Anticholinesterase constituents from the leaves of *Spondias mombin* L. (Anacardiaceae)

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Abstract: *Spondias mombin* has been used in traditional medicine for the management of several diseases, including memory loss. This study aimed to evaluate the cholinesterase inhibitory activity of the methanol extract of the leaves and its derived fractions, as well as carry out detailed phytochemical investigations leading to the isolation and characterization of bioactive compounds from the plant. The acetyl cholinesterase (AChE) and butyryl cholinesterase (BuChE) inhibitory activities were evaluated by colorimetric and thin-layer chromatography bioautographic assay techniques. The ethyl acetate fraction was most active against both enzymes, with percentage inhibition of $58.10 \pm 1.08\%$ and $52.66 \pm 1.34\%$ against AChE and BuChE, respectively. Three compounds, namely, botulin, campesterol and phytol, with IC_{50} of 0.88 $\mu\text{g/mL}$ (AChE), 4.67 $\mu\text{g/mL}$ (BuChE); 1.89 $\mu\text{g/mL}$ (AChE), 4.08 $\mu\text{g/mL}$ (BuChE) and 12.51 $\mu\text{g/mL}$ (AChE), 23.89 $\mu\text{g/mL}$ (BuChE), respectively, were isolated from the supernatant of the ethyl acetate fraction. The isolated cholinesterase inhibitory compounds correlate with the known memory-enhancing property of the plant and thus support one of its uses in ethnomedicine.

Keywords: Alzheimer's disease, acetylcholinesterase, butyryl cholinesterase, neurodegenerative, *Spondias mombin*

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by low levels of acetylcholine (ACh) in the brain, with associated cognitive deficit and memory impairment.¹ Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are enzymes that catalyze hydrolysis of ACh and butyrylcholine, respectively. The activities of these enzymes increase progressively in AD patients as the severity of dementia progresses,² and their inhibition results in an increase in the levels of ACh and butyrylcholine in the brain, as well as a corresponding increase in cholinergic functions in AD patients.³ Thus, most drugs available today for the management of AD are cholinesterase inhibitors, such as galantamine, rivastigmine and donepezil⁴ even though their effectiveness in long-term treatment is debatable.⁵ Moreover, the primary targets recommended for AD treatment are both AChE and BuChE, but some inhibitors are more selective than others.⁶

There is still the need to search for newer anticholinesterase treatment for AD due to the serious side effects associated with available drugs. Natural products, especially medicinal plants, have been considered valuable sources of drugs for many conditions, including AD, especially since plants have been used to enhance memory traditionally.⁷

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Spondias mombin, a medium-sized, occasionally large deciduous tree with long compound leaves, large panicles of small white flowers and yellow plumb-like fruits,⁸ is a common inclusion in remedies used in ethnomedicine in southwestern Nigeria as a memory enhancer and/or an antiaging agent.⁹ All parts of the *S. mombin* tree are of medicinal importance in traditional medicine. The leaves have been used as an oxytocic agent,¹⁰ particularly for the expulsion of placenta in goats and women when normal delivery of such is delayed or impossible and as an ingredient in postpartum medication.^{11,12} It is useful as an antidiarrheal agent for the treatment of wounds and as an astringent.^{13–15} It is also used in treating inflammatory and arthritic conditions.¹⁶ In Nigeria, it is used in treating intestinal disorders, particularly those associated with typhoid, diarrhea and dysentery.¹⁷ It is also a component of traditional antituberculosis recipes.¹⁵ The fruit decoction is used as a diuretic and febrifuge. The bark and leaves are used as an emetic and for hemorrhoids, gonorrhea and leucorrhea.¹⁸ A decoction of the leaves and flower is taken as a relief for stomach ache, various inflammatory conditions and wound healings.¹⁹ In southwestern Nigeria, the leaves are used traditionally for the treatment of psychiatric disorders.²⁰

Several biological activities of the plant have been reported, including antiviral,^{21–23} antibacterial and molluscicidal,^{15,23} β -lactamase inhibitory,²⁴ anti-inflammatory,¹⁶ wound healing,¹⁹ antipsychotic, anticonvulsant and sedative,^{18,20} abortifacient,¹¹ oxytocic,²⁵ antimicrobial,²⁶ antifertility,²⁷ antigonadotrophic,²⁸ hematinic,²⁹ antioxidant,³⁰ antidiabetic³¹ and anticholinesterase activities.³²

