



Anti-inflammatory Effects and Acute Toxicity of Methanol Stem Bark Extract of *Morus mesozygia* Stapf.

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Authors' contributions

This work was carried out in collaboration between all authors. Author AMA designed the study, wrote the protocol and wrote the first draft of the manuscript. Author KOO identified the species of plant. Authors KOO, OAO and AMA managed the literature searches and performed the experimental process. All authors read and approved the final manuscript.

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ABSTRACT

Aim: *Morus mesozygia* is a specie of the *Morus* family in Africa that is traditionally used for the treatment of rheumatism. Until this moment, there is no available literature on the scientific evidence of the in vivo anti-inflammatory effects. The aim is to determine the safety of this extract after acute exposure and evaluate the anti-inflammatory effects *in vitro* and *in vivo*.

Methodology: The phytochemical screening of the powdered stem bark was determined following standard procedures. Extraction in methanol yielded a brownish extract, which was subjected to total phenolic and flavonoid contents estimation. Antioxidant activity was determined by DPPH free radical scavenging assay. Acute oral toxicity effects was evaluated in female mice and signs of

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toxicity was observed for 14 days. Anti-inflammatory activity was evaluated using the carrageenan-induced paw oedema and in vitro membrane stabilizing activity assay in rat erythrocytes.

Results: Preliminary phytochemical analysis revealed the presence of flavonoids, saponins, tannins, alkaloids, anthraquinones, and cardiac glycosides. Ratio of total phenolic to total flavonoid content was determined to be 0.53. MEMm showed strong antioxidant activity comparable to ascorbic acid in DPPH free radical scavenging activity assay. No signs of acute toxicity was observed indicating the LD₅₀ is greater than 5000 mg/kg. MEMm (200 and 400 mg/kg) significantly ($p < 0.05$) reduced oedema formation in rat paw, and showed concentration dependent membrane stabilizing activities in rat erythrocytes.

Conclusion: The present study shows that stem bark methanol extract of *Morus mesozygia* is safe and contains bioactive constituents with antioxidant, anti-inflammatory and membrane stabilizing properties.

Keywords: Safety; phytochemicals; antioxidants; membrane stabilizing.

1. INTRODUCTION

Inflammatory diseases such as rheumatoid arthritis continued to be longstanding medical problem and a major cause of morbidity throughout the world. Rheumatoid arthritis is an incurable disease and life - long therapy is required [1]. Anti-inflammatory herbal medicine and its constituents are being proven to be a potent protector against various pro-inflammatory mediators in both acute and chronic inflammations [2]. There is a growing patronage of open herbs markets for herbs used in the treatment of rheumatic disorder and other inflammatory conditions [3]. Among the active constituents of plant extracts, flavonoids are a family of substances whose members have many interesting biological properties including anticancer, antimicrobial, antiviral, anti-inflammatory, immunomodulatory, and antithrombotic activities. Many investigations have shown that a variety of flavonoid molecules exhibit anti-inflammatory activity both, in vitro and in various animal models of inflammation [4].

Morus mesozygia Stapf., is an endemic species of Morus family (Moraceae) that grows in tropical Africa. It is a small to medium sized forest tree, its leaves and fruits are sources of food for the Monkey and Chimpanzee of West and Central African forests [5]. Morus species have several medicinal activities and have been the subject of many phytochemical and pharmacological studies. *Morus alba* leaves are dried and used in infusion in many Asian countries and particularly in China [6,7]. According to folk medicine, the stem bark, leaves, and/or roots of *M. mesozygia* are prepared as decoctions and used against inflammatory conditions. The aqueous maceration of the stem bark has been cited to be

traditionally useful in different part of Africa in the treatment of arthritis [8], stomach disorders and ulcers [9]; pain [10], and malarial [11].

The stem bark of *M. mesozygia* is reported to be frequently sold in popular herbal markets in Abeokuta South West Nigeria [12]. Phytochemical investigation of the stem bark methanol extract has led to isolation of arylbenzofuran and flavanones with anti-oxidant and anti-microbial properties [13]. Dzoyem and Eloff [14] had recently reported the antioxidant, anticholinesterase and in vitro anti-inflammatory activity of the acetone leaf extract of South African *M. mesozygia*. In spite of extensive searching, no literature has demonstrated the *in vivo* anti-inflammatory activity of the stem bark extract of *M. mesozygia* extract. The present study therefore report our preliminary findings on the anti-inflammatory activity of the methanol extract of *M. mesozygia* stem bark.

2. MATERIALS AND METHODS

2.1 Plant Collection and Extract Preparation

The stem bark of *Morus mesozygia* (family Moraceae) was collected from Ibadan, Oyo State in December 2013. The plant was authenticated by Plant Taxonomist at the Forest Herbarium, by comparing with existing herbarium voucher specimen FHI 107677. The air dried powdered stem bark (1 kg) was soaked in 80% methanol for 72 hours with occasional shaking. The extract was concentrated under reduced pressure at 40°C using a rotary evaporator and dried in the oven at 40°C to obtain a brown solid powder with percentage yield of 5.6%. A freshly prepared solution in distilled water was used for pharmacological studies.

