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A new bicyclic azepinoindole alkaloid from the seed cotyledon of *Chrysophyllum albidum* G. Don-Holl. (Sapotaceae)

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ABSTRACT

Background: Previous investigation in our laboratory revealed the presence of beta carbolines in the seed cotyledon of *Chrysophyllum albidum* (G. Don), Sapotaceae. Recent developments underscore the utility of repurposing antifungal compounds for anticancer activities. As a continuation of our search for antcancer compounds from Nigerian medicinal plants, the seed cotyledons of *Chrysophyllum albidum* (G. Don), Sapotaceae was re-investigated for the expression of anti-tumour compounds.

Methods: The seed cotyledons of *C albidum* were air-dried under shade for two weeks after which they were powdered using Hammer mill. The powdered seed cotyledons was extracted with methanol (100 %). The methanol (MeOH) extract was defatted and subjected to solid phase extraction using Strata C-18-E cartridge (Phenomenex, USA) (20 g), to obtain four fractions (CA1-CA4). Further purification of CA1 on silica gel (mesh 200-400) gave pure compounds **1 and 2**. Both compounds were subjected to extensive spectroscopic analysis using NMR (^{1}H- and ^{13}C-NMR, ^{1}H–^{1}H COSY, DEPT, HMQC, HMBC) and ESI-MS spectra. Cell viability assay was carried out on the identified compounds using MTT assay.

Results: Compounds **1** and **2** were named (provisionally) as albidumine, a new azepinoindole alkaloid, and bamindolinol, a new fused beta carboline, respectively. The compounds did not show any significant effect against human cervical adenocarcinoma (HeLa) cell line in the cytotoxicity assay.

Conclusion: Two new beta carbolines were identified from the seed cotyledon of *C albidum* for the first time.

Introduction

The term 'azepinoindole' is used to describe a class of natural products which can either be monoterpenoid or non-monoterpenoid in origin, containing a fused tricyclic ring system bearing both an indole and azepine heterocycle, and all the oxygenation patterns. Azepinoindoles have been found in plants, fungi, bacteria and marine life. This natural product class has been a veritable scaffold for many synthetic studies, which has offered new synthetic methodology in addition to many important structural and stereochemical revisions[1]. Azepinoindole has been observed to have interesting activity, especially their high affinity for the brain[2]. They possess tryptamine like activity. Many compounds in the class have been found to have antimalarial, antitrypanosomal, antitumor, and many other biological activities. The non-monoterpenoid azepinoindole class, especially the ones containing a tricyclic ring system with both an indole and an azepine heterocycle, have not been extensively described. In literature, only azepino[5,4,3-c,d] indoles *e.g.* clavicipic acid, aurantoclavine[3]; azepino [4,3-b] indole *e.g.* hinckdentine A, pseudocerosine[4] and azepino [3,2-b]

skeleton, represented by cryptohepine isolated from the West African medicinal plant *Cryptolepis sanguinolenta* have been previously described[5]. Also, azepinoindole N-oxide has been reported *e.g.* fargesine[6]. However, unlike the monoterpenoid azepinoindole class, the chemistry of non monoterpenoid class has not been fully explored, as well as the medicinal potential of the class[1].

Chrysophyllum albidum G. Don-Holl (Sapotaceae) (African Star Apple), the plant of this study, is a small to medium buttressed tree species, up to 25-37 m in height with a mature girth varying from 1.5 to 2 m[7,8]. It is known for its seasonal fruit that is available during the dry season (December to March). Previously, investigation of the methanol extract of the seed cotyledon of *C. albidum* in our laboratory, to validate its antifungal applications in ethnomedicine, led to the identification of eleagnine (1, 2, 3, 4-tetrahydro-1-methyl-β-carboline) and tetrahydro-2-methylharman (1, 2, 3, 4-tetrahydro-1, dimethyl-β-carboline[9] as the antifungal constituents. The recent developments where antifungal compounds were repurposed for anticancer therapy[10] led us to investigate compounds isolated from *C. albidum* seed cotyledon extract for possible expression of cytotoxic activities in continuation of our search for compounds with antitumour effect from Nigerian medicinal plants.