The compounds isolated from this plant include caryophyllene, myrcene, hexanal, 3-hexenol and (*e*)-2-hexenal from the fruits,³³ cinnamic acid, 4-hydroxycinnamic acid, 3-methoxy-4-hydroxycinnamic acid, 3-methoxy-4-hydroxycinnamic acid, benzaldehyde, linalool, hexanoic acid, alpha-terpineol, palmitic acid and octanoic acid,³⁴ as well as anacardic acid.²⁴ The leaves and stems contain two ellagitannins galloylgeraniin and geraniin and two caffeoyl esters 5-*O*-caffeoylquinic acid and 2-*O*-caffeoyl hydroxycitric acid, which have also been reported.^{21,22} Pelandjuaic acid and 6-(8¹Z,11¹Z-heptadecadienyl) salicylic acid have been reported from the ethanolic extract of the leaves and stems of *S. mombin*.²³ Two new phytosterols (mombintanes I and II),³⁵ one new coumarin and three new flavonoids (mombinrin, mombincone, mombinoate and mombinol, respectively) have also been reported.³⁶ The plant also contains estra-17-propoxy, 3,4-dimethoxy-3-yl benzoate.³⁷

This study assessed the cholinesterase inhibitory activity of *S. mombin* leaves, and isolated and characterized its anticholinesterase compounds.

Materials and methods

Chemicals

The chemicals used were as follows: acetylthiocholine iodide (ATChI), butyrylcholine chloride (BuChCl), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), physostigmine (eserine) salicylate (Sigma-Aldrich, St Louis, MO, USA); and electric eel AChE (EC 3.1.1.7, type VI-s) and horse butyrylcholinesterase (EC 3.1.1.8) (Fluka Co, Germany). The other reagents and buffers, which include disodium hydrogen orthophosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) and sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$), were of analytical grade. Silica gel for vacuum liquid chromatography (VLC) (American Society for Testing and Materials [ASTM]) and precoated thin-layer chromatography (TLC) plates with silica gel G_{60} PF_{254} (EMD Millipore, Billerica, MA, USA).

Plant material collection and authentication

S. mombin was identified by Mr Oladele of the Department of Pharmacognosy, Faculty of Pharmacy, and was authenticated by Dr H Illoh of the Botany Department, Obafemi Awolowo University, Ile Ife, where herbarium specimen with herbarium number IFE 9572 was deposited. The leaves were collected from the Medicinal farm of the Obafemi Awolowo University Campus in August 2005.

Preparation of extract and fractions

The powdered leaves were extracted with 80% methanol by maceration for 72 hours, and the extract was concentrated to dryness at 40°C on a rotary evaporator. The crude extract was partitioned into *n*-hexane, ethyl acetate and water. Both the extract and the fractions were screened for their AChE and BuChE inhibitory activities.

Bulk extraction with ethyl acetate and precipitation studies

The powdered leaves of the plant were extracted with 100% ethyl acetate. Nonpolar lipid components were precipitated out by gradual addition of methanol. Both the filtered precipitate and the supernatants were then assessed for their cholinesterase inhibitory activities.

Phytochemical and cholinesterase analyses

TLC of both precipitates and supernatant was done with chloroform-hexane (7:3, v/v) as the solvent system. Some of the developed plates were sprayed with different

phytochemical screening reagents, such as vanillin/sulfuric acid, antimony trichloride, Dragendorff's reagent and anisaldehyde spray reagents. The other plates were used for the TLC bioautographic enzyme assay.

