GSC Biological and Pharmaceutical Sciences
eISSN: 2581-3250 CODEN (USA): GBPSC2
Cross Ref DOI: 10.30574/gscbps
Journal homepage: https://gscbps.gsconlinepress.com/

(RESEARCH ARTICLE)

BIOACTIVITY OF *Uncaria callophylla* LEAVES: PHYTOCHEMICAL, ANTIOXIDANT ACTIVITY, AND TOXICITY ASSAY

M. Rizky Wardani, Eva Marliana, Subur P. Pasaribu, Winni Astuti, Rudi Kartika, Rita Hairani and Chairul Saleh*

Department of Chemistry, Faculty of Mathematics and Natural Sciences, Mulawarman University, Jl. Barong Tongkok, Kampus Gunung Kelua, Samarinda, 75123, Indonesia.
* Corresponding Author

GSC Biological and Pharmaceutical Sciences, 2026, 37(02), 074–080

Publication history: Received on 27 September 2026; revised on 03 November 2026; accepted on 05 November 2026

Article DOI: https://doi.org/10.30574/gscbps.2026.37.2.0378

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ABSTRACT

Uncaria callophylla is a plant that has been traditionally used by the Dayak tribe as a medicinal plant for treating various diseases. This study aimed to investigate the content of phytochemicals, toxicity, antioxidant activity, and total flavonoid content (TFC) of the crude methanol extract, *n*-hexane, ethyl acetate, and aqueous fractions of *U. callophylla* leaves. The methods used included maceration to extract the leaves, liquid-liquid partitioning, phytochemicals screening with specific color reagents, determination of TFC using the $AlCl_3$ colorimetric method, antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay, and toxicity assay against *Artemia salina*. The results showed that the crude methanol extract, *n*-hexane fraction, ethyl acetate fraction, and aqueous fraction of *U. callophylla* leaves exhibited antioxidant activity, with IC_{50} values of 5.36, 84.90, 10.62, and 5.75 µg/mL, respectively, and TFC values of 215.39, 64.56, 163.50, and 252.77 mg QE/g, respectively. In the toxicity assay, all of tested extract and or fractions of *U. callophylla* leaves showed toxicity, with LC_{50} values of 43.79, 27.40, 65.95, and 10.41 µg/mL, respectively. In conclusion, the extract and fractions of *U. callophylla* leaves possessed strong antioxidant activity and preliminary toxicity indicating potential cytotoxic activity.

Keywords: *Uncaria callophylla*, Phytochemical, Total Flavonoid Content, Antioxidant, Toxicity

1 INTRODUCTION

The forests of Kalimantan (Indonesia) are rich in biodiversity and contain a wide variety of plant species with potential as sources of bioactive compounds. One of the plant genera commonly found in this region is *Uncaria* (Rubiaceae). Empirically, Dayak tribe refer to these plants as bajakah and have used the water contained in their stems to quench thirst during activities in the forest. In addition, a decoction of the stems is traditionally believed to be used to treat various diseases and health conditions, including fever, hypertension, asthma, headache, digestive disorders, and microbial infections [1, 2].

Research on the biological activities of *Uncaria* species has been conducted. Ethanol extracts of the stems of *U. cordata* and *U. gambir* showed antioxidant activity, with IC_{50} values of 9.16 and 29.84 µg/mL, respectively [3, 4]. In toxicity tests against *Artemia salina* larvae, ethyl acetate extract of *U. cordata* leaves and methanol extracts of bark and stem bark of *U. nervosa* showed LC_{50} values of 2.57, 1.76, and 2.66 µg/mL, respectively [1, 5]. In an antibacterial activity assay, *n*-

hexane extract of *U. tomentosa* stems showed a Minimum Inhibitory Concentration (MIC) of 50 µg/mL against *Escherichia coli* [6]. In addition, ethanol extract of *U. cordata* leaves was reported to have MIC values of 4% against *Staphylococcus aureus* and *Salmonella typhi* [7]. Based on these findings, *Uncaria* species are potential sources of bioactive compounds that warrant further investigation of their phytochemical constituents and biological activities.

