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RESEARCH ARTICLE

Effects of *Alchornea cordifolia* on elastase and superoxide anion produced by human neutrophils

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Abstract

The ability of *Alchornea cordifolia* (Schum. and Thonn.) Müll. Arg. (Euphorbiaceae) leaves to inhibit human neutrophil elastase (HNE) and superoxide anion ($O_2^{\bullet-}$) activities was evaluated on aqueous and ethyl acetate extracts as they allow for a targeted extraction of polyphenols. The direct effect of *A. cordifolia* extracts on HNE and $O_2^{\bullet-}$ was assessed in an acellular system. Results showed that extracts scavenge HNE and $O_2^{\bullet-}$ in a dose-dependent manner. Better activity was exhibited by the ethyl acetate extract with lower IC_{50} (2.2 and 4.1 mg/L for HNE and $O_2^{\bullet-}$, respectively) than for the aqueous extract. Cellular systems including isolated human polymorphonuclear neutrophils (PMN) were investigated to assess the effect of extracts on PMN metabolism. PMN were stimulated with 4β-phorbol-12-myristate-13-acetate (PMA), calcium ionophore (CaI), or *N*-formyl-methionyl-leucine-phenylalanine (fMLP), each stimulant having its own stimulation pathway. From the IC_{50} obtained, it can be concluded that *A. cordifolia* reduces HNE and $O_2^{\bullet-}$ liberation. Furthermore it was demonstrated that *A. cordifolia* extracts have no cytotoxic activity on PMN by measuring release of the cytosolic enzyme lactate dehydrogenase. As the ethyl acetate extract offers a higher rate of total phenols than the aqueous extract as well as better scavenging activity, it can be supposed that polyphenols, which are well known for their potent antioxidant and antielastase activity, are implicated in the activity of the plant. Phenolic substances such as quercetin, myricetin-3-glucopyranoside, myricetin-3-rhamnopyranoside, and proanthocyanidin A2 were identified in the ethyl acetate extract. In conclusion, the study provides proof of ethnomedical claims and partly explains the mechanisms of the anti-inflammatory action of *A. cordifolia* leaves.

Keywords: Alchornea cordifolia*; elastase; human polymorphonuclear neutrophils; superoxide anion*

Introduction

From Senegal to Uganda the leaves of *Alchornea cordifolia* (Schum. and Thonn.) Müll. Arg. (Euphorbiaceae) are successfully used by healers practicing traditional African medicine. The plant is used internally in the treatment of gastrointestinal disorders, malaria, and respiratory and urinary tract infections and externally as an anti-inflammatory in numerous ailments such as toothache, piles, arthritis, and rheumatism (Adjanohoun, 1994; Adjanohoun & Aké Assi, 1979; Iwu, 1983).

Research was done on the antielastase and antioxidant actions of this plant to try to elucidate its anti-inflammatory mechanism. Human neutrophil elastase (HNE) has a high affinity for cartilage tissue and can deteriorate major cartilage tissue components (Siedle et al., 2007). The production of superoxide anion ($O_2^{\bullet-}$) has been implicated in the pathology of a number of inflammatory conditions, including inflamed arthritic joints (Afonso et al., 2007; Henrotin et al., 2005). HNE and $O_2^{\bullet-}$ have therefore been the subject of intensive research to find inhibitors that target their destructive and proinflammatory action.

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Neutrophils have a central role in inducing and regulating the inflammatory process. Cells have been shown to liberate potent proteinases such as elastase and generate some reactive oxygen species (ROS) such as $O_2^{\bullet-}$. HNE, which is a serine protease primarily stored within granules in polymorphonuclear leukocytes, has been shown to deteriorate all the components in the extracellular matrix and also cleave the protein elastin, the connective tissue which is widely distributed in vertebrate tissue, and is particulary abundant in the lung, arteries, skin, and ligaments (Bode et al., 1989; Nenan et al., 2005). $O_2^{\bullet-}$ is transformed into a reactive secondary oxidant and will then damage intracellular macromolecules, oxidizing lipids, DNA, or proteins (Roos, 1991). These reactive substances (ROS and HNE) can be beneficial, acting as defense mechanisms such as phagocytosis and in eradicating microorganisms, and they have an important role in cell signaling and in controlling the inflammatory process (Bieth, 1998; Wiedow et al., 1992). But in the case of overload, the production and liberation of HNE and $O_2^{\bullet-}$ may damage cells. HNE and $O_2^{\bullet-}$ are implicated in many illnesses related to inflammation: rheumatoid arthritis, pulmonary emphysema, chronic bronchitis, respiratory distress syndrome of adults (Chabot et al., 1998), atherosclerosis, cardiovascular diseases (Dhalla et al., 2000), glomerulonephritis, psoriasis, tumoral invasion, and cerebral ischemia (Chan, 2001).