In this report, we present the isolation and structural elucidation of two new beta carboline alkaloids isolated from the seed cotyledon of *C. albidum*

Materials and Methods

General methods

HPLC grade Chromatographic solvents were purchased from Fisher Scientific, United Kingdom. ^{1}H and ^{13}C NMR spectra were obtained on the Bruker AV400 (IconNMR) Spectrometer at 300 and 75 MHz, respectively while the mass determination was carried out on a Waters LCT Premier™ ESI-TOF Mass Spectrometer (Waters, UK) at the School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool, United Kingdom. Analytical HPLC was carried out on Dionex Ultimate 3000 UHPLC with UV/DAD detector (Agilent, UK) with the chromatogram monitored at 230 nm. For the HPLC analysis, the sample concentration was 1 mg/ml, flow rate was 1 mL/min with mobile phase being water (A) and methanol (B), both containing 0.1 % Trifluoro acetic acid. The gradient was 20-100 % B (0-15.0 minutes), 100 % B (15.0-17.0 minutes), 20 % B (17.0- 20.0 minutes) and equilibration for the next two minutes at 20 % B. Adsorption chromatography was carried out on silica gel (ASTM 230–400 mesh, Merck). Thin layer chromatographic analysis of the eluate was effected at room temperature on silica gel 60 GF254 pre-coated aluminum backed plates (Merck, 0.25 mm thick). Spots on TLC plates were visualized under UV light (254 nm) and detected using 1% vanillin/H_2SO_4spray reagent.

Collection of the fruits and isolation of compounds

The fruits of *C. albidum* were collected from the Research and Experimental Farm of the Obafemi Awolowo University, Ile Ife, in the month of December 2017. The fruits were identified by Mr. I.I. Ogunlowo of the Department of Pharmacognosy, Obafemi Awolowo University and a voucher specimen with number IFE 17797 was deposited in IFE Herbarium. The fruits were cut opened and the pulp removed leaving the seeds. The seed coats were manually removed to harvest the seed cotyledon. The seed cotyledons were air-dried under shade for two weeks after which they were powdered using Hammer mill. The powdered seed cotyledons (2.4 kg) was extracted with methanol 100 %. The methanol (MeOH) extract was concentrated *in-vacuo* to a constant weight using rotary evaporator to give a sticky mass 0.48 kg (crude methanol extract). The crude methanol extract (20 g) each time, was dissolved in 100 mL of water and defatted by partitioning with saturated *n*-Hexane (4 x 100 mL), using a 1 L separating flask. The aqueous portion was concentrated *in vacuo* to give hygroscopic flaky powder. This was repeated many times to give a pooled defatted extract (192 g).

The defatted MeOH extract (2.0 g) was suspended in 5 mL of 20% MeOH-water and loaded on to a Strata C-18-E cartridge (Phenomenex, USA) (20 g), which was previously washed with MeOH (50 mL), followed by equilibration with water (100 mL). The cartridge was subsequently eluted with MeOH-H_2O mixture of decreasing polarity using 200 ml each of: 20, 50, 80 and 100% MeOH in water to obtain four fractions (CA1-CA4, respectively). CA1 was subjected to repeated purification on silica gel using different proportions of solvents of increasing polarity from *n*-Hexane 100 % to ethyl acetate (100 %) to methanol : ethyl acetate (1:1). The eluate was monitored by analytical thin layer chromatography (TLC) to give a pure CA1b, compound **1**. CA2 was also repeatedly subjected to purification on silica gel as CA1, to give

another pure compound CABS 2a (compound **2)**. Both CA1b and CABS 2a were subjected to spectroscopic analysis and cell viability assay.

Cell culture

Immortalized human adenocervical carcinoma cell line (HeLa cell line) was employed as model of cancer. The cell line was obtained from the European Collection of Authenticated Cell Cultures (ECACC), Salisbury, UK. They were were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% Foetal Calf Serum (FCS), 1% L Glutamine (2mM) and 1% antibiotic-antimycotic solution (penicillin/streptomycin/amphotericin B) and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cell viability assay was carried out as described by Taiwo et al.[11] with modification, to determine cytotoxicity activity of the isolated compounds.

