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Abstract

Background: *Toddalia asiatica* (Linn) Lam is a woody vine that is used medicinally in China, India, and East Africa. The aim of the present study was to examine the antioxidant and antibacterial activity of the roots of *Toddalia asiatica* (Linn) Lam (TA).

Materials and Methods: The antioxidant capacity of TA roots was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS), and the ferric reducing antioxidant potential (FRAP) assay. The antimicrobial activity of TA against *Staphylococcus aureus* (SA), methicillin-resistant *S. aureus* (MRSA), and extended-spectrum β -lactamase positive *S. aureus* (ESBLs-SA) was screened.

Results: Methanol and ethyl acetate extracts of TA exhibited strong antioxidant activity. The methanol extract had the highest antioxidant activity (DPPH, $IC_{50}=41.45 \mu\text{g/mL}$; ABTS, $IC_{50}=8.34 \mu\text{g/mL}$; FRAP= $1304.8 \pm 60.38 \mu\text{mol Trolox equivalent (TE)/g}$), which was close to that of the positive control, butylated hydroxytoluene (BHT). The petroleum ether extract of TA showed the highest antimicrobial activity (SA, minimum inhibitory concentration (MIC) = $250 \mu\text{g/disc}$; MRSA, MIC= $125 \mu\text{g/disc}$) when compared with that of ethyl acetate extract (SA, MIC= $250 \mu\text{g/disc}$) and methanol extract.

Conclusions: Investigation of methanol, petroleum ether, and ethyl acetate extracts of TA root revealed robust antioxidant activity in methanol extracts and strong antimicrobial activity against SA and MRSA in petroleum ether extracts.

Key words: *Toddalia asiatica* (Linn) Lam; antioxidant; antibacterial

Introduction

Toddalia asiatica (Linn) Lam is a woody vine that is used medicinally in China, India, and East Africa (Orwa et al., 2008). Also known as 'wild orange tree' and 'forest pepper,' *Toddalia asiatica* (TA) has medicinal properties attributed to its leaves, roots, flowers, and fruit. TA is widely distributed throughout the eastern and southeastern regions of Asia and Africa (Katuura et al., 2007). The fruit is traditionally used to treat malaria and cough; roots are used to treat indigestion and influenza, and the leaves are used to treat lung diseases and rheumatism (Karunai Raj et al., 2012). In addition, the stems and leaves of *T. asiatica* have been used for the treatment of diabetes (Pullaiah et al., 2003). The identification, isolation, and characterization of biologically active components of TA may provide therapeutic agents useful for treating a variety of conditions.

Phytochemical research has shown that TA contains coumarins, alkaloids, terpenoids, and other constituents (Chu, 2010; Huang et al., 2005; Muo et al., 2010; Shi et al., 2011). Pharmacological investigations have identified a variety of biological activities in TA extracts. Ethanol and water

extracts have anti-inflammatory and analgesic effects (Wang et al., 2007). The water extract of TA also has a vasodilatory effect that protects against myocardial infarction and myocardial ischemia (Ren & He, 1998; Ye et al., 2000). The methanol extract of TA promotes coagulation and stypcity (Shi et al., 2010a).

Of particular pharmacological interest are the reported anti-microbial and antioxidant actions of TA components. The increase in microbial strains resistant to antibiotics necessitates the search for novel and potent therapeutic agents to treat bacterial and fungal infections (Bolivar et al., 2011). Extracts of TA leaves and stems inhibit the growth of *Bacillus subtilis*, *Shigella*, Beer yeast, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus niger* (Ding et al., 2007; Narod et al., 2004). In addition, *in vitro* antioxidant activity has been observed in TA ethanol extracts (Balasubramaniam et al., 2012; Tien et al., 2011) reported that polysaccharides isolated from TA have strong antioxidant properties (Tien et al., 2011). Antioxidants scavenge free radicals, preventing the oxidative damage involved in the pathogenesis of many diseases, including diabetes, cancer, and cardiovascular disease (Valko et al., 2007). Thus, these potential pharmaceutical applications of TA warrant further characterization of its active constituents.