Besides synthetic HNE and $O_2^{\bullet-}$ inhibitors, other drugs or human proteins such as albumin (Imai et al., 2003; Kouoh et al., 2002; Obayashi, 2005; Quilan et al., 2004) and various plant compounds, namely phenolic compounds such as flavonoids and procyanidins, have been shown to possess inhibitory activity against HNE and $O_2^{\bullet-}$. The aim of this study was to investigate the capacity of *A. cordifolia* to inactivate HNE and $O_2^{\bullet-}$ activity. Two extracts, ethyl acetate and aqueous, were tested to assess the contribution of polyphenols to the activity of the plant.

First, inhibition of HNE and $O_2^{\bullet-}$ was tested in an acellular model. Second, the effect of *A. cordifolia* on HNE and $O_2^{\bullet-}$ release was assessed in a cellular model. The stimuli used [4β-phorbol-12-myristate-13-acetate (PMA), *N*-formyl-methionyl-leucine-phenylalanine (fMLP), or calcium ionophore (CaI)] possess differing transductional mechanisms. PMA directly activates protein kinase C (PKC) which induces NADPH (reduced nicotinamide adenine dinucleotide phosphate)-oxidase activation, and so produces elastase. Another stimulus is fMLP, which activates phospholipase C and leads to the production of inositol triphosphate and diacylglycerol. These two mediators are responsible for PKC activation (Baggliolini et al., 1993). The third and last stimulant used, CaI, induces calcium translocation by creating new pores in the cell membrane, causing calcium influx, followed by HNE and $O_2^{\bullet-}$ release.

Materials and methods

Plant material

Fresh leaves of *Alchornea cordifolia* (Schum. and Thonn.) Müll. Arg. (Euphorbiaceae), Herbarium N° 183, were harvested at the Floristic National Center of Abidjan and authenticated by an expert botanist (Professor Aké Assi, Department of Botany, University of Abidjan) to be identical to the sample in the Herbarium at the Center. Voucher specimens were deposited in the Botanical Department of the Centre National de Floristique at the Cocody University of Abidjan (Côte d'Ivoire). The leaves were collected and dried in a 25°C air-conditioned room.

Chemicals and biochemicals

PMA, fMLP, CaI, superoxide dismutase, methoxy-Suc-(Ala)2-Pro-Val-pNa, phenylmethylsulfonyl fluoride, ferricytochrome C, hypoxanthine, ethylenediamine-tetraacetic acid (EDTA), KOH, xanthine oxidase, solvents, and chemical reagents were obtained from Sigma and elastase from Biosys SA.

Instrumental analysis

Ultraviolet (UV) absorbance was measured with a Kontron Uvikon 860 spectrophotometer. One-dimensional (1D) and 2D nuclear magnetic resonance (NMR) spectra were recorded in the Laboratoire d'Application de RMN (LARMN), at the University of Lille 2, on a Bruker Avance 300 MHz spectrometer, using tetramethylsilane (TMS) as an internal standard. Electrospray ionization-mass spectrometry (ESI-MS) was carried out on an API 3000 instrument (PerkinElmer Sciex), in the Laboratoire d'Application de Spectrométrie de Masse, at the University of Lille 2.

Preparation of ethyl acetate and aqueous extracts

Ethyl acetate extract

The dried leaves (50 g) were powdered and macerated for 24 h at a temperature of 4°C in 500 mL methanol/acetone/water (70:70:30, v/v/v). The filtrates were then low-pressure concentrated at 30°C. After centrifugation the aqueous phase was washed with dichloromethane to remove lipophilic pigments and extracted three times in ethyl acetate. The final phase was evaporated to dryness and the dried extract maintained at 4°C. For further assays this powder was dissolved in the appropriate solvents.