One of the less studied *Uncaria* species in terms of its phytochemical constituents and biological activities is *U. callophylla*. This species is distributed in tropical rainforests in Kalimantan, Sumatra, Maluku, Papua, Malaysia, Philippines, Thailand, and Queensland. Ahmad et al. (2011) reported that methanol extracts of the stem and leaves of this species exhibited antioxidant activity, with IC_{50} values of 20 and 35 µg/mL, respectively, as well as α-glucosidase inhibition of 99.4% and 55.3%, respectively [8]. In addition, Siregar et al. (2023) reported that the phytochemical profile of the methanol stem extract of *U. callophylla* contained 14 compounds, including terpenoids, alkaloids, tannins, glycosides, and fatty acids [9]. Most studies have focused on the stems of this plant; therefore, further investigation is needed to explore the phytochemical constituents and biological activities of *U. callophylla* leaves extracts and fractions.

2 MATERIALS AND METHODS

2.1 Materials and equipments

The equipment used were standard laboratory glassware, analytical balance, micropipettes, microplate, vortex, incandescent lamps, stand, clamp, cuvettes, rotary evaporator Buchi R-II, and UV-Vis spectrophotometer Thermo Scientific Orion AquaMate 8100. The material used were leaves of *U. callophylla*, aquadest, methanol, *n*-hexane, ethyl acetate, Mg powder, HCl 37%, $FeCl_3$ 1%, NaOH 5%, chloroform-ammonia solution, H_2SO_4 2 N, Dragendorff's reagent, H_2SO_4 98%, glacial acetate acid, $AlCl_3$ 10%, $NaNO_2$ 5%, *Artemia salina* cysts, sea salt, dimethyl sulfoxide (DMSO) 1%, 2,2-diphenyl-1- picrylhydrazyl (DPPH), and quercetin.

2.2 Research Period and Location

This study was conducted at Laboratory of Organic Chemistry and Laboratory of Analytical Chemistry, Faculty of Mathematics and Natural Sciences, Mulawarman University, from September 2025 to January 2026. Leaves of *U. callophylla* were collected from Kembang Janggut, Kutai Kartanegara, East Kalimantan, Indonesia (0°13′48.4″N, 116°11′19.0″E). The sample was identified at the Herbarium Wanariset, Balai Penerapan Standar Instrumen Lingkungan Hidup dan Kehutanan (BPSILHK) Samboja, East Kalimantan, Indonesia, with specimen numbers PK 2246 and VB 6079.

2.3 Research Procedure

2.3.1 Extraction and Phytochemical Screening

A total of 1.3 kg of powdered *U. callophylla* leaves was extracted by maceration using methanol for 3 days and then the maceration was repeated for another 3 days until a clear macerate was obtained. The macerated extract was concentrated using a rotary evaporator to obtain the crude methanol leaves extract of *U. callophylla*. The crude methanol extract was then partitioned successively with *n*-hexane and ethyl acetate to obtain *n*-hexane, ethyl acetate, and aqueous fractions. The three fractions were concentrated using a rotary evaporator. The crude methanol extract and the three fractions were dissolved in methanol and subjected to qualitative phytochemical screening using specific color reagents according to the method described by Harborne (1998) for the detection of phenolics, flavonoids, quinones, alkaloids, triterpenoids, steroids, and saponins [10].

2.3.2 Determination of Total Flavonoids Content

Total flavonoids content (TFC) was testing with the $AlCl_3$ colorimetric method, using quercetin as the flavonoid standard [11]. Each 2 mL of quercetin standard solutions (20, 40, 60, and 80 µg/mL) and 2 mL of the sample test solution (100 µg/mL) were separately transferred into 10 mL volumetric flasks. Subsequently, 1 mL of $AlCl_3$ 10% and 0.3 mL of $NaNO_2$ 5% were added, and the mixtures were diluted to volume with methanol. The reaction mixtures were allowed to stand for approximately 1 hour at room temperature before measuring their absorbance at 432 nm using a visible spectrophotometer. The TFC was calculated using the following equation:

$$C = \frac{Cq \times V}{m} \times Df$$

with C = total flavonoid content (mg QE/g); Cq = concentration of quercetin equivalent (µg/mL); V = volume solution (L); m = mass (g); and Df = dilution factor.