Most investigations of TA have used a single method to assay antioxidant activity. A variety of antioxidant assays are available, each based on different oxidizing agents and reaction mechanisms. Each of these assay methods is best suited to a particular type of antioxidant. Thus, no single assay will accurately reflect the activity of all antioxidants in a mixture (Prior et al., 2005). Because plant extracts are a complex mixture of components, multiple assays are required to accurately assess the range of antioxidant activities therein. The aim of this study is to compare the antioxidant activity of different TA root extracts using 3 different assay methods, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-Azino-bis(3-ethylbenzo-thiazoline-6-sulfonicacid) (ABTS), and ferric reducing antioxidant potential (FRAP), and to assess the antimicrobial activities of these extracts.

Materials and Methods

Plant and Extract Preparation

TA was collected in September 2008 in Guizhou province, China, and its identity confirmed by Professor Zhiyou Guo (Qiannan Normal College for nationalities). A voucher specimen was deposited in the Institute of Chinese Materia Medica, Henan University.

TA roots (1.3 kg) were extracted 3 times (7 days/extraction) with methanol at $25 \pm 2^\circ\text{C}$ under 45–55% humidity. After evaporation of the solvent *in vacuo*, the concentrated extract was mixed with silica gel and then eluted with petroleum ether, ethyl acetate, or methanol. The solutions were concentrated under reduced pressure to yield the respective extracts.

Chemicals

Dimethyl sulfoxide (DMSO), 6-Hydroloxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonicacid) (ABTS), butyl-p-hydroxyanisole (BHA), Propyl gallate (PG) and butylated hydroxytoluene (BHT) were purchased from Sigma Aldrich Co. (Beijing, China). DPPH was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan), and 2,4,6-Tripyridyl-S-triazine (TPTZ) was purchased from Acros organics (Geel, Belgium).

Test Microbial Strains

S. aureus (SA25923) was purchased from Shanghai Tiancheng Bio-information and Technology Co. (Shanghai, China). Methicillin resistance *S. aureus* (MRSA) and extended-spectrum β -lactamase (ESBLs) were acquired from Huaihe Hospital of Henan University (Henan province, China) and their identities confirmed using the Vitek-AMS (Automated Microbic System, Biomerieux Co. Lyon, France).

DPPH Assay of Antioxidant Activity

DPPH radical scavenging activity was assayed according to the literature (Stephen et al., 2012). Petroleum ether, ethyl acetate, and methanol extracts of TA and the positive control were serially diluted to yield concentrations of 2, 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL with 0.1 mL methanol as previously described (Duraipandiyar et al., 2006). The 3 extracts and the positive control were mixed with 3.5 mL DPPH/methanol solution (0.06 mM). The solution was measured at 515 nm after 30 min at room temperature with PG, BHA, and BHT as positive control. The antioxidant activity was expressed as the IC₅₀ value (the concentration [μ g/mL] that inhibits DPPH \cdot absorption by 50%) as calculated using the concentration-effect linear regression curve. All experiments were repeated in triplicate.

ABTS Assay of Antioxidant Activity

The scavenging activity of the TA extracts on the ABTS radical was evaluated as previously described (Gakunju et al., 1995). The 3 extracts and positive control were serially diluted to concentrations of 2, 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL with methanol. Aliquots of TA extracts (0.15 mL) were mixed with ABTS stock solution (2.85 mL), incubated at 37°C for 10 min, and the absorbance at 734 nm measured. Positive controls were PG, BHA, and BHT. The % inhibition of ABTS radical formation was calculated as follows: $\text{ABTS}^{\cdot+}\text{Inhibition (\%)} = [(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the blank control and A_1 is the absorbance of the sample.

FRAP Assay of Antioxidant Activity

The 3 TA extracts and the positive control were formulated into concentration of 2, 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL with methanol (Lu et al., 2005). Extract fractions (0.2 mL) were mixed with freshly prepared TPTZ stock solution (3.8 mL), incubated at 37°C for 30 min, and the absorbance measured at 593 nm. Trolox was used as a reference standard. All samples and the positive control were tested 3 times in parallel and the results averaged to determine the inhibition rate. Results are expressed as Trolox equivalents (TE)/g sample.