Cholinesterase inhibition assay

Cholinesterase inhibitory activities of the crude extract, fractions, precipitate, supernatant and isolated compounds were analyzed in a 96-well microplate reader according to the modified method of Ellman.^{38,39}

The reaction mixture was made up of 2000 mL 100 mM phosphate buffer (pH 8.0), 100 mL of test sample stock solution in methanol (at 42.5 µg/mL final concentration), 100 mL enzyme, either AChE or BuChE at a final concentration of 0.003 µ/mL or 0.001 µ/mL, respectively, and 100 µL of DTNB (0.3 mM) prepared in 100 M phosphate buffer pH 7.0 containing 120 mM sodium bicarbonate. Preincubation of the assay mixture was done on a water bath at 37°C for 30 minutes following proper mixing, and the reaction started by the addition of 100 µL of ATChI or butyrylthiocholine chloride (BTChCI) at a final concentration of 0.5 mM. Methanol was used as the negative control, while eserine ((-)-physostigmine) was used as the positive control. Change in absorbance at $\lambda_{\max}$ 412 was recorded at ambient temperature every 30 seconds for 5 minutes. All determinations were done in triplicate, and percentage inhibition was calculated as follows:

$$\frac{a - b}{a} \times 100$$

where a is the $\Delta A/\text{min}$ of control, b is the $\Delta A/\text{min}$ of test sample and ΔA is the change in absorbance.

TLC bioautographic assay method was also used to monitor active spots.⁴⁰ The various samples were spotted on precoated aluminum TLC plates (G_{60} PF₂₅₄) and developed in appropriate solvent systems. The developed plates were air-dried, sprayed with 2.55×10^{-3} units/mL of the cholinesterase enzyme till saturation and then incubated at 37°C for at least 20 minutes before spraying with 0.5 mM of the substrate (ATChI or BTChCI, respectively) and DTNB. Positive result was indicated by white spots on a yellow background.

Isolation of bioactive components

VLC of *S. mombin* supernatant (19.20 g) was done on silica gel 60 (Sigma-Aldrich), using *n*-hexane, dichloromethane and methanol as solvents. Fractions were monitored using TLC on precoated G_{60} PF₂₅₄ (0.25 mm) plates with vanillin/sulfuric acid reagent and heating at 100°C for a few minutes. A total of 103 subfractions collected were bulked into six

based on their TLC patterns. The six bulked samples were tested for their AChE inhibitory activity using TLC bioautographic method. Active subfractions were bulked together and purified further using VLC, leading to the isolation of three bioactive compounds through preparative TLC (PTLC).

Analysis of bioactive compounds

The isolated compounds were subjected to a number of analyses, including different spectroscopic analyses such as ¹H-nuclear magnetic resonance (NMR) and ¹³C-NMR (CDCl₃, 300 Hz), TLC analysis in different solvent systems, solubility in water and determination of IC₅₀.

Results

Medicinal plants are known to contain different classes of chemical compounds called secondary metabolites, which are responsible for their various biological activities. Phytochemical analysis of *S. mombin* was carried out on developed TLC plates. Partial purification of the methanol extract was done by precipitation. Thus, spraying the developed TLC plates of precipitate and supernatant of *S. mombin* with different phytochemical reagents is shown in Figure 1A–D for vanillin/H₂SO₄, Dragendorff's reagent, antimony trichloride and anisaldehyde spray, respectively. Various colors were observed for the spots with the different reagents, indicating the possible nature of these chemical constituents. Organic compounds generally show color reactions to concentrated sulfuric acid⁴¹ and could be indicative for detecting steroidal and terpenoidal compounds.⁴² Figure 1A gave colors with vanillin/H₂SO₄, which are more prominent in the supernatant than in the precipitate. Alkaloids are detected with Dragendorff's reagent as an orange–brown zone against a yellow background.⁴² This seems to be absent in the spotted samples, as seen in Figure 1B. Cardiac glycosides, saponins, terpenoids and flavonoids give colored spots with antimony trichloride, and this can be seen in this plant (Figure 1C), while terpenoids can also be detected with anisaldehyde spray giving purple, blue or red spots.⁴² Again, more colors were detected in the supernatant with anisaldehyde when compared with the precipitate (Figure 1D). Several bioactive constituents belonging to various classes have been previously reported in *S. mombin*. These include coumarins, flavonoids,³⁶ sterols,³⁵ phenols^{33,34} and tannins.^{21,43}

The precipitate and the supernatant were subjected to cholinesterase inhibitory analysis. Qualitative TLC bioautographic (Figure 2) as well as quantitative Ellman³⁹ colorimetric assays (Table 1) showed that cholinesterase inhibitory activity of the supernatant was better than that of the precipitate.

