-hypertension effect in Sprague-Dawley rats via intravenous injection [11]. Oral administration of methanolic *A. altilis* leaf extract conferred anti-inflammatory property to Wistar rats after carrageenan-induced paw edema [12]. Recent works in Wistar rats also points toward the

hepatoprotective and renoprotective activities of methanolic *A. altilis* extract against cadmium-induced liver and kidney dysfunction via antioxidant and radical scavenging activities [13]. Oral administration of aqueous *A. altilis* extract provided cardioprotective effect in myocardial injury via the antioxidant activity of the phenolic and flavonoid compounds in the extract [14]. Furthermore, the ethanolic extract of *A. altilis* leaf were demonstrated to be of very low toxicity as demonstrated by the LD50 value > 10,000 mg/kg BW [15]. Due to the low toxicity of the extract, *A. altilis* leaf extract was employed in a 21-day clinical trial involving diabetes mellitus type 2 outpatient. The oral administration of *A. altilis* leaf extract induced a lower fasting blood sugar in the treatment group as compared to the control group without any severe side effects [16].

The bioactivity of *A. altilis* leaf extract has been attributed to the numerous phytoconstituents present in the *A. altilis* leaves. Much work on the phytochemical profiles of *A. altilis* leaves has been carried out and several classes of phenolic and flavonoid compounds have been identified and isolated [3, 7, 9-11, 14]. Experimental and computational approaches have demonstrated that there is a relationship between the antioxidative property with the flavonoid and phenolic contents of *A. altilis* extract [5, 7, 9-11, 13, 14, 16-18]. Despite evidence pointing toward the responsibility of the phenolic and flavonoid content in modulating the bioactivity of *A. altilis* extract, there is a gap in the knowledge in the optimal extraction conditions to maximize the amount of such phytochemicals in *A. altilis* extract.

In an attempt to identify the optimal extraction parameters for the Soxhlet extraction of *A. altilis* leaves, the extraction time and the sample-to-solvent ratio were optimized by one-factor-at-a-time methodology. Soxhlet extraction was employed as a simple and safe extraction method with potential to upscale at an industrial level [19]. Aqueous ethanol (ethanol:water, 60:40, v/v) was chosen as the solvent system for the extraction of phenolic and flavonoid compounds to maximize the extractive power of the solvent system toward both phenolic and flavonoid compounds present in *A. altilis* leaves [20-23]. The *A. altilis* extract was evaluated by their total phenolic content and total flavonoid content. The antioxidant potential of the extract was assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay.

Materials and methods

Collection and preparation of *Artocarpus altilis* leaves

Fresh *A. altilis* leaves were collected in October 2018 in Tam Binh district, Vinh Long, Viet Nam. The leaves were detached, washed thoroughly, cut into small pieces and shade-dried for seven days. They were then homogenized to a fine powder with an electronic blender. Afterwards, *A. altilis* leaf powder was transferred into transparent plastic packaging and stored in an air-tight container.

Soxhlet extraction of *Artocarpus altilis* leaves

The *A. altilis* leaf powder was extracted with ethanol:water (60:40, v/v) at 75 °C by conventional Soxhlet extraction with a serial extraction unit (Behr Labor-Technik, Germany). After the extraction, the crude extract was concentrated by rotary evaporation under low pressure, 180 RPM at 50 °C to dryness to achieve semi solid extract. The dried *A. altilis* extract was stored in the refrigerator until further analysis.

One-factor-at-a-time optimization of extraction conditions of *Artocarpus altilis* leaves

The extraction time and the sample-to-solvent ratio were optimized by one-factor-at-a-time optimization. The extraction time was varied (3, 4.5, and 6 hours) while the sample-to-solvent ratio (3:160, g:mL) was kept constant. Then, the sample-to-solvent ratio was screened (3:160, 4:160, 5:160, and 6:160, g:mL) at the optimal extraction time.

Determination of total phenolic content

The total phenolic content (TPC) was estimated with Folin-Ciocâlteu reagent according to the method by Al-Owaisi *et al*. with some modifications [24]. 375 µL Folin-Ciocâlteu reagent (10:1 dilution with distilled water) was added to 150 µL of the extract (2 mg/mL). Then, 375 µL Na_2CO_3 (7.5% w/v) was added. The reaction mixture was kept away from light exposure at room temperature for 30 min with intermittent agitation for color development. The absorbance was measured at 765 nm. A standard calibration curve was generated with known concentrations of gallic acid (6.25-200 µg/ml). The TPC was expressed as milligram gallic acid equivalent (GAE) per gram of dry weight of the extract (mg GAE g^{-1} DW).