Antimicrobial Assay

Disc diffusion tests were carried out under a sterile hood as previously described (Wei, 2010). Briefly, test samples were serially diluted two-fold to obtain a range of concentrations. Whatman filter paper discs (6 mm) were impregnated with 5 μ L of each concentration and evaporated using a hair dryer. Two discs were prepared for each sample. Discs prepared with only the corresponding volume of methanol were used as negative controls. Berberine hydrochloride (purity, 99.9%) was used as the positive control (Chinese National Institute for the Control of Pharmaceutical and

Biological Products, batch number 110713-200609). Microbial strains were plated onto agar medium in culture plates. The discs were placed at equidistant points on top of the agar medium. The plates were incubated at 37°C for 24 h. Antimicrobial activity was evaluated by measuring the diameter of the inhibition zone (IZ) around the disc. Samples that created an inhibition zone were diluted 50%; each sample was diluted in this manner 2 times, and the average concentration was then calculated. The corresponding solvent was used as a control. MIC was defined as the lowest sample concentration that exhibited IZ. All were performed under sterile conditions.

Statistical Analysis

The antioxidant activity is presented as IC₅₀, the concentration required to inhibit 50% absorption, as determined by dose-response curves. ABTS inhibition (%) = $[(A_0 - A_1)/A_0] \times 100\%$, where A₀ is the absorbance of the blank control and A₁ is the absorbance of the sample. For the FRAP assay, the results are presented in μmol Trolox equivalents (TE)/gram sample as the antioxidant activity; Trolox was used as a standard reference. Each sample was assayed 3 times, and the data were averaged to determine the FRAP assay inhibition rates. The IC₅₀ was determined using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Characteristics and Chemical Composition of TA Extracts

Petroleum ether extraction yielded 113g of a yellow compound that contained grossly observed needle-shaped crystals and was mainly composed of fatty acids (Shi et al., 2012).

Ethyl acetate extraction yielded 150g of a brown compound that contained bulk crystals and was composed primarily of alkaloids (Shi et al., 2013). Methanol extraction yielded 179g of a black compound that was more viscous and contained more glycosides and polysaccharides than the petroleum ether or ethyl acetate extracts (Shi et al., 2014b). We isolated and characterized the chemical components of all 3 extracts in the previous studies cited above. Our previous pharmacological experiments showed that all 3 of these extracts have hemostatic activity (Shi et al., 2010b; Shi et al., 2014a).

Antioxidant Activity of TA Extracts

The antioxidant activity of TA as determined by DPPH, ABTS, and FRAP assay is presented in Table 1. According to the DPPH assay, the antioxidant activity of the TA methanol extract was lower than that of BHA, BHT, and PG (IC₅₀, 38.11 vs. 2.77, 17.78, and 0.77 μg/mL respectively). Using the ABTS assay, the antioxidant activity of the TA methanol extract was higher than that of the TA ethyl acetate extract (IC₅₀, 7.15 vs. 16.28 μg/mL), similar to that of BHT (IC₅₀, 7.15 vs. 6.14 μg/mL), and lower than that of BHA and PG (IC₅₀, 7.15 vs. 1.62 and 0.68 μg/mL, respectively). According to the FRAP assay, the antioxidant activity of the TA methanol extract was higher than that of petroleum ether and ethyl acetate extracts (1484.8 vs. 107.55 and 343.2 μmol TE/g, respectively) and lower than that of BHA, BHT, and PG (1484.8 vs. 8584, 2902.8, and 13514 μmol TE/g, respectively) (Table 1).