Aqueous extract

Powdered dried leaves (50 g) were extracted by maceration for 48 h in 500 mL distilled water. After filtration, the aqueous solution was evaporated to dryness and the

dried extract was maintained at 4°C. A stock solution was obtained by dissolving small samples of the extract in water.

Isolation of pure compounds

The ethyl acetate extract of *A. cordifolia* was dissolved in H_2O and submitted to column chromatography, using Sephadex LH-20, Silicagel 60H, and Silicagel 60, under nitrogen pressure with a step gradient (H_2O–CH_3OH), yielding four pure compounds. Compounds were identified by means of 1D (^1H, ^{13}C) and 2D NMR (correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond correlation (HMBC)) experiments and mass spectrometry.

Isolation of human polymorphonuclear neutrophils

Human polymorphonuclear neutrophils (PMN) were purified from fresh heparinized venous blood from healthy human subjects. Briefly, PMN were isolated by a gradient density technique over Ficoll Histopaque, followed by isotonic ammonium chloride hemolysis (Cabanis et al., 1994). Cells isolated in this way were viable as confirmed by the Trypan blue exclusion test, and cells were suspended in Hank's balanced salt solution-HEPES (HBSS-H) pH 7.4 buffer.

Lactate dehydrogenase assay

The potential toxicity of *A. cordifolia* extracts toward PMN was measured by releasing the cytosolic enzyme lactate dehydrogenase (LDH) (Srinivas et al., 1993). PMN (2×10^6/mL) were stimulated for 15 min by 160 nM PMA after 30 min incubation at 37°C with *A. cordifolia* extracts at different concentrations (from 0.1 to 1.0 g/L). After centrifugation and lysis of the cells by isotonic shock, LDH activities released by PMN were determined in the supernatant using a conversion rate of NADH (reduced nicotinamide adenine dinucleotide) to NAD by UV spectrophotometry at 340 nm in the presence of pyruvate.

Control PMN were not incubated with *A. cordifolia* leaf extracts, and control LDH activity released in the reaction mixture was determined after centrifugation in the supernatant (S_{Ref}). After lyzing control pellets by hypotonic shock and centrifuging, LDH activity (S_{LyzRef}) was also measured as above.

The results are expressed as the percentage of LDH release by the chemical compounds as follows:

$$\%P = Y \times 100/X$$

where $X = S_{Ref}$ LDH activity + S_{LyzRef} LDH activity and $Y = S$ LDH activity – S_{Ref} LDH activity, as previously reported (Daels-Rakotoarison et al., 2003).

Dosage of human neutrophil elastase

Cell-free system

Neutrophils were incubated for 30 min at a concentration of 2×10^6/mL in HBSS-H buffer in the presence of PMA (1 μM). The tubes were then centrifuged at 4°C for 5 min in order to collect elastase from the supernatant; 400 IU of supernatant was added to 100 IU of increasing concentrations of plant extracts at 37°C for 15 min. After incubation, 400 IU of specific substrate (methoxy-Suc-(Ala)2-Pro-Val-pNa) was added to reaction mixtures in a final volume of 1 mL for 5 min. The reaction was stopped with phenylmethylsulfonyl fluoride (PMSF) (0.1 U/mL) and absorbance was measured at 405 nm in a spectrophotometer. The concentration of HNE was calculated from a standard curve using various concentrations of HNE. The results are expressed as the percentage of elastase inhibition (Khalfi et al., 1996).

Cellular system

Neutrophils were incubated for 15 min at a concentration of 2×10^6/mL in HBSS-H buffer, pH 7.4, in the presence of increasing concentrations of plant extracts at 37°C. Then PMA (160 nmol/L), fMLP (1 μmol/L), or CaI (4 μmol/L) was added and the reaction mixtures incubated for 30 min at 37°C. After incubation, the tubes were centrifuged for 5 min at 4°C. Cell-free supernatants were assayed for elastase activity. Briefly, 400 μL supernatant was added to 400 μL specific substrate methoxy-Suc-(Ala)2-Pro-Val-pNa (0.5 mmol/L in 0.1 mol/L HEPES–0.5 mol/L NaCl pH 7.5 buffer) in a final volume of 1 mL. After 5 min incubation at 37°C, the reaction was stopped with PMSF and absorbance was measured at 405 nm. The elastase concentration was obtained from a standard curve using various concentrations of reagent grade elastase. Results are expressed as the percentage of elastase activity inhibited by plant extracts (Daels-Rakotoarison et al., 2003).