Figure 1 Spray analysis of the TLC plates of precipitate and supernatant with phytochemical reagents.

Notes: Phytochemical analysis of precipitate and supernatant is shown. Samples: a = precipitate, b = supernatant; solvent system: *N*-hexane:chloroform (3:7); the different spray reagents: **(A)** vanillin/ H_2SO_4 , **(B)** Dragendorff's reagent, **(C)** antimony trichloride and **(D)** anisaldehyde spray.

Abbreviation: TLC, thin-layer chromatography.

Figure 2 TLC bioautographic assay of precipitate and supernatant.

Notes: **(A)** AChE; **(B)** BUCHE; samples: a = precipitate, b = supernatant; solvent system: *N*-hexane:chloroform (3:7).

Abbreviations: AChE, acetyl cholinesterase; BUCHE, butyryl cholinesterase; TLC, thin layer chromatography.

Table 1 Percentage cholinesterase inhibitory activity of *Spondias mombin* samples

Fraction	% inhibition of AChE	% inhibition of BUCHE
Hexane fraction	36.16±1.11	24.86±1.09
Ethyl acetate fraction	58.10±1.08	52.66±1.34
Aqueous fraction	23.00±1.63	18.84±1.96
Precipitate	71.52±2.01	ND
Supernatant	87.33±1.98	ND

Note: Values shown as mean ± standard deviation.

Abbreviations: AChE, acetyl cholinesterase; BUCHE, butyryl cholinesterase; ND, not determined.

The phytochemical investigations, which showed more constituents in the supernatant when compared with the precipitate, corroborate the observed better cholinesterase inhibitory activity of the supernatant. Thus, activity-directed

fractionation using VLC with TLC bioautography of the supernatant was carried out. Figure 3A shows the spots of the various bulked fractions after VLC, while Figure 3B shows the AChE assay results, from which it was observed that subfractions A–C were active. These were subjected to a combination of repetitive VLC and PTLC to isolate the active compounds, which were then identified through spectroscopic analysis.

Compound 1

Spectral data

The 1H -NMR spectrum ($CDCl_3$, 300 Hz) showed signals at δ 7.8 (m), δ 7.75 (m), δ 5.45 (t), δ 4.6 (s) and δ 4.5 (d). The ^{13}C -NMR spectrum ($CDCl_3$, 300 Hz) showed signals at 38.71 (C-1), 20.90 (C-2), 78.83 (C-3), 35.57 (C-4), 55.24 (C-5), 18.30 (C-6), 34.06 (C-7), 39.35 (C-8), 54.96 (C-9), 37.34 (C-10), 27.22 (C-11), 24.92 (C-12), 37.83 (C-13), 39.99 (C-14), 27.19 (C-15), 29.48 (C-16), 47.08 (C-17),

Figure 3 Bulk samples from the VLC of *Spondias mombin* supernatant.

Notes: TLC bioautographic assay of VLC fractions is shown. Solvent system: chloroform 100%; **(A)** vanillin/ H_2SO_4 spray; **(B)** enzyme, ATChI and DTNB spray.

Abbreviations: ATChI, acetylthiocholine iodide; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); VLC, vacuum liquid chromatography.

Table 2 TLC profiles of the isolated compounds

Compounds	Detecting agent	Solvent system	Color	R _f
Compound 1	Vanillin/H ₂ SO ₄	Chloroform (100%)	Purple	0.35
	Anisaldehyde	Hexane:chloroform (3:7)	Purple	0.46
Compound 2	Vanillin/H ₂ SO ₄	Hexane:chloroform (2:8)	Purple	0.20
	Anisaldehyde	Chloroform (100%)	Purple	0.27
Compound 3	Vanillin/H ₂ SO ₄	Hexane:chloroform (1:1)	Purple	0.64
	Anisaldehyde	Chloroform (100%)	Pink	0.51

Abbreviations: R_f, retardation factor; TLC, thin-layer chromatography.

50.22 (C-18), 48.97 (C-19), 150.8 (C-20), 29.66 (C-21), 36.65 (C-22), 27.92 (C-23), 15.96 (C-24), 15.46 (C-25), 16.64 (C-26), 14.33 (C-27), 59.41 (C-28), 109.40 (C-29) and 19.70 (C-30).