Table 1: Antioxidant activity of TA extracts

	Antioxidant Assay		
	DPPH	ABTS	FRAP*
	IC50 (µg/mL)	IC50 (µg/mL)	(µmol TE/g)
Petroleum ether extract	-	-	107.5
Ethyl acetate extract	-	16.28	343.2
Methanol extract	38.11	7.15	1484.8
BHA	2.77	1.62	8584.0
BHT	17.78	6.14	2902.8
PG	0.77	0.68	13 514.0

* data represent the average of triplicate experiments

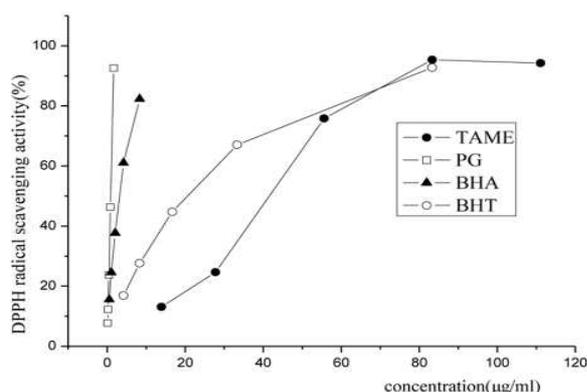
- Low activity

BHA, Butyl-p-hydroxyanisole; BHT, Butylated hydroxytoluene; PG, Propyl gallate

DPPH- and ABTS-Radical-Scavenging Activity of TA Extracts

The DPPH radical scavenging activity of TA extracts is presented in Figure 1. In the methanol extract, scavenging activity increased in a concentration-dependent manner up to 80µg/mL; a similar trend was observed for BHT at concentrations of 0–80 µg/mL. The scavenging activity of BHA and PG increased dramatically over a narrow range of low concentrations (Figure 1).

Fig. 1 DPPH free radical scavenging by TA methanol extract

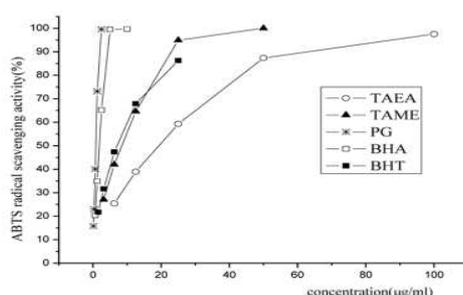


Note: TAME= methanol extract of TA, PG= Propyl gallate, BHA= butyl-p-hydroxyanisole, BHT= butylated hydroxytoluene

Figure 1: Concentration-response curve of the DPPH-radical-scavenging activity of the methanol extract. The methanol extracts exhibited strong antioxidant activity that increased with increasing extract concentrations. Using Origin 6.0, the IC₅₀ was calculated according to the curve generated from the inhibition rates at 5 different concentrations. Thus, the SD was not calculated

The ABTS radical scavenging activity increased in a concentration-dependent manner up to 60µg/mL for the ethyl acetate extract and up to 20µg/mL for the methanol extract and BHT (Figure 2). The ABTS scavenging activity of BHA and PG increased dramatically over a narrow range of low concentrations (Figure 2).

Fig. 2 ABTS free radical scavenging by TA extracts



Note: TAEA = ethyl acetate extract of TA. TAME = methanol extract of TA. PG= Propyl gallate, BHA= butyl-p-hydroxyanisole, BHT= butylated hydroxytoluene

Figure 2: Concentration-response curve of the ABTS-radical-scavenging activity of methanol and ethyl acetate extracts. The ethyl acetate and methanol extracts exhibited strong antioxidant activity that increased with increasing extract concentrations. Using Origin 6.0, the IC₅₀ was calculated according to the curve generated from the inhibition rates at 5 different concentrations. Thus, the SD was not calculated.

Antimicrobial Activity of TA Extracts

The zone of inhibition of MRSA for the petroleum ether extract was 9 mm at 50 mg/mL and 8 mm at 25 mg/mL; that of SA for petroleum ether and ethyl acetate extracts were 8 mm at 50 mg/mL. The zone of inhibition for the positive control (berberine hydrochloride, 2.5 mg/mL) was 15 mm for SA, 16 mm for MASA, and 16 mm for ESBLs-SA (Table 2).

