Antioxidant, Anti-inflammatory, Analgesic Properties, and Phytochemical Characterization of Stem Bark Extract and Fractions of Anthocleista nobilis

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ABSTRACT
Background: Anthocleista nobilis (Loganiaceae) is used by Mbano people of Imo State, Nigeria, for the treatment of various ailments. Objective: The aim of this study is to evaluate the antioxidant, anti-inflammatory, and analgesic properties of the methanol extract, fractions, and subfractions of A. nobilis. Materials and Methods: The powdered stem bark was extracted with methanol and sequentially fractionated into n-hexane, ethyl acetate, and butanol fractions. The constituents of the fractions were analyzed using high-pressure liquid chromatography (HPLC), and the components were identified by dereplication. Antioxidant potential of the extracts and fractions was investigated using 2,2-diphenyl-1-picrylhydrazyl free-radical scavenging method. Anti-inflammatory and analgesic activities of the extract and fractions were also investigated using xylene-induced inflammation and acetic acid-induced writhing models, respectively. Results: A total of five compounds were identified: vitexin, isovitexin, isoquercitrin, eriptol, and apigenin monoglycoside. Conclusion: A. nobilis could be a potential source of anti-inflammatory and analgesic lead compounds. Key words: Analgesic, Anti-inflammatory, Antioxidant, high-pressure liquid chromatography analysis

INTRODUCTION
Oxidative stress plays a significant role in the pathogenesis of several human diseases including inflammatory conditions, neurological disorders, cardiovascular complications, and cancers among other ailments. Management of pains and inflammatory-mediated diseases with conventional therapeutic agents has not always produced the most desired result because of the numerous adverse effects such as gastric erosion and liver toxicity associated with some of the agents. Following the limitations posed by existing and mostly synthetic agents, the search for alternative therapies from natural sources including medicinal plants has received greater attention recently. Historically, medicinal plants have played significant role in the management of numerous disease conditions, especially in the rural areas, due to their availability and affordability. Interestingly, good numbers of modern drugs were derived from natural sources, mostly based on their ethnomedical relevance.
found in tropical African habitats such as the Mascarene Islands and Madagascar as well as Southern, Western, and Eastern part of Nigeria. The bark is smooth and pale grey. The inner bark is cream-yellow and granular, whereas the twig has 2 spines above the leaf axis. The leaves are opposite, crowded at the end of branches, and petiole is 1–6 cm long.[14] It is commonly called candelaurum, cabbage tree, cabbage palm, or palma chrísti in English language. It is also locally known as Uko nkiri in Igbo language. Conventionally, A. nobilis is used in the treatment of fever, stomach ache, diarrhea, and gonorrhea. It is also used as strong purgative, diuretic, and as poultice for treating sores in parts of West Africa.[15] It is used as vapor bath for the treatment of leprosy, veneral diseases, and dysmenorrheal. Its root decoction is usually taken to regulate menstruation and also as an abortifacient. In Mbanio community in Imo State, Nigeria, the root bark decoctions are mostly used in the treatment of diabetes mellitus, gastrointestinal worms, malaria, jaundice, and pruritis.[3] The studies had shown that the root bark of A. nobilis possessed anti-diabetic activity,[16] antiviral and anti-plasmodial activities,[6,7] anti-Leishmanial activity,[16] antibacterial, antioxidant activity, and wound healing properties.[15] Recent studies has shown that its acetone and methanol stem bark extracts possess moderate free-radical scavenging activities.[18] Phytochemical analyses also revealed the presence of polyphenols, flavonoids, tannins, and triterpenoids, coumarins and saponosides.[10,11] The present study was designed to evaluate the anti-inflammatory and analgesic properties of methanol extract, fractions, and ethyl acetate chromatographic fractions of A. nobilis which to the best of our knowledge has not been previously reported. We also report for the first time the identification of Vitexin and it is derivatives in the stem bark of A. nobilis.

MATERIALS AND METHODS

Chemicals and reagents

Analytical grade methanol, n-hexane, ethyl acetate, and n-Butanol were obtained from JHD, Shantou, Guangdong, China), 2,2-diphenyl-1-picrylhydrazyl (DPPH), xylene, and ascorbic acid were obtained from Sigma-Aldrich, Germany. Indomethacin and Diclofenac sodium were obtained from Hovid (Bhd) Pharmaceuticals, Malaysia. All laboratory reagents were freshly prepared when required.