Determination of total flavonoid content

The total flavonoid content (TFC) was estimated using the aluminum chloride colorimetric method by Madaan *et al*. with some modifications [25]. 300 µL methanol and 20 µL CH_3COOK 1 M were added to 100 µL of the extract (5 mg/mL). After 6 min, 20 µL $AlCl_3$ (10% w/v) and 560 µL distilled water were added. The reaction mixture was incubated at room temperature for 30 min with intermittent agitation for color development. The absorbance was measured at 420 nm. A standard calibration curve was generated with known concentrations of quercetin (20-200 µg/ml). The TFC was expressed as milligram quercetin equivalent (QE) per gram of dry weight of the extract (mg QE g^{-1} DW).

Assessment of antioxidant activity

The antioxidant activity of *A. altilis* extract was estimated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay [26]. Ascorbic acid, which was used as the reference standard to estimate the antioxidant capacity, was prepared at different concentrations (0.78-12.5 µg/ml). 500 µL DPPH solution 0.007% was added to 500 µL of the extracts (3.91-62.5 µg/mL) or the ascorbic acid. A blank solution was prepared identically without the extracts or the ascorbic acid. The mixtures were agitated vigorously and kept at room temperature for 30 min in the dark. The absorbance was measured at 517 nm. The antioxidant activity is expressed as DPPH radical scavenging activity (%):

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

In which,

A_{blank} is the absorbance of the blank sample, A_{sample} is the absorbance of the extracts or the ascorbic acid.

The IC50 value (µg/mL) was estimated from fitting the logarithm of either the extract or the ascorbic acid concentrations against the DPPH radical scavenging activity

(%) with the dose-response relationship in OriginPro (OriginLab, USA). The dose-response relationship is established by the following equation:

$$y = A1 + \frac{A2 - A1}{1 + 10^{(logx0 - x)p}}$$

In which,
$A1$ and $A2$ is the bottom and top asymptote, respectively; $logx0$ is the IC50 value; p is the Hill slope.

Statistical analysis
All tests were performed in triplicate, and these values were presented as the mean±standard deviation (SD). One Way Analysis of Variance (ANOVA) and Tukey post hoc test were used for statistical analysis, and the differences were considered significant at the 95% confidence level (p-value < 0.05).

Results and discussion
Effect of extraction time on total phenolic and total flavonoid contents of *A. altilis* extract
The impact of extraction time on the total phenolic and total flavonoid content was investigated by performing extraction with varied extraction time (3, 4.5, and 6 hours) at 75 °C with ethanol:water (60:40, v/v) as the extraction solvent. The total phenolic content (TPC) and the total flavonoid content (TFC) of the *A. altilis* extract obtained with varied extraction time are presented in Table 1 and Figure 1. There was a statistically significant difference in the TPC as determined by One-Way ANOVA (F = 164.065, p = .000). Tukey post hoc test revealed that the yield of TPC obtained after 6-hour extraction was significantly higher (71.47±0.7545 mg GAE g^{-1} DW, p = .000) compared to those of 4.5-hour extraction (67.85±0.6048 mg GAE g^{-1} DW) and 3-hour extraction (60.08±0.9594 mg GAE g^{-1} DW). Similarly, there was a significant difference in the TFC as determined by One-Way ANOVA (F = 290.197, p = .000). Tukey post hoc test revealed that the yield of TPC was significantly higher after 6-hour extraction (30.±0.368mg QE g^{-1} DW, p = .000) compared to those of 4.5-hour extraction (26.±0.509 mg QE g^{-1} DW) and 3-hour extraction (21.±0.139 mg QE g^{-1} DW).
Overall, increased extraction time had a positive impact on the TPC and TFC of the *A. altilis* extract. Increasing the extraction duration from 3 hours to 4.5 hours resulted in a 12.9% and 28.2% increase in TPC and TFC of *A. altilis* extract, respectively. When extraction time was prolonged to 6 hours, the TPC and TFC of *A. altilis* extract increased by 19.0% and 46.8%, respectively. The *A. altilis* extract obtained from 6-hour extraction achieved the highest TPC (71.47±0.7545 mg GAE g^{-1} DW) as well as the highest TFC (30.±0.638 mg QE g^{-1} DW). Interestingly, the TPC of *A. altilis* from this work was approximately 2.7-times higher than that of Sikarwar *et al.* 2014.[26] Under the experimental conditions, there was a positive correlation between the extraction time and the total amount of phenolic and flavonoid compounds being extracted. Therefore, the extraction time of 6 hours was chosen for the experiments to proceed to surveying the effect of sample-to-solvent ratio on the phenolic and flavonoid contents of *A. altilis* extract.