Cell viability assay to determine toxicity of compounds

Cell viability assay was carried out based on the method of Taiwo et al.[11] Briefly, compounds 1 and 2 at concentrations ranging from 6.25 to 100 µg/ml were evaluated for their ablity to alter the viability of HeLa cells. Cells were seeded into opaque, flat bottom, microclear 96-well plates at 7.5 x 10^5 cells/ml ($7.5x10^4$ cells per well at 100 µl/well) and incubated for 24 h at 37°C and 5% CO_2. After 24 h, the medium was discarded and each well was treated with 100 µl of extracts dissolved in the growth medium. A set of negative control wells was included in each plate as well as cells treated with doxorubicin (positive control) at a concentration 20 µM. After incubation for 48 hours, 10 µl of the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT), prepared as 5 mg/ml in Phosphate Buffered Saline (PBS)) was added to each well. After 2 hours of incubation with MTT at 37°C, the medium was discarded and 100 µl of DMSO was added to each well to dissolve the insoluble formazan formed. The absorbance at 570 nm was then determined with a microplate reader (CLARIO Star Microplate reader, BMG Labtech, UK). Each experiment was run in triplicate and repeated three different times.

Data presentation and analyses

Statistical analyses were conducted with the GraphPad Prism Software (Version 8.0.1) (GraphPad Software Inc., CA, USA). To assess statistically significant differences between means, analysis of variance (ANOVA) was used. This was followed by a post-hoc test for multiple comparisons (Tukey test), with a $p<0.05$ considered statistically significant.

Results and Discussion

Compound **1** was isolated pure (Figure 3) as a brown amorphous powder. The UV spectrum showed absorption maxima (Figure 4) at 280 nm, due to an indole chromophore, which is diagnostic for an unsubstituted aromatic ring of an indole moiety[12]. The ^{1}H NMR spectrum showed the presence of a deshielded NH signal as a singlet at δ11.10 and five aromatic signals at δ_H: 7.57 (1H, d, J = 8 Hz), 7.35 (1H, d, J = 8 Hz), 7.23 (1H, s), 7.06 (1H, t), 6.97 (1H, t), while the other signals were deshielded aliphatic methine signals at δ_H 3.50 (1H, dd, J = 8, 3 Hz), and methylene proton signal at δ_H 3.32 (1H, dd, J= 15, 4 Hz), and δ_H 3.00 (1H, dd J=8, 15 Hz). ^{13}C NMR displayed 13 carbon signals, edited in the Distortionless Enhancement Proton Transfer (DEPT) spectrum as six quaternary carbon signals and five aromatic methine carbon signals, an aliphatic methine carbon signal at 54.7 and a methylene carbon signal at δc 27.1 (Table 1). The long range correlation observed in the heteronuclear multiple bond correlation (HMBC) spectrum, between the set of methylene protons at δc 3.32 and 3.00 ppm with the carbon signals at δc 54.7, 109.5, 124.1, 127.3 and 170.5 ppm suggested a bicyclic azepine ring arrangement fused to the indole moiety. The ESI Q-TOF mass spectrum of the compound gave a signal at *m/z* 224.0768 $[M+1]^+$ for a molecular formula $C_{13}H_{10}N_3O$ (calculated 224.0824). Therefore compound **1** is proposed as 4,5-dihydro-4,1-(azenometheno) azepino [3,4-b]indol-3(10H)-one (**Albidumine,** Figure 1). To the best of our knowledge, this is the first report of the occurrence of azepinoindole alkaloid representing the [3,4-b] indole class, in the Sapotaceae family.

Compound **2** was isolated pure (Figure 5) as a brown hygroscopic powder. The UV spectrum shows two absorption bands at 220.7 and 273.9 nm (Figure 6). The ^{1}H NMR (300 MHz, CD_3OD) showed four aromatic signals at δ_H: 7.39 (1H, d, J=7.6 Hz), 7.29 (1H, d, J=12.6 Hz), 7.02 (1H, t) and 6.96 (1H, t) with multiplicity values consistent with unsubstituted phenyl ring of an indole moiety. In the aliphatic region, the proton spectrum showed four deshielded signals at δ_H 4.43, 4.25, 4.07 and 3.79. The signals between 3.48 and 3.68 appeared as multiplet. In the aliphatic region, the COSY spectrum revealed cross-peak between the following pairs of signals at δ_H: 4.53/4.09; 4.25/3.79, 3.16/2.52; 2.86/2.76. Carbon 13 spectrum for compound **2** (75 MHz, CD_3OD) indicated 18 carbon