Plant collection and authentication

Stem barks of A. nobilis were collected from Ezza Community in Ebonyi State Nigeria, in March, 2014. They were identified and authenticated by Mrs. Emezie A, a plant taxonomist in the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria. A voucher specimen PHC0098 was deposited in the herbarium of the same Department for reference purposes.

Experimental animals

Swiss Albino mice (25–30 g) were obtained from the animal house of the Department of Pharmacology, Nnamdi Azikiwe University, Agulu. The animals were housed in standard laboratory conditions of 12 h light, room temperature, and 40%–60% relative humidity. They were allowed free access to food (Guinea feeds Nigeria Ltd) and water ad libitum. All animal experiments were conducted in compliance with NIH guide for care and use of laboratory animals (National Institute of Health (2011) pub No: 85-23).

Preparation of plant extract

Air-dried stem bark of A. nobilis ground coarse consistency using a locally fabricated mechanical grinder. Exactly 900 g of the pulverized stem bark was macerated in methanol with intermittent shaking for 72 h. The extract was removed every 12 h and fresh solvent introduced. At the end of 72 h, the extracts were pooled together, strained through muslin cloth, and filtered with filter paper (Whatman No. 1). The filtrate was evaporated to dryness using rotary evaporator (RE300 Model, United Kingdom) at 40°C. The extract was stored at 4°C until when required.

Liquid-liquid partitioning

The methanol extract of A. nobilis was sequentially portioned n-hexane, ethyl acetate, and butanol. All the fractions obtained were filtered using Whatman No. 1 filter paper and were concentrated with a rotary evaporator at 40°C. The fractions obtained were stored at 4°C. The fractions were all subjected to bioactivity testing and high-performance liquid chromatography (HPLC) analysis. Based on the result of the bioactivity testing of the fractions, ethyl acetate fraction (1.39 g) was further fractionation using vacuum liquid chromatography (VLC).

Vacuum liquid chromatographic separation of ethyl acetate fraction

The ethyl acetate fraction (1.39 g) mixed with 20 g of silica gel (200–400 mesh) was loaded on top of the glass column already packed with silica gel (mesh size, 200–400). The sample was gradually eluted with mobile phase in increasing order of polarity from nonpolar to polar solvents. Different fractions were collected and concentrated using rotary evaporator.

Analytical high-performance liquid chromatography analysis

Each of the dried crude extract and fractions (2 mg) was dissolved 2 mL of HPLC grade methanol and the mixture was centrifuged at 3000 rpm for 5 min. Then, 100 μL of the dissolved samples was transferred into HPLC vials containing 500 μL of HPLC grade methanol.

HPLC analysis was conducted on the samples with a Dionex P580 HPLC system coupled to a photodiode array detector. Detection was at 235, 254, 280, and 340 nm. The separation column (125 mm × 4 mm; length × internal diameter) was prefilled with Eurosphere C-18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as the eluent. Compounds were detected using diode array and identified based on similarity with data in the inbuilt library.

2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

The DPPH free-radical scavenging activities of the extract, fractions, and ethyl acetate chromatographic fractions of A. nobilis was evaluated by the method described by Patel and Patel[12] with some modifications. Half milliliter (0.5 mL) of DPPH solution (0.6 mM) was added to 0.5 mL of different concentrations of the extract and fractions (15, 63, 31.25, 62.5, 125, 250, 500, and 1000 μg/mL). The volume of the solution was adjusted with methanol to a final volume of 5 mL. The mixture was incubated in the dark for 30 min at room temperature and absorbance of the mixtures was obtained at 517 nm using VIS spectrophotometer (Model 752, China). All the tests were performed in duplicate and ascorbic acid was used as standard. The absorbance of the control (containing 0.5 mM of DPPH solution and 4.5 mL of methanol) was used to calculate the free radical scavenging activities. The percentage radical scavenging potentials of the extracts, fractions and standard (ascorbic acid) were calculated using the equation below.