Table 2: Zone of inhibition in antimicrobial assays of TA extracts

TA extract	Zone of inhibition (mm)*		
	SA	MRSA	ESBLs-SA
Petroleum ether (50 mg/mL)	8	9	-
Petroleum ether (25 mg/mL)	nd	8	nd
Ethyl acetate (50 mg/mL)	8	-	-
DMSO (50 mg/mL)	-	-	-
Berberine hydrochloride ** (2.5 mg/mL)	15	16	16

* data represent the average of duplicate experiments

** positive control

- , low activity

nd, not done; SA, Staphylococcus aureus; MRSA, Methicillin-resistant S. aureus; ESBLs-SA, Extended-spectrum β-lactamase positive S. aureus

MIC of TA Extracts

The MIC of TA petroleum ether and ethyl acetate extracts were all 250 µg/disc for SA, while that of petroleum ether for MRSA was 125µg/disc. The MIC of berberine hydrochloride was 0.156 for SA, MRSA, and ESBLs-SA (Table 3).

Table 3: MICs of TA extracts

TA extract	MIC (µg/disc)		
	SA	MRSA	ESBLs-SA
Petroleum ether	250	125	0
Ethyl acetate	250	0	0
Methanol	0	0	0
Berberine hydrochloride*	0.156	0.156	0.156

* positive control

SA, Staphylococcus aureus; MRSA, Methicillin-resistant S. aureus; ESBLs-SA, Extended-spectrum β-lactamase positive S. aureus

Supplement Table: The scavenging rate of DPPH and ABTS

	ABTS		DPPH	
	Concentration (µg/mL)	Scavenging rate (%)	Concentration (µg/mL)	Scavenging rate (%)
Positive control				
PG	2.5	99.56	1.67	92.6
	1.25	73.15	0.83	46.3
	0.625	40.03	0.42	23.75
	0.3125	23.01	0.21	12.22
	0.15625	15.8	0.1	7.75
	10	99.67	8.33	82.3
BHA	5	99.51	4.17	61.06
	2.5	65.18	2.08	37.7
	1.25	34.9	1.04	24.6
	0.625	20.31	0.52	15.58
	25	86.25	83.33	92.78
BHT	12.5	67.88	33.33	67.07
	6.25	47.34	16.67	44.72
	3.125	31.59	8.33	27.64
	1.5625	21.73	4.17	16.9

PG, Propyl gallate; BHA, Butyl-p-hydroxyanisole; BHT, Butylated hydroxytoluene

Discussion

TA root extracts exhibited antioxidant and antimicrobial activity that varied with the extraction solvent and assay method used. The methanol and ethyl acetate TA root extracts exhibited strong antioxidant activity. The methanol extract had the highest antioxidant activity, with the FRAP assay showing results closest to that of the positive control (DPPH, IC_{50} = 38.11 μ g/mL vs. BHT control, 17.78 μ g/mL; ABTS, IC_{50} = 7.15 μ g/mL vs. BHT control, 6.14 μ g/mL; FRAP = 1484.8 μ mol TE/g vs. BHT control, 2902.8 μ mol TE/g). The petroleum ether extract of TA showed the highest antimicrobial activity (SA, MIC = 250 μ g/disc; MRSA, MIC = 125 μ g/disc) when compared with that of ethyl acetate and methanol extracts (SA, MIC = 250 μ g/disc).

Accurate assessment of the antioxidant activity of plant extracts requires the parallel use of several assay methods that are based on different mechanistic principles. This study is the first to assess the antioxidant activity of *T. asiatica* (Linn) Lam. extracts using the following 3 assay methods: DPPH, ABTS, and FRAP. DPPH are stable and free radicals that can accept an electron or hydrogen radical to become a stable, diamagnetic molecule (Ani et al., 2006). The DPPH assay sensitively detects scavenging of the DPPH radical by antioxidants in the test solution (Noipa et al., 2011) and is independent of substrate polarity. The ABTS assay is used for determining the antioxidant capacity of hydrogen-donating antioxidants (Lee et al., 2011). The FRAP assay is an easy and accurate electron-transfer-based method that measures the antioxidant capacity of natural extracts (Müller et al., 2011).