Structure elucidation

Compound 1 (35 mg) was isolated as a white powder with retardation factor (R_f) 0.46 in hexane:chloroform 3:7 and R_f 0.35 in 100% chloroform (Table 2). It gave purple color with both vanillin/H₂SO₄ and anisaldehyde spray reagent, indicating its steroidal nature,⁴⁴ and had a melting point range of 256°C–258°C (Table 3).

The ¹³C-NMR spectrum in the distortionless enhancement by polarization transfer experiment showed that there were 6CH₃, 11CH₂, 6CH and 7C. Thus, Compound 1 is a C-30 carbon compound.

The ¹H-NMR showed a proton at δ4.5 (d) germinal to the hydroxyl group and had a corresponding carbon chemical shift at δ59.41. There was also an olefinic proton at δ4.6, which resided on the carbon at δ109.40. This proton was assigned to C-22, which is a terminal CH₂ (Figure 4).

Compound 2

Spectral data

The ¹³C-NMR data are as follows: 36.92 (C-1), 34.35 (C-2), 72.22 (C-3), 42.73 (C-4), 141.17 (C-5), 122.14 (C-6), 28.67 (C-7), 32.80 (C-8), 50.53 (C-9), 32.33 (C-10), 21.50 (C-11), 37.66 (C-12), 40.18 (C-13), 57.17 (C-14), 23.42 (C-15), 26.45 (C-16), 56.45 (C-17), 12.26 (C-18), 19.82 (C-19), 36.56 (C-20), 19.44 (C-21), 32.31 (C-22), 24.72 (C-23), 46.23 (C-24), 29.54 (C-25), 20.25 (C-26), 19.20 (C-27) and 12.40 (C-28).

Structure elucidation

Compound 2 (20 mg) had R_f values of 0.2 and 0.27 in hexane:chloroform 2:8 and 100% chloroform, respectively, with purple color in both vanillin/H₂SO₄ and anisaldehyde spray reagent (Table 2).

Table 3 Analysis of isolated compounds

Properties	Compound 1	Compound 2	Compound 3
Solubility in water	Not determined	Soluble	Insoluble
Boiling point	—	—	202°C–204°C
Melting point	256°C–258°C	156°C–158°C	—
IC ₅₀ (BuChE)	4.67 µg/mL	4.08 µg/mL	23.89 µg/mL
IC ₅₀ (AChE)	0.88 µg/mL	1.89 µg/mL	12.51 µg/mL

Abbreviations: AChE, acetyl cholinesterase; BuChE, butyryl cholinesterase.

Figure 4 Compound 1: betulin.

The ¹³C-NMR spectrum of Compound 2 showed that it is a C-28 compound. The attached proton test (APT) revealed 3 quaternary (3 C), 10 methylene (10 CH₂), 6 methyl (6 CH₃) and 9 methine (9 CH) carbons. The ¹H-NMR showed one olefinic proton at δ5.40, with a corresponding carbon chemical shift of δ121.14 in the heteronuclear multiple quantum coherence spectrum. It also revealed the presence of one oxygenated methylene proton at δ3.5, as confirmed by the downfield chemical shift at δ72.22. In the heteronuclear multiple bond coherence spectrum, the diagnostic olefinic proton and the proton germinal to the OH showed connectivity with the quaternary carbon resonating at 141.17. From the combined ¹H-NMR, ¹³C-NMR and APT experiments, as well as comparison with literature data, Compound 2 was identified to be campesterol (Figure 5).^{45,46}

Compound 3

Spectral data

^{13}C -NMR spectra of Compound 3 showed signals at 59.85 (C-1), 123.48 (C-2), 130.92 (C-3), 40.29 (C-4), 25.55 (C-5), 33.21 (C-6), 30.13 (C-7), 37.78 (C-8), 24.89 (C-9), 37.08 (C-10), 33.11 (C-11), 37.70 (C-12), 25.22 (C-13), 39.79 (C-14), 28.40 (C-15), 23.15 (C-16), 23.05 (C-17), 20.17 (C-18), 20.14 (C-19) and 16.86 (C-20).