Dosage of superoxide anion

Acellular model

Superoxide anion ($O_2^{\bullet-}$) was generated by the hypoxanthine–xanthine oxidase system (Gressier et al., 1995). Reaction mixtures contained 50 μL of EDTA (30 mmol/L) in KOH (50 mmol/L), 5 μL of hypoxanthine in KOH (30 mM), 50 μL of ferricytochrome C (0.5 mmol/L), and 150 μL of *A. cordifolia* extracts (in concentrations

ranging from 0.5 to 20 mg/L) in a final volume of 1.5 mL buffered in PBS, pH 7.4. The reaction was started by adding 100 µL of xanthine oxidase (1 U/mL) and the rate of reduced ferricytochrome C was measured at 550 nm against a reference cuvette.

The amount of $O_2^{\bullet-}$ generated was calculated using the Beer–Lambert law with an extinction coefficient $\varepsilon_{550} = 2.1 \times 10^{-2}\,\mu M^{-1} cm^{-1}$. The percentage of inhibited $O_2^{\bullet-}$ production by *A. cordifolia* extracts was then calculated and results expressed as IC_{50} values, as previously reported (Hennebelle et al., 2007).

Cellular model

$O_2^{\bullet-}$ production by human PMN stimulated by PMA (160 nmol/L), fMLP (1 µmol/L), or CaI (4 µmol/L) was measured by reducing ferricytochrome C (Cohen & Chovianiec, 1978). 400 µL of 2.5×10^6 PMN/mL stimulated by one of these three agents was incubated for 5 min at 37°C in the presence of increasing concentrations of *A. cordifolia* extracts and 0.3 mg ferricytochrome C in HBSS-H buffer, pH 7.4. After centrifugation at 4°C, the absorbance of the supernatants was measured by UV spectrophotometry at 550 nm against a reference cuvette containing 310 U superoxide dismutase. The amount of $O_2^{\bullet-}$ was calculated as described for the acellular system (Daels-Rakotoarison et al., 2002).

Statistical analysis

Results are expressed as mean ± SEM, and comparison of fraction values was established using the Wilcoxon test. Differences were considered to be statistically significant when $p < 0.05$.

Results

Cellular viability

The potential cytotoxicity of leaf extracts of *A. cordifolia* was tested at concentrations from 0.1 to 1.0 g/L. PMN viability, assessed by LDH liberation, was not modified after 1 h of incubation in the presence of ethyl acetate or aqueous extract of *A. cordifolia*.

Effect of Alchornea cordifolia *extracts on human neutrophil elastase*

In the cell-free system, direct contact between neutrophil elastase and various concentrations of *A. cordifolia* extracts showed that they have the capacity to limit neutrophil elastase activity in a dose-dependent manner. IC_{50} values were 2.2 and 4.7 mg/L for ethyl acetate extract and aqueous extract, respectively (Figures 1 and 2).

In the cellular experiment, *A. cordifolia* ethylacetate extracts decreased elastase activity with IC_{50} values of 8.6, 5.9, and 7.4 mg/L when PMN were stimulated by PMA, fMLP, and CaI, respectively. *A. cordifolia* aqueous extracts decreased elastase activity with IC_{50} values of 12.1, 7.3, and 9.2 mg/L when PMN were stimulated by PMA, fMLP, and CaI, respectively (Figures 1 and 2).

Effect of Alchornea cordifolia *extracts on superoxide anion*

In acellular experiments, direct contact between $O_2^{\bullet-}$ and various concentrations of *A. cordifolia* extracts showed that each extract had the capacity to limit $O_2^{\bullet-}$ activity in a dose-dependent manner. IC_{50} values obtained were 4.1 and 5.0 mg/L for ethyl acetate and aqueous extracts, respectively (Figures 3 and 4).