Percentage of inhibition of free radical = \[
\left(\frac{A_b - A_i}{A_b}\right) \times 100
\]
Where $A_0$ is the absorbance of the control, $A_t$ is the absorbance of the test/standard. The 50% inhibitory concentration ($IC_{50}$) was determined from a plot of percentage scavenging potentials against concentration.

**Anti-inflammatory activity of extracts and fractions**

The effect of the extract on xylene-induced acute topical inflammation was evaluated with modification of the method adopted by Atta and Alkohafi.[13] Adult Swiss albino mice (5 per group) were used for the study. The extract and fraction treatment groups received 250 and 500 μg/anterior surface of the right ear. Negative control group received the vehicle, 50 μL of 5% Tween-80, whereas the positive control group received 250 and 500 μg of Indomethacin/anterior surface of the right ear. Immediately after treatment, topical inflammation was induced by application of 50 μL of xylene on the posterior surface of the same ear. Two hours after induction of inflammation, the mice were sacrificed by cervical dislocation and sections of both (right and left) ears were punched out with the aid of circular cork borer (4 mm diameter) and were weighed with the aid of analytical weighing balance (Ohaus, China). Edema was quantified as the weight difference between the two earplugs. The anti-inflammatory activity was evaluated as percentage edema reduction/inhibition in the treated animals relative to control animals[16] using the relation:

\[
\text{Percentage edema reduction (\%)} = 1 - \frac{(\text{RET} - \text{LET} / \text{REC} - \text{LEC})}{100}
\]

where,

- RET = Mean weight of right earplug of treated animals
- LET = Mean weight of left earplug of treated animals
- REC = Mean weight of right earplug of control animals
- LEC = Mean weight of left earplug of control animals.

**Anti-inflammatory activity of ethyl acetate chromatographic fractions**

Similar method and procedure above were used in the screening of the anti-inflammatory activity of various ethyl acetate chromatographic fractions from ethyl acetate main fractions.

**Analgesic activity on extract, ethyl acetate fraction**

This study was carried out using acetic acid-induced abdominal writhing reflex pain model as described by Smahane et al.[15] with some modifications. Adult mice (25–30 g) were fasted 12 h before the experiment and were randomly divided into 4 groups of 4 mice each, and treated as follows, group 1 (negative control group) received 10 mL/kg normal saline, group 2 and 3 (positive control groups) received 100 and 200 mg/kg of diclofenac sodium; groups 4 and 5 received 100 and 200 mg/kg of *A. nobilis* methanol extract, whereas group 6 and 7 received 100 and 200 mg/kg of ethyl acetate fraction of *A. nobilis* using gastric gavages. One hour after administration, 10 mL/kg of 0.6% glacial acetic acid was administered intraperitoneally (L.P) to all the mice to induce pain. The pain responses by animals characterized by abdominal writhing was counted were recorded and analgesic activity was calculated using the following equation:

\[
\text{Analgesic activity} = \frac{\text{Abdominal writhing in control} - \text{Abdominal writhing in test group}}{\text{Abdominal writhing in control}} \times 100
\]

**Statistical analysis**

Results were presented as mean ± standard error of mean. Statistical comparisons among and between group means was performed using one-way analysis of variance followed by post hoc Turkey’s test for multiple analyses using Statistical Package for the Social Sciences (SPSS-20, IBM corporation, USA). P values ($P < 0.05$) were considered to be statistically significant. $IC_{50}$ of the extracts and fractions were calculated from the graph of regression equation using Microsoft Excel, 2010.

**RESULTS**

**High-performance liquid chromatography identification of compounds from extract and fractions of Anthocleista nobilis**

The HPLC chromatogram of the methanol extract [Figure 1] revealed the presence of isovitexin ($R_t = 18.77$ min) and isovitexin-2''-O-xyl ($R_t = 19.68$ min. N-hexane fraction [Figure 2] revealed the presence of p-Hydroxybenzoic acid ($R_t = 11.88$ min) and Sarasinside L ($R_t = 19.64$ min). Ethyl acetate fraction [Figure 3] revealed isovitexin ($R_t = 18.77$), whereas butanol fraction [Figure 4] revealed the presence of apigenin monoglycoside ($R_t = 19.64$ min).