Structure elucidation

Compound 3 (19 mg) was isolated as a yellowish liquid with R_f of 0.64 in hexane:chloroform 1:1 and 0.51 in 100% chloroform. It gave a purple color with vanillin/ H_2SO_4 and a pink color with anisaldehyde spray reagent (Table 2).

The ^{13}C -NMR spectrum revealed 5CH_3 , 10CH_2 , 4CH and $1\text{C}=\text{C}$, indicating a C-20 compound. The ^1H -NMR spectrum showed a signal at δ 5.4 (t), representing an olefinic proton, assigned to C-2. The signal at δ 4.1 (d) is an alcoholic proton assigned to the proton residing on C-1. A triplet at δ 1.98 was assigned to the proton on C-4, while the multiplets at δ 1.44 and δ 1.35 are the methine protons on C-7 and C-11. The other methine proton on C-15 had its signal at δ 1.52. In addition, the multiplets at δ 1.30– δ 1.03 were assigned to the protons on C-6, C-8, C-9, C-10, C-12 and C-13, while the signal at δ 1.65 (s) was assigned to the methyl proton on C-20. The OH group had a signal at δ 1.66. Analysis of the spectra and comparison with literature values showed that Compound 3 is phytol (Figure 6).⁴⁷

Discussion

Three compounds with cholinesterase inhibitory activity were successfully isolated from *S. mombin* and identified

in this study. *S. mombin* exhibited potent cholinesterase inhibitory activity attributable to the presence of the various isolated compounds and of significance in the management of neurodegenerative disorders such as Alzheimer's disease. This plant has been used for enhancement of memory in traditional medicine,⁹ and plants with such history have been previously reported by various researchers to possess cholinesterase inhibitors.^{48–50}

On comparison with data in literature,^{51–53} Compound 1 was identified as betulin. Betulin has been previously reported in several plants for its different biological activities.⁵¹ However, it is being linked with the cholinesterase inhibitory activity for the first time, with an IC_{50} of 0.88 $\mu\text{g/mL}$ against AChE and 4.67 $\mu\text{g/mL}$ against BuChE.

Compound 3, which is phytol, has been previously reported by us for its cholinesterase inhibitory activity.⁵⁴ Phytol is a diterpene alcohol, while betulin is a lupane-type triterpene. Several reports have implicated terpenoids as good cholinesterase inhibitors.^{55–57} In 2004, dihydrotanshinone, cryptotanshinone, tanshinone I and tanshinone IIA were identified as the first example of diterpenoids that inhibit AChE.⁵⁸ Several others have since been reported. Triterpenes have also been documented as potent cholinesterase inhibitors.^{59–61} Some other oleanane triterpene saponin compounds have also been implicated in the treatment of dementia and mild cognitive impairment by previous researchers who are already seeking patency in the USA.⁶²

Compound 2 identified as campesterol has been previously reported in several plant species, including rapeseed oil (*Brassica napa*),⁶³ soybean oil (*Glycine max*)⁶⁴ and wheat germ oil (*Triticum* spp.).⁶⁵ Campesterol, though not new, is being reported for cholinesterase inhibitory activity for the first time, with an IC_{50} of 1.89 $\mu\text{g/mL}$ (AChE) and 4.08 $\mu\text{g/mL}$ (BuChE). It is a phytosterol, and several phytosterols have been reported to possess cholinesterase inhibitory activity to varying extents.^{66–68} However, others such as stigmasterol and β stigmasterol were reported in a molecular docking experiment to have weak bonding with AChE proteins when compared with Aricept®–AChE complex, even though they had comparable Glide score.⁶⁹

Figure 5 Compound 2: campesterol.

Figure 6 Compound 3: 3,7,11,15-tetramethyl-2-hexadecen-1-ol.

Conclusion

Studies relating to the identification of naturally occurring secondary metabolites from medicinal plants have allowed the discovery of important drugs, including inhibitors of several enzymes such as AChE. This has been useful in the development of new drugs for clinical use. We isolated and identified three compounds from *S. mombin* with good cholinesterase inhibitory ability. These compounds could be candidates for further studies in the development of new drugs for the treatment of disorders such as AD.

Disclosure

The authors report no conflicts of interest in this work.

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