Table 1: Total phenolic and total flavonoid contents of aqueous ethanol *A. altilis* extract obtained with varied extraction time.

Extraction time (hour)	Total phenolic content (mg GAE g^{-1} DW)	Total flavonoid content (mg QE g^{-1} DW)
3	60.08±0.9594 [a]	20.50±0.2407 [a]
4.5	67.85±0.6048 [b]	26.29±0.5092 [b]
6	71.47±0.7545 [c]	30.10±0.6375 [c]

Data are presented as mean±standard deviation (N = 3). Different letter notations indicate that there was a significant difference between the groups analyzed by ANOVA with Tukey post hoc test at a 95% confidence level.

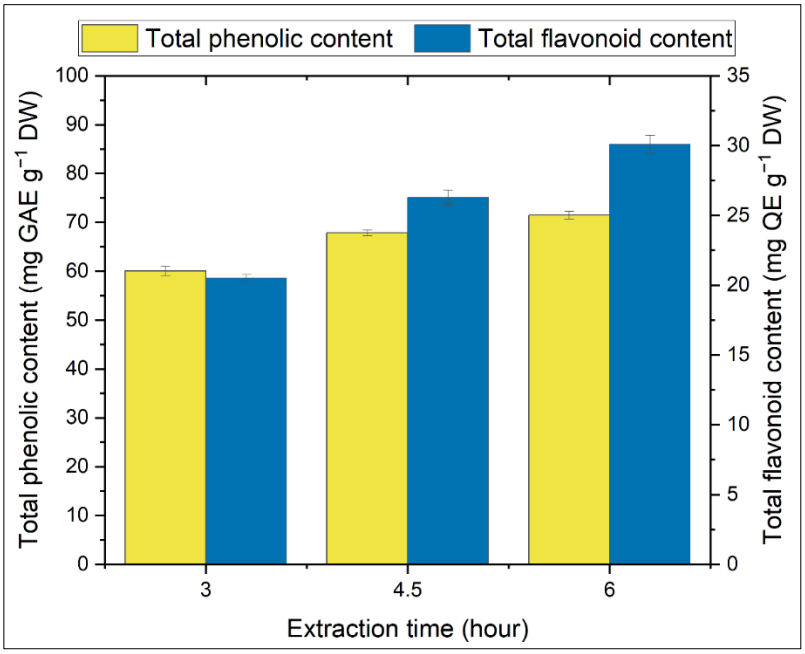

Fig 1: Effect of extraction time on total phenolic and total flavonoid contents of aqueous ethanol *A. altilis* extract

Effect of sample-to-solvent ratio on total phenolic and total flavonoid contents of *A. altilis* extract

The effect of solvent-to-sample ratio on the total phenolic and total flavonoid content was investigated by performing extraction of *A. altilis* with varied solvent-to-sample ratio (3:160, 4:160, 5:160, and 6:160, g:mL) using ethanol:water (60:40, v/v) as the extraction solvent for 6 hours. The TPC and the TFC of the *A. altilis* extract obtained with varied solvent-to-sample ratio are presented in Table 2 and Figure 2. There was a statistically significant difference in the TPC as determined by One-Way ANOVA ($F = 130.353$, $p = .000$). Tukey post hoc test revealed that the yield of TFC was significantly higher for extraction with the sample-to-solvent ratio (g:mL) of 4:160 (86.96±0.2130 mg GAE g^{-1} DW, $p = .000$) compared to those of 3:160 (71.47±0.7545 mg GAE g^{-1} DW), 5:160 (84.05±1.117 mg GAE g^{-1} DW) and 6:160 (78.88±1.545 mg GAE g^{-1} DW). Similarly, there was a statistically significant difference in TFC as determined by One-Way ANOVA ($F = 59.556$, $p = .000$). Tukey post hoc test revealed that the yield of TFC was prominently higher for the extraction with sample-to-solvent ratio (g:mL) of 4:160 (33.76±0.2991 mg QE g^{-1} DW, $p = .000$) compared to 3:160 (30.10±0.6375 mg QE g^{-1} DW), 5:160 (32.32±0.4631 mg QE g^{-1} DW) and 6:160 (29.40±0.3204 mg QE g^{-1} DW).