Strong antioxidant activity was observed in methanol and ethyl acetate TA extracts. The highest activity was seen in the methanol extract, as indicated by all 3 assays (DPPH: IC_{50} = 38.11 μ g/mL; ABTS: IC_{50} = 7.15 μ g/mL; FRAP: 1484.8 μ mol TE/g) (Table 1). The antioxidant activity of this extract demonstrated dose dependence (Figure 1). Of the 3 methods used to assay the methanol extracts, the FRAP assay showed results closest to that of the positive control (1484.8 vs. 2902.8 μ mol TE/g respectively).

Our observations support reports that ethanol extracts of TA root exhibit antioxidant activity (IC_{50} , 26 μ g/mL) as evidenced by DPPH assay (Madhavan, 2010), and *in vivo* studies indicating that ethyl acetate extracts of TA leaves are efficacious in attenuating oxidative stress in the liver of diabetic rats (Duraipandiyani & Ignacimuth, 2009). Subsequent experiments have shown that the TA methanol fractions with high anti-oxidant activity have additional alkaloids and glycosides as compared to petroleum ether extracts. Further studies of methanol extracts identified 9 compounds in active fractions which were not found in ethyl acetate extract or petroleum ether extract: dictamine, hesperetin, 4-hydroxy-N-methylproline, 8-hydroxy-dihydrochelerythrine, hesperetin-7-O- β -D-glucopyranoside, daucosterol, hesperidin, diosmin, and neohesperidin (Shi, 2014). Whether the anti-oxidant activity of methanol extracts is related to these compounds requires confirmation by future studies.

The petroleum ether extract of TA roots strongly inhibited the growth of *S. aureus* (Zone of inhibition: 50 mg/mL, 8 mm; MIC, 250 μ g) and methicillin-resistant *S. aureus* (Zone of inhibition: 25 mg/mL, 8 mm; 50 mg/mL, 9 mm; MIC, 125 μ g) (Tables 2 and 3). Methanol extracts had no inhibitory effects on any of the bacteria tested. None of the extracts inhibited ESBLs. These results are similar to previous reports of antimicrobial activity in various TA extracts from different parts of the plant. TA leaves are reported to have antimicrobial activity against *B. subtilis*, *S. aureus*, and *S. epidermidis* (Kang & Wang, 2010). Methanol: chloroform extracts of TA leaves were also shown to have potent antimicrobial activity against *E. coli*, *P. aeruginosa*, *S. typhimurium*, *S. aureus*, and *A. niger* (Narod et al., 2004). In addition, sequential extracts of TA root bark showed different levels of antimalarial activity against 3 Kenyan strains of *Plasmodium falciparum* (Kang & Wang, 2011); with the methanol extract yielding the highest activity. Ethanol extracts of TA also have antiviral activity against influenza type A virus (Kang et al., 2010). The antimicrobial activity of

TA extracts clearly depends on the solvent and plant structures used and is specific to particular organisms.

While the constituents responsible for the antimicrobial action observed in this study are unknown, several labs have begun isolating compounds with such activity from TA. Flindersine isolated from TA inhibits the growth of Gram-positive bacteria such as *B. subtilis*, *P. aeruginosa*, resistant *Acinetobacter baumannii*, as well as the fungi *Trichophyton mentagrophytes*, and *Trichophyton simii* (Chang et al., 2009). Ulopterol, a coumarin isolated from TA leaves, shows activity against *Staphylococcus epidermidis*, *Enterobacter aerogenes*, *Shigella flexneri*, *Klebsiella pneumoniae*, *E. coli*, *Aspergillus flavus*, *Candida krusei*, and *Botrytis cinerea* (Karunai Raj M et al., 2012). Further investigations are needed to identify and characterize the constituents responsible for the antimicrobial activities of TA root observed here.

This study has several limitations. The antioxidant activity tests were all *in vitro*; this activity can be quite different *in vivo*. In addition, specific active components of TA extracts were not identified here. Both of these issues should be addressed in further studies of TA extracts.

Conclusion

Investigation of methanol, petroleum ether, and ethyl acetate extracts of TA root revealed robust antioxidant activity in methanol extracts and strong antimicrobial activity against SA and MRSA in petroleum ether extracts. Studies are thus warranted to isolate, identify, and characterize the active constituents of these extracts.

Acknowledgements

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Conflicts of Interest: None

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