signals, which in the HSQC were displayed as 8 aromatic carbon signals, of which four are methine signals and four were quaternary carbon signals. The rest signals were displayed as 4 deshielded aliphatic methine carbon signals and five methylene carbon and one quaternary carbon signal. A quick examination of the proton NMR of the compound revealed that the compound belongs to the tetrahydro β-carboline (THBC) class. However unlike the classical THBC compound with 12 carbon signals, this compound has 18 signals. The COSY spectrum showed the presence of two separate sets of coupled proton signals at δ_H: 4.53/4.09; 4.25/3.79. The signal at δ_H:0 4.53 which coupled with carbon 1 of the basic carboline skeleton indicated the position of attachment of the substituent at carbon 1. The presence of a ketal carbon signal at δ_c 104.6, as well as the coupling proton signals at δ_H 4.25/3.79 indicate a spirocyclic substitution on the beta carboline compound. Two groups of NCH_2 signals were observed from the HSQC spectrum with each correlating with δ_c50.3 (which belongs to the THBC skeleton and) and 50.4 (which belongs to the spirocyclic substituent). The spirocyclic NCH_2 was differentiated from the THBC NCH_2 by observing a germinal coupling between the spirocyclic NCH_2 proton pair with *J* value of 10.4 and 10.5 Hz respectively. In the HMBC spectrum, a long range correlation was observed between the spirocyclic NCH_2 proton signal at δ_H 2.86 and the carbon signals at δ_C: 56.8 and 104.6 while the other spirocyclic NCH_2 proton signal at δ_H 2.76 had a long range correlation with the carbon signals at δ_C: 50.3., 85.5, 104.6, confirming the attachment of the spirocyclic substituent. The HRESIMS gave a signal at *m/z* 367.1508 $[MNa]^+$ for a molecular formula $C_{18}H_{22}N_3O_4Na$ (calculated 367.1501). Thus we propose compound **2** to be 2'-amino-1,2,3,5,7,8,13,13b-octahydrospiro[azepino[1',2':1,2]pyrido[3,4-b]indole-4,2'-[1,3]dioxolane]-1,3-diol (**Bamindolinol,** Figure 1).

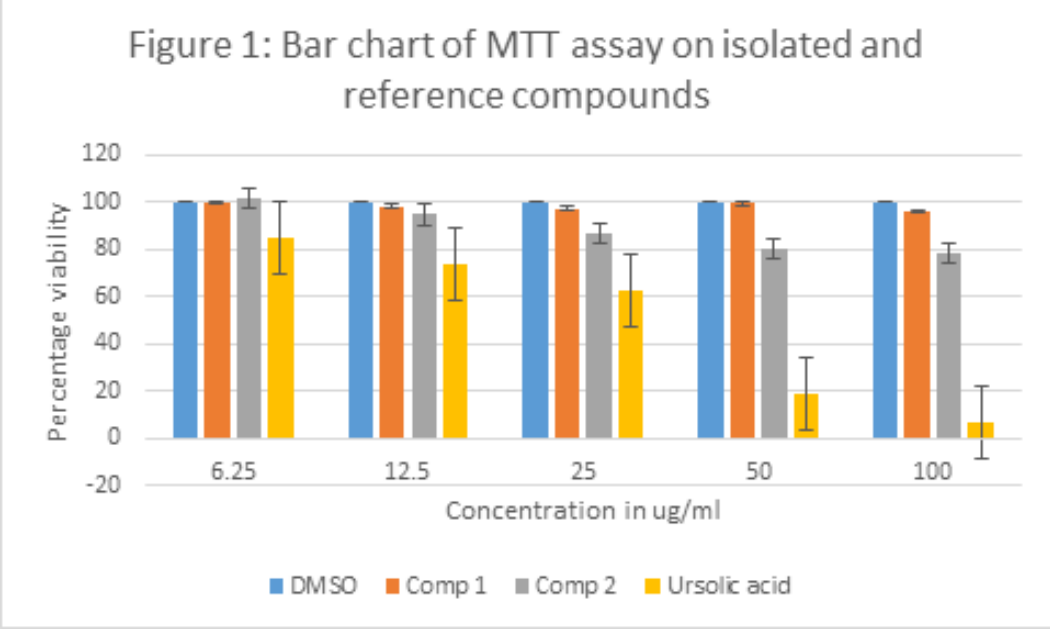
The two identified compounds were subjected to viability assay using HeLa cell line. Incidentally, none of the compounds evaluated, tested positive, indicating they have no antitumour effect. Interestingly, ginsenine, isolated from the berries of Panax ginseng C. A. Meyer[13] and its epimeric derivative, isoginsenine isolated from the Soybean plant Glycine max L[14]. which were close analogues of compound 1 and 2 displayed no significant inhibition of Con-A-activated lymphocyte proliferation, thus corroborating our bioassay results[14].