The results suggested that the optimal sample-to-solvent ratio for the extraction of *A. altilis* with regards to the TPC and TPC was 4:160 g:mL. The variations in the sample-to-solvent ratio modulated the yield of the TPC and the TFC in the *A. altilis* extract. At first glance, the increase in the sample-to-solvent ratio could increase the TPC and TFC by providing more raw material for the extraction and thereby providing a greater concentration gradient which facilitates mass transfer [27]. This phenomenon abided by the mass transfer principles where higher sample-to-solvent ratio correlated with an increased concentration gradient which would result in an increased diffusion rate of the phenolic and flavonoids from the dry material into the solvent.[28] Our results are consistent with previous works in which the increase of sample-to-solvent ratio resulted in the increase in values of TPC and TFC among the experimental sample-to-solvent ratios [28, 29]. However, as the sample-to-solvent ratio grew to 5:160 and 6:160 (g:mL), there was a halt in the increase of phytochemical extraction of the *A. altilis* indicated by the decline in both TPC and TFC. The reduction in the TPC and TFC could be attributed to the fact that once the sample-to-solvent reached a specific threshold, the phytochemicals became fully dissolved in the solvent, leading to saturation and hindering any further extraction.[30] To sum up, the optimal sample-to-solvent ratio for *A. altilis* was 4:160 (g:mL) given the experimental condition. Notably, the TPC in the *A. altilis* extract obtained from the optimized conditions was approximately 3.3-times higher than that reported by *Sikarwar et al*. (26.22 mg GAE g^{-1} DW).

Table 2 Total phenolic and total flavonoid contents of aqueous ethanol *A. altilis* extract obtained with varied sample-to-solvent ratio.

Sample-to-solvent ratio (g:mL)	Total phenolic content (mg GAE g^{-1} DW)	Total flavonoid content (mg QE g^{-1} DW)
3:160	71.47±0.7545 [a]	30.10±0.6375 [a]
4:160	86.96±0.2130 [b]	33.76±0.2991 [b]
5:160	84.05±1.117 [c]	32.32±0.4631 [c]
6:160	78.88±1.545 [d]	29.40±0.3204 [a]

Data are presented as mean±standard deviation (N = 3). Different letter notations indicate that there was a significant difference between the groups analyzed by ANOVA with Tukey post hoc test at a 95% confidence level.

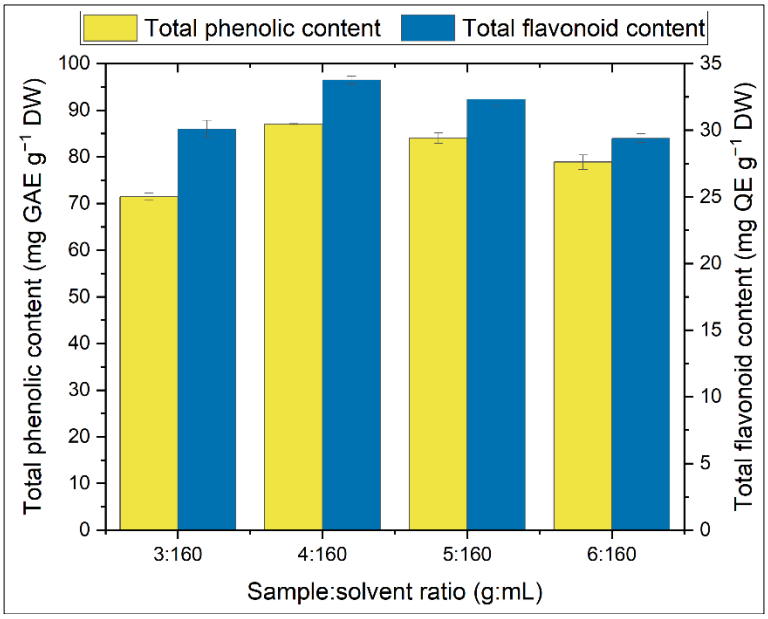

Fig 2: Effect of sample-to-solvent ratio on total phenolic and total flavonoid contents of aqueous ethanol *A. altilis* extract