**Antioxidant result of crude extract and various fractions**

All the samples tested showed radical scavenging activity against DPPH. Methanol extract (AC1), n-hexane fraction (AC2), ethyl acetate fraction (AC3), butanol fraction (AC4) manifested antioxidant activities with $IC_{50}$ values of 528, 570.5, 220, and 529.4 μg/mL, respectively. Ethyl acetate fraction showed moderate antioxidant activity compared to the other fraction. However, when compared with the standard ascorbic acid ($IC_{50}$, 23.39 μg/mL), the antioxidant activity of ethyl acetate fraction was about ten-fold less. The order of scavenging activity of the test samples was AC3 > AC1 > AC4 > AC2 [Table 1].

**Antioxidant result of ethyl acetate chromatographic fraction**

From Table 2, AV2 showed the highest antioxidant activity ($IC_{50}$ 220 μg/mL) after ascorbic acid ($IC_{50}$ 60 μg/mL) while AV1 showed the least activity with ($IC_{50}$ 1564.1 μg/mL). The order of...
the scavenging activity of the ethyl acetate subfraction include AV2 > AV7 > AV9 > AV5 > AV10 > AV8 > AV1 [Table 2].

Effect of methanol extract and fractions on xylene-induced topical inflammation
The results presented in Table 3 demonstrate that the methanol extract and fractions exhibited significant ($P < 0.05$) anti-inflammatory activity in a nondose-dependent manner. Ethyl acetate fraction showed the highest activity, and at doses of 250 and 500 μg/ear decreased the ear edema rate at 83.49 and 68.40%, respectively, similar to indomethacin. The ethyl acetate, n-hexane, and butanol fractions had better activity at a lower dose of 250 μg/ear compared to 500 μg/ear.

Anti-inflammatory activity of ethyl acetate chromatographic fractions of *Anthocleista nobilis*
The results presented in Table 4 showed that ethyl acetate chromatographic fraction elicited a significant ($P < 0.05$) anti-inflammatory activity. Subfraction AV9 showed the highest activity by decreasing ear edema by 55.03% and 48.52% at doses of 250 μg/ear and 500 μg/ear respectively [Table 4].

Effects of methanol extract and ethyl acetate fraction of *Anthocleista nobilis* on acetic acid-induced writhing reflex of on mice
The oral administration of 100 mg/kg and 200 mg/kg of methanol extract significantly ($P < 0.05$) inhibited writhing response induced by acetic acid, giving a percentage inhibition of 82.4% and 75.7%, respectively, as compared with the negative control [Table 5]. Significant ($P < 0.05$) inhibition was also recorded with the ethyl acetate fraction. At the same doses, the activities of the extract and ethyl acetate fractions were comparable to diclofenac sodium.

**DISCUSSION**
Oxidative stress, inflammation, and pain are closely associated processes that can be simultaneously present in many pathological conditions. [54]
Substances effective against these conditions may mediate their effect through pathophysiological processes common among them. Findings from this experiment revealed that *A. nobilis* showed activity against free radicals, inflammation, and pain. The HPLC chromatogram of the extract and fractions of *A. nobilis* stem bark showed the abundance of Vitexin (a glycoside of the flavones class) and its derivatives. Apigenin-6-C-β-D-glucoside (isovitexin) has previously been isolated from *Polygonatum odoratum* (Mill) [17] and has also been reported by Qiulan et al. [18] as an efficient antioxidant. A research carried out by Liu and Jan [19] revealed that isovitexin protected DNA from the Fenton reaction-induced breakage in a dose-dependent manner. Isovitexin has also been documented to have protected HL-60 cells from the ROS damage induced by the xanthine/xanthine oxidase reaction. [20] Its radioprotective effect and antihyperglycemic activity have been reported. [21,22] Isovitexin and isovitexin 2'-O-xyl compounds identified in this study may have contributed to the antioxidant and anti-inflammatory activities of *A. nobilis*.