2.3.3 Antioxidant Activity Test

Antioxidant activity was testing using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay according to the method described by Erwin et al. (2018) [12]. Crude methanol extract and three fractions of *U. callophylla* leaves dissolved in methanol, and test solutions were prepared at various concentration. Each 2 mL of the sample test solutions and quercetin solutions as the positive control, were transferred into dark bottles. Subsequently, 2 mL of 100 mg/L DPPH solution was added to each sample, and the mixtures were incubated at room temperature for approximately 30 min. Methanol served as the negative control. After incubation, the absorbance of each solution was measured at 518 nm using a visible spectrophotometer. Antioxidant activity was expressed as the inhibition concentration 50% (IC_{50}), which was determined using a nonlinear regression equation between the natural logarithm (ln) of the concentration and the percentage inhibition. The equation used to calculate the percentage inhibition was as follows:

$$\%\text{Inhibition} = \frac{\text{Absorbance control (DPPH)} - \text{Absorbance sample}}{\text{Absorbance control (DPPH)}} \times 100\%$$

2.3.4 Toxicity Assay

The toxicity assay was testing using a modified Meyer brine shrimp lethality assay [13]. Crude methanol extract and three fractions of *U. callophylla* leaves dissolved in DMSO 1%, and test solution were prepared in a microplate at concentrations of 1000, 500, 250, 125, 62.50, 31.25, 15.63, and 7.81 µg/mL. A 100 µL of seawater containing 10 *Artemia salina* larvae at 48 h post-hatching was transferred into a microplate, followed by the addition of 100 µL of each test solution. The number of dead larvae was recorded after 24 h of exposure, with DMSO 1% used as the negative control. The toxicity against *A. salina* was expressed as the 50% lethal concentration (LC_{50}), which was determined by probit analysis using IBM SPSS software.

3 RESULT AND DISCUSSION

3.1 Extraction and Phytochemical Screening

Extraction was carried out using the maceration method with methanol as the solvent. This method enables the extraction of plant bioactive compounds while minimizing structural degradation caused by heat. Methanol was selected because of its semipolar nature, possessing both a hydroxyl group (-OH, polar) and a methyl group ($-CH_3$, nonpolar), making it effective for extracting a wide range of compounds with different polarities [14]. A total of 130 g of crude methanol extract was obtained, of which 120 g was subjected to liquid-liquid partitioning to produce fractions with different polarities. The *n*-hexane, ethyl acetate, and aqueous fractions were obtained in amounts of 12, 56, and 50 g, respectively. The extraction yields of the crude methanol extract, *n*-hexane fraction, ethyl acetate fraction, and aqueous fraction were 10.00, 10.00, 46.67, and 41.67%, respectively. The crude methanol extract and three fractions of *U. callophylla* were subjected to phytochemical screening to obtain information on their secondary metabolite constituents, and the result is shown in **Table 1**.

Tabel 1: Result of Phytochemicals Screening of *U. callophylla* Leaves

Sample Test	Phytochemicals Group					
	Phenolics	Flavonoids	Quinones	Alkaloids	Triterpenoids/ Steroids	Saponins
Crude methanol extract	+	+	+	+	+	+
n-Hexane fraction	-	-	-	-	+	-
Ethyl acetate fraction	+	+	+	+	+	-
Aqueous fraction	+	+	+	+	+	+

Note: (+) detected; (-) not detected.

3.2 Total Flavonoid Content (TFC)

Total flavonoid content (TFC) describes the overall concentration of flavonoids present in a sample and is commonly expressed as an equivalent of a standard flavonoid, such as quercetin. In this study, the determination of TFC was based on the formation of a yellow-colored complex between Al^{3+} and flavonoids. The intensity of the resulting yellow color increases proportionally with the flavonoid concentration in the sample. Therefore, the TFC can be quantitatively determined using spectrophotometric analysis [15]. The reaction scheme between quercetin and $AlCl_3$ used in the determination of TFC is depicted in **Figure 1.**