Table 1: NMR data for compound **1**

SN	^{1}H (ä) (300 MHz, DMSO-*d*$_6$)	COSY (ä)	^{13}C (ä) (75 MHz, DMSO-*d*$_6$)	HMBC
1	-	-	136.4	-
3	-	-	170.5	-
4	3.50,(1H,dd, *J*=8, 3 Hz)	3.32	54.7	27.1,109.5, 170.5
5á	3.32 (1H,dd, *J*=15, 4 Hz)	-	27.1	54.5,109..5,124.1,127.3, 170.5
5â	3.00 (1H,dd *J*=8, 15 Hz)	-	27.1	-
5a	-	-	109.5	-
5b	-	-	127.3	-
6	7.06 (1H, t)	7.35, 6.97	120.9	111.3, 118.4, 136.4
7	6.97 (1H, t)	7.06, 7.57	118.3	111.3, 127.3
8	7.35 (1H, d, *J* = 8 Hz)	7.06	111.3	118.4, 127.3
9	7.57 (1H, d, *J* = 8 Hz)	6.97	118.4	109.5, 120.9, 136.4
9a	-	-	136.4	-
9b	-	-	127.3	-
10	7.23	-	124.1	109.5, 127.3, 136.4

Table 2: NMR data for compound **2**

SN	^{1}H (ä) 300 MHz, (DMSO-d_6)	COSY (ä)	^{13}C (ä) (75MHz, DMSO-d_6)	HMBC
1	4.07 (1H, s)	4.53	56.8	50.4, 108.6, 132.3
3a	3.20 (1H, m)	-	50.3	17.3,50.4,56.8, 108.6
b	2.81 (1H, m)			
4	3.05 (1H, m)	2.52	17.3	
4a	-	-	108.6	
4b	-	-	127..3	
5	7.35 (1H, d, *J* = 7.6 Hz)	6.96	118.5	108.6, 122.0, 127.3, 137.9
6	6.96 (1H, t)	7.35, 7.05	119.6	111.9, 118.5, 127.3, 137.9
7	7.05 (1H, t)	6.96, 7.29	122.0	118.5, 137.9
8	7.29 (1H, d, *J* = 8.0 Hz)	6.98	111.9	118.5, 122.0, 127.3
8a	-	-	137.9	
8b	-	-	132.3	
1'	4.53 (1H, s)	4.05	81.8	81.9, 85.5, 104.6
2'	-	-	104.6	-
3'	4.26 (1H, s)	3.66	81.9	56.8, 85.5
4'	4.05	4.55	56.8	50.3, 107, 132.3.
5'	3.66 (1H, m)	4.26	73.8	64.4
7'a	2.86 (1H, d, *J*=10.5 Hz)	2.76	50.4	56.8, 104.6
b	2.76 (1H, d, *J*=10.4 Hz)	2.86	50.4	50.3., 85.5, 104.6
8'	3.58 (2H, m)		64.4	73.8
9'	3.54 (2H, m)		64.4	64.4