### Table 3: Effect of methanol extract and fractions of Anthocleista nobilis on xylene-induced topical inflammation

| Treatment            | Dose (µg/ear) | Mean left ear (mg) | Mean right ear (mg) | Mean edema (mg) | Inhibition (%) |
|----------------------|--------------|--------------------|---------------------|----------------|---------------|
| Methanol extract     | 250          | 0.40±0.02          | 0.77±0.09           | 0.37±0.09      | 11.32         |
|                      | 500          | 0.53±0.03          | 0.80±0.14           | 0.27±0.11      | 15.09         |
| n-hexane fraction    | 250          | 0.39±0.02          | 0.68±0.11           | 0.29±0.09      | 32.55         |
|                      | 500          | 0.43±0.02          | 0.74±0.08           | 0.31±0.08      | 30.19         |
| Ethyl acetate fraction | 250        | 0.40±0.02          | 0.50±0.04           | 0.10±0.04      | 83.49         |
|                      | 500          | 0.41±0.02          | 0.54±0.05           | 0.13±0.05      | 68.4          |
| Butanol fraction     | 250          | 0.45±0.01          | 0.68±0.07           | 0.23±0.07      | 44.34         |
|                      | 500          | 0.45±0.01          | 0.69±0.12           | 0.24±0.12      | 40.09         |
| Aqueous fraction     | 250          | 0.41±0.01          | 0.65±0.09           | 0.24±0.08      | 43.34         |
|                      | 500          | 0.42±0.01          | 0.65±0.07           | 0.23±0.08      | 45.28         |
| Indomethacin         | 250          | 0.46±0.02          | 0.52±0.03           | 0.06±0.02      | 85.85         |
|                      | 500          | 0.41±0.01          | 0.46±0.01           | 0.05±0.01      | 89.15         |

n=5; *P<0.05 compared with 5% tween-80 (vehicle)-treated group

### Table 4: Effect of ethyl acetate chromatographic fraction of Anthocleista nobilis on xylene-induced topical inflammation

| Treatment | Dose (µg/ear) | Mean left ear (mg) | Mean right ear (mg) | Mean edema (mg) | Inhibition (%) |
|-----------|--------------|--------------------|---------------------|----------------|---------------|
| AV1       | 250          | 0.58±0.02          | 0.91±0.06           | 0.33±0.06      | 22.49         |
|           | 500          | 0.66±0.04          | 0.96±0.02           | 0.31±0.04      | 27.81         |
| AV5       | 250          | 0.53±0.04          | 0.69±0.07           | 0.16±0.07      | 63.91         |
|           | 500          | 0.53±0.05          | 0.81±0.14           | 0.28±0.10      | 33.14         |
| AV7       | 250          | 0.69±0.08          | 1.00±0.07           | 0.31±0.08      | 26.63         |
|           | 500          | 0.72±0.12          | 1.06±0.11           | 0.34±0.03      | 21.31         |
| AV8       | 250          | 0.52±0.04          | 0.76±0.08           | 0.24±0.06      | 44.97         |
|           | 500          | 0.60±0.05          | 0.88±0.05           | 0.28±0.09      | 33.14         |
| AV9       | 250          | 0.63±0.03          | 0.81±0.09           | 0.18±0.07      | 55.03         |
|           | 500          | 0.54±0.04          | 0.75±0.08           | 0.21±0.07      | 48.52         |
| AV10      | 250          | 0.48±0.04          | 0.80±0.06           | 0.32±0.05      | 25.55         |
|           | 500          | 0.40±0.00          | 0.94±0.02           | 0.54±0.02      | 15.38         |
| Indomethacin | 250     | 0.47±0.02          | 0.53±0.04           | 0.05±0.02      | 87.62         |
|           | 500          | 0.42±0.01          | 0.46±0.01           | 0.04±0.01      | 89.94         |

AV1 (n=5); *P<0.05 compared with 5% tween 80 (vehicle) treatment

### Table 5: Effects of methanol extract and ethyl acetate fraction of Anthocleista nobilis on acetic acid-induced writhing reflex of on mice

| Treatment            | Dose (mg/kg) | Mean writhing reflex±SEM | Inhibition (%) |
|----------------------|--------------|--------------------------|---------------|
| Methanol extract     | 100          | 10.50±4.09*              | 82.4          |
|                      | 200          | 8.25±2.36*               | 77.5          |
| Ethyl acetate fraction | 100        | 10.75±3.12*              | 73.8          |
|                      | 200          | 12.25±4.39*              | 77            |
| Diclofenac sodium    | 100          | 13.50±4.13*              | 81.3          |
|                      | 200          | 8.75±3.47*               | 71.1          |
| 5% tween 80          | 5 mL/kg      | 46.75±10.44*             | 85.0          |

n=5; *P<0.05 compared with 5% Tween-80 (vehicle-treated control) group. SEM: Standard error of mean