In the cellular experiment, *A. cordifolia* ethylacetate extracts decreased $O_2^{\bullet-}$ activity with IC_{50} values of 9.2, 6.5, and 6.7 mg/L when PMN were stimulated by PMA, fMLP, and CaI, respectively. *A. cordifolia* aqueous extracts decreased $O_2^{\bullet-}$ activity with IC_{50} values of 13.4, 8.5, and 10.3 mg/L when PMN were stimulated by PMA, fMLP, and CaI, respectively (Figures 3 and 4).

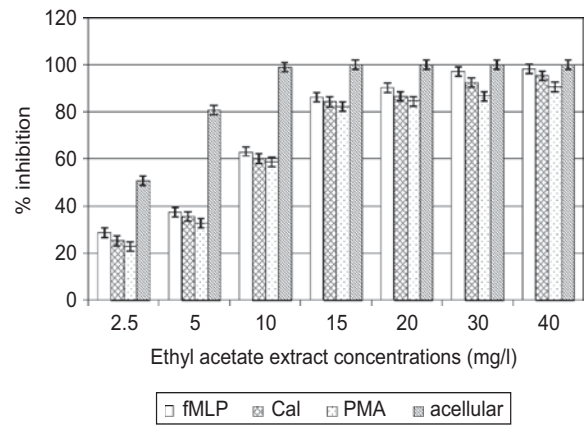

Figure 1. Effects of *A. cordifolia* ethyl acetate extract on elastase.

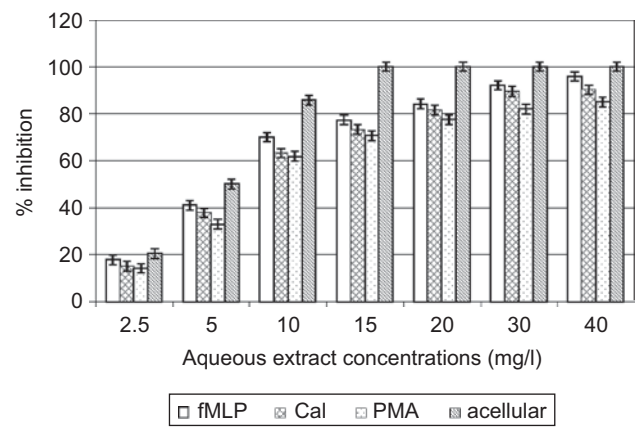

Figure 2. Effects of *A. cordifolia* aqueous extract on elastase.

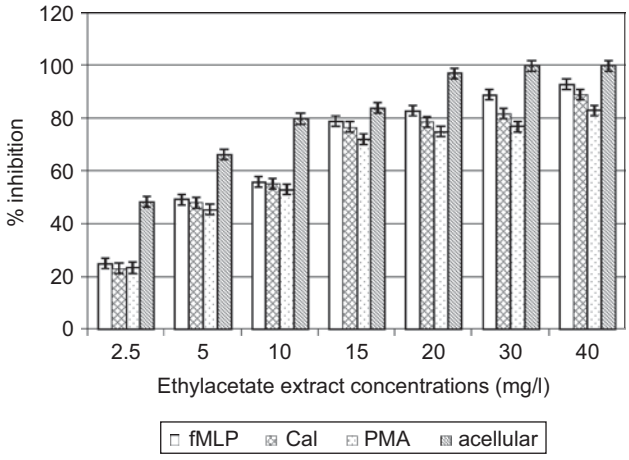

Figure 3. Effects of *A. cordifolia* ethyl acetate extract on superoxide anion.

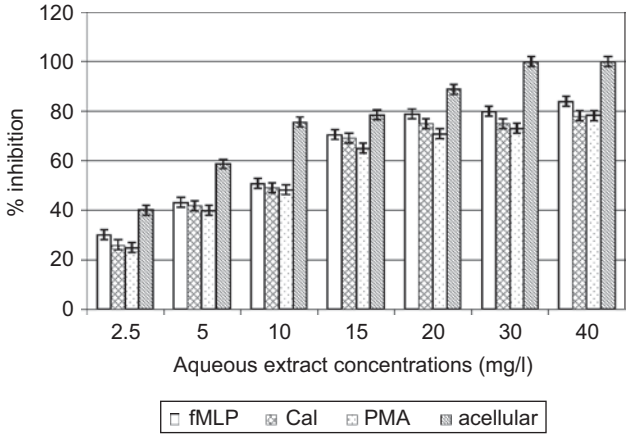

Figure 4. Effects of *A. cordifolia* aqueous extract on superoxide anion.