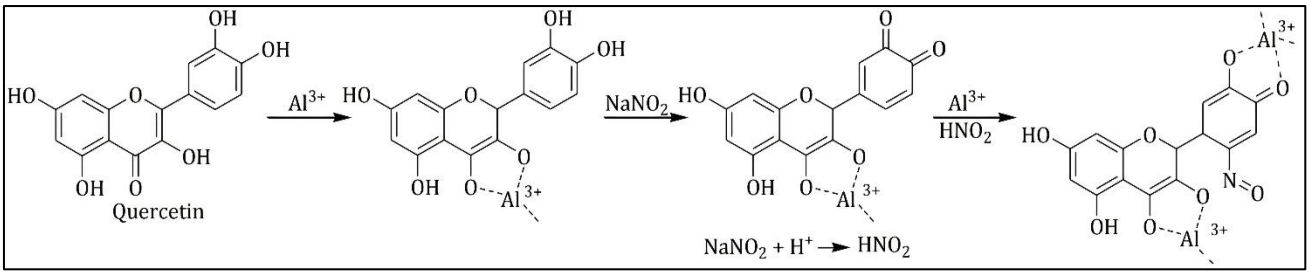

Figure 1: Reaction Between Quercetin and $AlCl_3$ Used in The Determination of TFC

Base on **Figure 1**, flavonoids form coordination complexes with Al^{3+} through the carbonyl and hydroxyl groups on the C ring. Complex formation enhances the conjugated π-electron system of the flavonoid molecule, resulting in a bathochromic shift of the absorption maximum into the visible region, with maximum absorbance observed at approximately 432 nm. This spectral shift is manifested by the development of a yellow color. In addition, $NaNO_2$ facilitates the deprotonation of hydroxyl groups on the flavonoid structure, thereby promoting coordination with Al^{3+} and enhancing the intensity of the yellow complex formed. Result of determination of TFC of *U. callophylla* leaves is depicted in **Figure 2.**

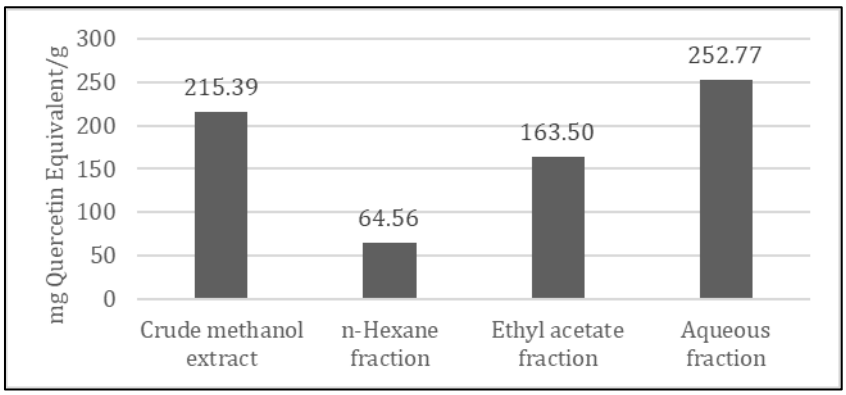

Figure 2: Diagram of Total Flavonoid Content of *U. callophylla* Leaves

The distribution of flavonoids among the fractions of *U. callophylla* leaves is influenced by the polarity of the solvent used. According to Wakeel et al. (2019), polar solvents such as methanol and ethanol are effective for extracting polar flavonoids, such as flavonoid glycosides, whereas ethyl acetate is a semipolar solvent that can extract flavonoids with intermediate polarity, such as flavonols and flavanones. The *n*-hexane fraction only small amounts of flavonoids because its nonpolar nature is less suitable for dissolving flavonoids and is mainly capable of extracting flavonoids with polymethoxylated aglycones. Flavonoids are phenolic compounds containing multiple hydroxyl (-OH) groups and may also contain glycoside groups, making them more soluble in relatively polar solvents [16].

3.3 Antioxidant Activity Test

Antioxidants are compounds capable of inhibiting the oxidation of substrates by donating electrons or hydrogen atoms to neutralize free radicals [12]. Base on antioxidant activity test on DPPH scavenging method, the test result for *U. callophylla* leaves and quercetin as a positive control is presented in **Table 2**.

Based on **Table 2**, crude methanol extract and three fractions of *U. callophylla* showed potential as sources of antioxidants. The total flavonoid content (TFC) showed a positive correlation with the antioxidant activity of the three fractions. Aqueous fraction and crude methanol extract had high TFC values and exhibited very strong antioxidant activity. In contrast, ethyl acetate and *n*-hexane fractions, which had moderate and low TFC values, showed lower antioxidant activity than the aqueous fraction and crude methanol extract. The main pharmacological effect of flavonoids is their antioxidant activity, which occurs through electron donation or hydrogen atom abstraction from •DPPH, thereby stabilizing the radical and rendering it less reactive. The 2 pathways of proposed mechanism for the reaction of flavonoids in scavenging •DPPH is depicted in **Figure 3**.