Antioxidant activity of *A. altilis* extract

The *A. altilis* extract exhibiting the highest TPC and TFC (6-hour Soxhlet extraction, sample-to-solvent ratio of 4:160 (g:mL), ethanol:water (60:40, v/v) as solvent) was used in the assessment for the antioxidant activity by *in vitro* DPPH assay. The DPPH radical scavenging ability of the *A. altilis*

extract is expressed as percentage (%) and shown in Table 3. The data was fitted using the dose-response curve and shown in Figure 3, the radical scavenging ability of ascorbic acid was shown for comparison. The IC50 value of *A. altilis* extract was estimated to be 24.41 µg/ml with the maximum scavenging activity of 88.45±0.12 % at 62.50 µg/ml. The low value of IC50 suggested *A. altilis* to possess a strong antioxidant activity [31]. In comparison, the reference antioxidant ascorbic acid exhibited an IC50 value of 32.18 µg/ml. Altogether, the *A. altilis* extract illustrated a strong antioxidant activity and was comparable with that of ascorbic acid. The potent antioxidant activity of *A. altilis* extract might be attributed to the high content of phenolic and flavonoid compounds. Our results substantiates previous findings in which antioxidant capacities of the *A. altilis* extract were strongly associated with its phenolic and flavonoid content [13, 14, 17].

Table 3: DPPH radical scavenging activity (%) of *A. altilis* extract

Concentration (µg/mL)	DPPH radical scavenging activity (%)
3.91	6.32±0.31
7.81	12.09±0.42
10.00	21.60±0.51
15.63	32.61±0.42
25.00	36.21±0.82
31.25	47.35±1.73
50.00	87.43±0.24
62.50	88.45±0.12

Data are presented as mean±standard deviation (N = 3).

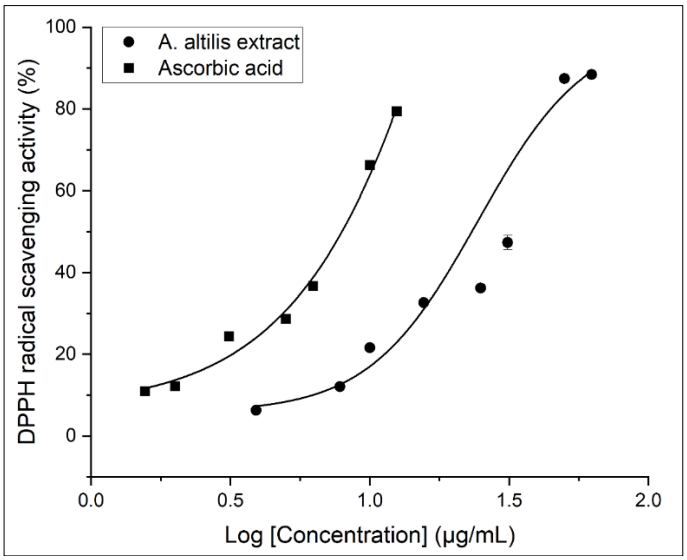

Fig 3: DPPH radical scavenging by different concentrations of *A. altilis* extract (3.91-62.50 µg/mL) and ascorbic acid (0.78-12.50 µg/mL)

Conclusion

The extraction parameters for the Soxhlet extraction of *A. altilis* with aqueous ethanol were optimized by one-factor-at-a-time method in order to achieve the highest value of total phenolic and total flavonoid content of the extract. The optimized Soxhlet extraction parameters with ethanol:water (60:40, v/v) as solvent were 6-hour extraction time, with sample-to-solvent ratio of 4:160 (g:mL). The obtained *A. altilis* extract exhibited a high total phenolic and total flavonoid content, 86.96±0.2130 mg GAE g^{-1} DW and 33.76±0.2991 mg QE g^{-1} DW, respectively. The *A. altilis* extract was assessed for their antioxidant activity and it demonstrated a strong potential as an alternative antioxidant source. Given the prevalence of *A. altilis* in the Asia-Pacific region, our findings could be beneficial for the valorization of breadfruit, notably the breadfruit leaves, in the development of nutraceutical and pharmaceutical products.

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Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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