Structure of pure compounds

Four compounds were isolated from the *A. cordifolia* ethyl acetate extract. They were identified by MS and NMR spectroscopy and their spectral data compared with those of reference compounds. Three of them were flavonoids: one flavonol aglycone, quercetin (**1**), two myricetin glycosides, myricetin-3-glucopyranoside (**2**) and myricetin-3-rhamnopyranoside (**3**), and the fourth was a dimeric proanthocyanidin, proanthocyanidin A2 (**4**). These are all known compounds, but this is the first time that **2**, **3**, and **4** have been isolated from *A. cordifolia* leaves, **1** being previously identified in the leaves of this plant (Lamikanra et al., 1990; Ogungbamila & Samuelsson, 1990).

Discussion

The antioxidant and antielastase activities of *A. cordifolia* in acellular and cellular systems, with predominant activity in the acellular system, are of considerable interest. In the acellular system, *A. cordifolia* antagonizes HNE and $O_2^{\bullet-}$ added to the medium. It is therefore possible that *A. cordifolia* reacts directly with HNE and $O_2^{\bullet-}$, either through a chemical effect (scavenging effect) or by an inhibition of HNE and $O_2^{\bullet-}$ activities.

In the cellular system, *A. cordifolia* extracts present no cytotoxic activity on PMN. Three stimuli were used to detect the ability of the plant to interfere with the liberation of HNE and other oxidants. These stimuli enhanced the exocytosis of oxidants and HNE by neutrophils through different pathways. But in the cellular system with PMA-, fMLP-, or CaI-stimulated human neutrophils, the activity was no better. On the contrary, these extracts were more active in the cell-free system. This action may be explained by a scavenging effect on the oxygen species ($O_2^{\bullet-}$).

Comparing the aqueous and ethyl acetate extracts, the more active extract was the ethyl acetate extract, with higher contents of polyphenols and/or flavonoids. It is well known that there is a good correlation between antioxidant activity and total phenol level (Hatano et al., 1989).

Our results support the ability of *A. cordifolia* to reduce topical inflammation induced by croton oil on mouse ear (Mavar-Manga et al., 2004) and egg albumin on rat paw (Osadebe & Okoye, 2003), and these studies showed a concentration of flavonoids related to anti-inflammatory activity.

Phenolics, such as flavonoids, have been reported to be direct HNE and oxidant inhibitors (Blackburn et al., 1987; Jovanovic et al., 1994; Sartor et al., 2002). Compounds with a catecholic structural element, including two neighboring phenolic hydroxyl groups, showed remarkable activity which is significantly decreased by the methylation of one of the phenolic groups. Inhibitory activity may also be dependent on the double C-3/C-2 bond in the flavonoid C-ring, and on the additional presence of both 3- and 5-OH groups (Bombardelli & Morazzoni, 1993). The molecules identified in our study, quercetin, myricetin rhamnoside, and myricetin glucoside, are flavonoids with structures favorable to antioxidant and antielastase activities. Previous studies identified quercetin in the leaves of *A. cordifolia* (Lamikanra et al., 1990; Ogungbamila & Samuelsson, 1990), but this is the first to identify myricetin-3-glucopyranoside, myricetin-3-rhamnopyranoside, and proanthocyanidin A2 in *A. cordifolia*.

It is now considered worthwhile to screen plant extracts for elastase and oxidant inhibitors (Perry et al., 1999; Siedle et al., 2007). With such data, *A. cordifolia* leaves can be expected to have a protective effect against oxidants and elastase, acting through scavenging activity rather than inhibition of release.

These antioxidant and antielastase activities confirm traditional applications in topical inflammatory

diseases, in the treatment of rheumatism, arthritis, chronic bronchitis, and other respiratory ailments, and in the effective healing of wounds and ulcers. The presence of flavonoids and proanthocyanidin surely testifies to the antioxidant and antielastase properties of *A. cordifolia*.

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***Declaration of interest*:** The authors report no conflicts of interest.

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