Table 2: Antioxidant Activity of *U. callophylla* Leaves

Sample Test	Concentration (µg/mL)	Absorbance			Inhibition (%)			IC_{50} Value (µg/mL)	Antioxidant Intensity [17]
		I	II	III	I	II	III		
Crude methanol extract	5	0.366	0.371	0.370	48.01	47.30	47.44	5.36 ±0.040	Very strong
	10	0.230	0.229	0.229	67.47	67.61	67.61		
	15	0.099	0.099	0.098	86.08	86.08	86.22		
	20	0.094	0.093	0.094	86.93	87.07	86.93		
n-Hexane fraction	40	0.510	0.512	0.510	27.98	27.70	27.98	84.90 ±0.262	Strong
	60	0.449	0.447	0.448	37.78	38.07	37.93		
	80	0.366	0.366	0.364	50.14	50.14	50.43		
	100	0.347	0.345	0.346	53.98	54.26	54.12		
Ethyl acetate fraction	10	0.369	0.368	0.370	47.59	47.73	47.44	10.62 ±0.013	Very strong
	15	0.253	0.253	0.251	64.20	64.20	64.49		
	20	0.144	0.144	0.141	79.83	79.83	80.26		
	25	0.089	0.090	0.089	87.78	87.64	87.78		
Aqueous fraction	4	0.637	0.635	0.636	34.98	35.19	35.08	5.75 ±0.021	Very strong
	6	0.493	0.490	0.488	49.90	50.21	50.41		
	8	0.372	0.371	0.368	62.35	62.45	62.76		
	10	0.230	0.225	0.220	77.06	77.57	78.09		
Quercetin	2	0.390	0.391	0.390	38.97	38.81	38.97	2.85 ±0.006	Very strong
	4	0.261	0.263	0.265	59.15	58.84	58.53		
	6	0.146	0.146	0.145	77.15	77.15	77.31		
	8	0.066	0.065	0.066	89.83	89.98	89.83		