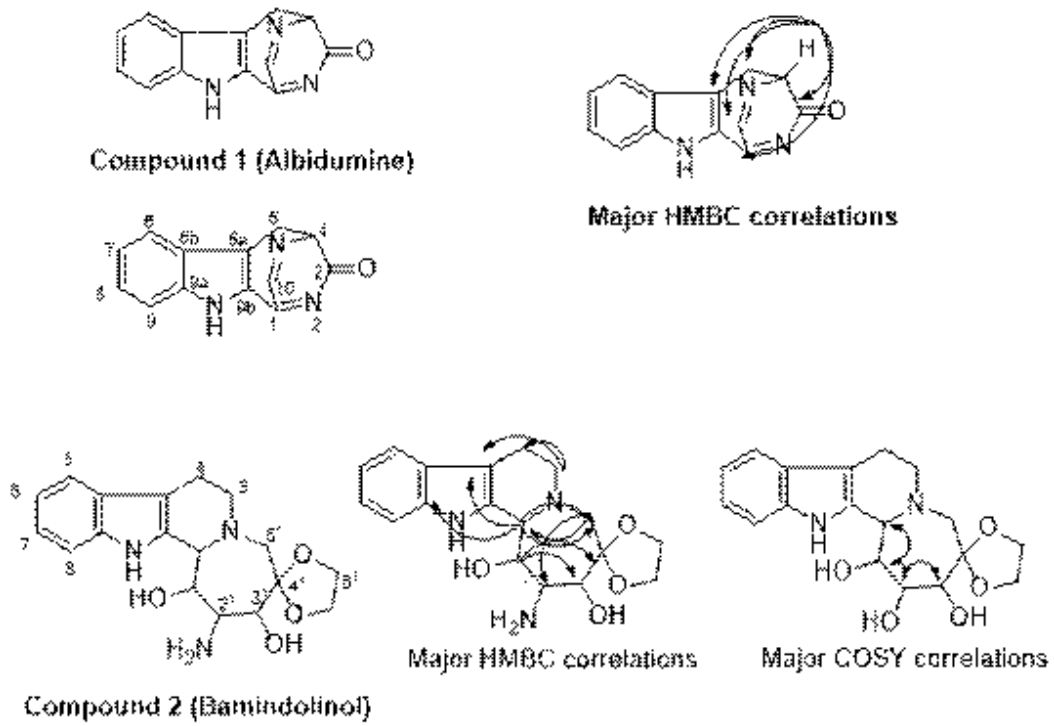


Figure 1: Structures of compound 1 and 2

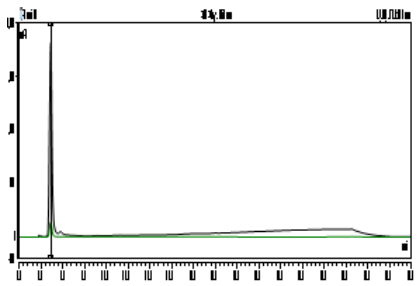


Figure 3: Chromatogram of compound **1**

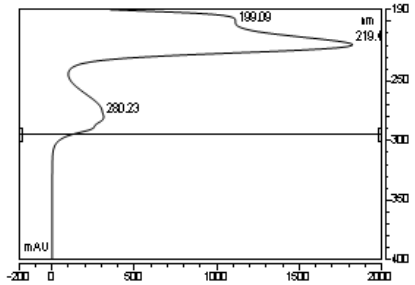


Figure 4: UV spectrum of compound **1**

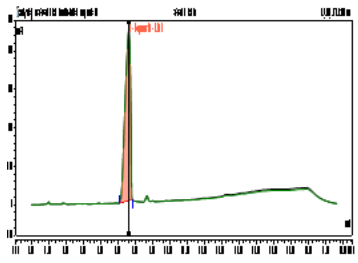


Figure 5: Chromatogram of compound **2**

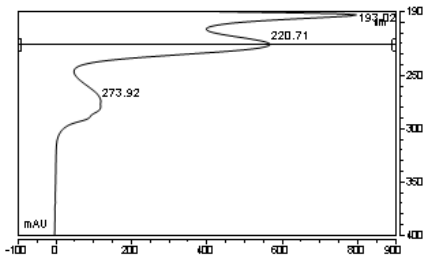


Figure 6: UV spectrum of compound **2**

Conclusion: The study identified two compounds for the first time in the seed cotyledon of *Chrysophyllum albidum*. The compounds were identified to belong to azepinoindole and beta carboline class. None of the two compounds had any significant effects on the viability of the Hela cell line evaluated.

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Conflict of interest: The authors have no conflict of interest to declare.

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