Figure 4: High-pressure liquid chromatography chromatogram of butanol fraction and ultraviolet chromatogram of apigenin monoglycoside. H: Apigenin monoglycoside (Rt = 19.64)
P-hydroxybenzoic acid identified from the n-hexane fraction is a phenolic derivative of benzoic acid. Several biological activities of this compound have been reported. Merkl et al.[25] reported that phenolic acid such as p-Hydroxybenzoic acid possesses good antioxidant activity. p-Hydroxybenzoic acid has also been known for its anti-inflammatory activity as reported by Luecha et al.[26] This compound may have also contributed to the analgesic, anti-inflammatory, and antioxidant activities exhibited by *A. nobilis*. The HPLC chromatogram of the ethyl acetate fraction revealed the presence of unidentified compounds (Peak 4 Rt. 14.29 min). From its intensity and peak area, it is clear that peak 4 is the major compound in the extract. The ethyl acetate fraction and its subfractions showed appreciable activity in all the tests carried out. Comparing the intensities of peak 4 in n-hexane and ethyl acetate fractions, the intensity of this peak is greater in ethyl acetate fraction (2000 mAU) than n-hexane fraction (1400 mAU). This suggests that peak 4 could have contributed to why ethyl acetate fraction gave the best activities among the fractions. Dereplication analysis identified peak 4 as triterpene acetate with hit of 981 which is below 990, the acceptable minimum. Further purification and spectroscopic experiment would be required to elucidate the structure and identity of peak 4. DPPH, a stable free radical, can accept an electron or hydrogen to become a stable molecule, and it can also be used as a substrate to evaluate the antioxidant activity of compounds.[25,26] The moderate antioxidant activity of ethyl acetate could be attributed to the presence of flavonoid especially isovitexin. Polyphenolic compounds, such as flavonoid and phenolic acids, which are mostly found in plants have been reported to have biological activities, including antioxidant activity.[23] Earlier report by Ngwoke et al.[10] also indicated that *A. nobilis* is rich in flavonoids, terpenoids, and tannins. Interestingly, the components of the crude extract and fractions of *A. nobilis* as revealed by HPLC chromatogram had been reported for their antioxidant activities as earlier stated.

Xylene, a phlogistic agent is a useful tool in the assessment of acute inflammation. Topical application of xylene causes irritation of the living tissues, thereby leading to fluid accumulation and edema. Suppression of xylene-induced topical edema by the extract and fractions is an indication that they possess anti-inflammatory activity. This activity was, however, not exhibited in a dose-dependent manner. Better activity shown at lower dose could be attributed to reduced solubility or poor permeability at higher concentration. Previous studies have also revealed that flavonoid, phenolic compounds, isovitexin, isovitexin 2’-O-xyly, apigenin glycoside, and p-Hydroxybenzoic acid possess anti-inflammatory activity due to their inhibitory effects against mediators of inflammation.[24,25] The presence of these compounds could account for the anti-inflammatory activity of *A. nobilis*. Acetic acid-induced pain is an experimental model of evaluating peripherally acting analgesics. The pain response is thought to be mediated by peritoneal mast cells and the prostaglandin pathways.[25] The organic acid has also been known to act indirectly by inducing the release of endogenous mediators, which stimulates the nociceptive neurons that are sensitive to analgesics.[26] Compounds such as flavonoids and steroids have been shown to possess anti-inflammatory and analgesic activity as reported by Pritam et al.[30] The potent analgesic activity of *A. nobilis* may be derived from its anti-inflammatory properties since the reduction in acute inflammation also mediates reduction in pain.[31]

### CONCLUSION

This study had revealed that crude extract, ethyl acetate fraction, and subfraction from the stem bark of *A. nobilis* showed potent anti-inflammatory and analgesic activities that could be attributed to the active compounds as revealed by the HPLC analysis. Outstandingly, the dose-dependent and potent inhibition of the acetic acid-induced writhing which was better than the inhibition observed for the positive control suggests that the extract has great potential for use as an analgesic and anti-inflammatory agent.

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

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