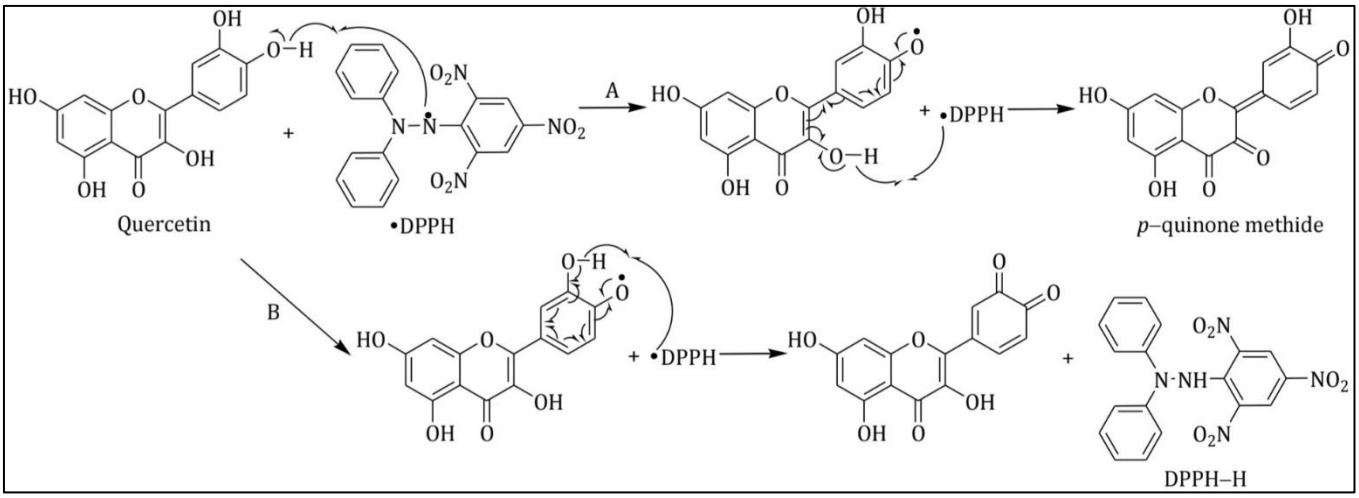

Figure 3: Proposed Mechanism Reaction of Flavonoids in Scavenging •DPPH

3.4 Toxicity Assay

Brine Shrimp Lethality Test (BSLT) is a toxicity assay that uses *Artemia salina* as the test organism and is often used as an alternative approach for preliminary bioactivity screening, as in vivo cytotoxicity assays using cancer cells can be costly. Although BSLT does not directly correlate with cytotoxicity assays, Meyer et al. (1982) reported that 14 of 24 plant species that were toxic to *A. salina* also exhibited cytotoxic activity. Therefore, BSLT remains relevant for

preliminary assessment of the potential bioactivity of plant extracts and compounds because it can be performed in a relatively short time and at lower cost than conventional cytotoxicity assays [13]. The test result for *U. callophylla* leaves is presented in **Table 3**.

Table 3: Toxicity Assay of *U. callophylla* Leaves

Sample Test	LC_{50} Value (µg/mL)	Toxicity Intensity [13]
Crude methanol extract	43.79	Toxic
n-Hexane fraction	27.40	Very Toxic
Ethyl acetate fraction	65.95	Toxic
Aqueous fraction	10.41	Very Toxic

The mortality of *A. salina* larvae induced by crude methanol extract and the three fractions indicated active toxicity; however, it was not directly correlated with the TFC. This difference was evident in the *n*-hexane fraction, which had a low TFC but exhibited very high toxicity. This finding may be associated with the presence of triterpenoids and steroids. These compounds have been reported to exhibit cytotoxic activity through the induction of apoptosis, cell cycle arrest, inhibition of cell proliferation, and modulation of various cellular targets and signaling pathways, depending on their chemical structures [18, 19]. Meanwhile, the high toxicity of the aqueous fraction may be associated with its relatively high flavonoid and alkaloid contents. Flavonoids have been reported to exhibit cytotoxic effects through various mechanisms, including the inactivation of carcinogens, inhibition of cell proliferation, cell cycle arrest, induction of apoptosis, suppression of angiogenesis, antioxidant activity, and reversal of multidrug resistance, either individually or through a combination of these mechanisms. Alkaloids found in *Uncaria*, including rhynchophylline, isorhynchophylline, corynoxeine, isocorynoxeine, hirsutine, and hirsuteine, have been reported to exhibit cytotoxic effects through the induction of apoptosis, cell cycle arrest, inhibition of proliferation and metastasis, and reversal of multidrug resistance in cancer cells through the modulation of various signaling pathways [20, 21]. In addition, differences in toxicity may also be influenced by interactions among compounds, including synergistic and antagonistic effects. Thus, the overall composition of secondary metabolites may contribute to the toxicity response observed in *A. salina*.

4 CONCLUSION

Based on the results of this study, crude methanol extract and three fractions of *U. callophylla* leaves contained various types of secondary metabolites, including phenolics, flavonoids, quinones, alkaloids, triterpenoids, steroids, and saponins, with varying total flavonoid content (TFC). The antioxidant activity was correlated with the TFC, with IC_{50} values of 5.36, 84.90, 10.62, and 5.75 µg/mL for the crude methanol extract, *n*-hexane fraction, ethyl acetate fraction, and aqueous fraction, respectively, indicating strong to very strong antioxidant activity. Meanwhile, the crude methanol extract, *n*-hexane fraction, ethyl acetate fraction, and aqueous fraction exhibited toxic to highly toxic effects against *A. salina*, with LC_{50} values of 43.79, 27.40, 65.95, and 10.41 µg/mL, respectively. In conclusion, the extract and fractions of *U. callophylla* leaves exhibited antioxidant activity and may have potential cytotoxic activity.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

Acknowledgments

The authors gratefully acknowledge the Laboratory of Organic Chemistry and Laboratory of Analytical Chemistry, Mulawarman University for providing facilities and support for this research.

Disclosure of conflict of interest

The authors declare no conflicts of interest regarding this manuscript.

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