2.2 Phytochemical Screening

The powdered samples of the stem bark of *Morus mesozygia* was screened for the following secondary metabolites using standard methods [15,16]. Secondary metabolites tested includes; tannins, saponins, anthraquinones, alkaloids, cardiac glycosides, flavonoids, steroids and cyanogenetic glycosides.

2.3 Estimation of Total Phenolic Content

Total phenolic content (TPC) in MEMm was estimated using the spectrophotometric method with Folin Ciocalteu reagent [17]. The reaction mixture was prepared by mixing 0.1 mL of MEMm (1 mg/mL) with 0.2 mL of Folin Ciocalteu reagent (FCR), after which 2 ml of 7.5% Sodium carbonate was added, and 2.7 ml of distilled water and then allowed to stand for 2 hours. The absorbance was read at 725 nm using a UV-VIS spectrophotometer. The analysis was carried out in triplicate. Gallic acid was used as standard for the calibration curve ($0.001x - 0.02$; $R^2 = 0.991$). The TPC was expressed as mg Gallic Acid Equivalents (GAE) per gram of sample.

2.4 Estimation of Total Flavonoid Content

Estimation of total flavonoid content (TFC) in MEMm was carried out by the aluminium chloride spectrophotometric method as described by Suntana et al. [18]. 1 mL of MEMm (1 mg/mL) was mixed with 0.3 mL of 10% (w/v) NaNO_2 for 5 minutes, 0.3 mL of 10% (w/v) AlCl_3 was added and left for 1 minute to react. Thereafter, 2 mL of 1 M NaOH was added and the mixtures shaken. The absorbance of the mixture was read at 510 nm using a UV-VIS spectrophotometer. The analysis was carried out in triplicate. Rutin was used as a standard for the Calibration curve ($y = 0.001x + 0.002$; $R^2 = 0.993$). The TFC was expressed as mg Rutin Equivalents (RE) per gram of sample.

2.5 Free Radical Scavenging Activity Assay

The electron donating ability of MEMm was measured by bleaching in a purple solution of 1,1-Diphenyl-2-picrylhydrazyl (DPPH). 1 mL MEMm (25 - 400 $\mu\text{g/mL}$) and Ascorbic acid (25 - 400 $\mu\text{g/mL}$) in methanol was prepared in triplicates, then 2 mL DPPH (0.1 mM) was added and the mixture was incubated for 30 min at room temperature in a dark cupboard. The

absorbance was read at 517 nm in a spectrophotometer. The percentage free radical scavenging activity was calculated using the following equation:

$$\% \text{ inhibition} = \frac{[(\text{absorbance of control} - \text{absorbance of test sample}) / \text{absorbance of control}] \times 100\%}{}$$

The antiradical activity of MEMm and ascorbic acid was expressed in terms of EC_{50} (micromolar concentration required to inhibit DPPH radical formation by 50%), which was estimated from the non-linear regression curve using version 5 GraphPad prism® [19].

2.6 Experimental Animals

Wistar rats weighing 150 to 200 g and Swiss albino mice weighing 20 to 25 g were used throughout this study. The animals were housed in cages maintained under standard conditions and fed with standard pellets (Vital Feeds Ltd, Ibadan, Nigeria) and received water *ad libitum*. All experiments were carried out with strict compliance to The "Principle of Laboratory Animal Care" (NIH Publication No. 85-23) and ethical guidelines for investigation of experimental pain in conscious animals [20,21].

2.7 Acute Toxicity Test

The acute toxicity of MEMm was determined in female mice according to the method of Lorke [22] with slight modification using the oral route. In phase 1, twelve mice which were fasted for 12 hours were randomly divided into four groups of three mice per group. Three groups were administered MEMm (10, 100, 1000 mg/kg) and the fourth group received vehicle (10 mL/kg) body weight. The mice were observed hourly for behavioral alterations or death in a Perspex chamber for duration of 24 hours. In Phase 2, three mice each received 1600, 2900 and 5000 mg/kg body weight. The mice were observed for signs of adverse effects and death for 24 h and then weighed daily for 14 days. The geometric mean of the least dose that killed mice and the highest dose that did not kill mice was taken as the median lethal dose.

2.8 Carrageenan – Induced Rat Paw Oedema

Inflammatory oedema in female wistar rats (weighing 150 – 200 g) was produced by

injecting carrageenan according to the method described by Winter et al. [23]. Rats were divided into four groups (n=5) and pretreated with vehicle (10 mL/kg distilled water), MEMm (200, and 400 mg/kg) and indomethacin (10 mg/kg). One hour after pretreatment, 0.1 ml of 1% carrageenan was injected into the right hind foot of each rat under the subplantar aponeurosis. Paw volumes were measured before carrageenan injection and hourly for five hours after carrageenan injection with UgoBasile (7134) digital plethysmometer. The increase in paw volume was calculated as percentage and plotted against time (hour). The area under the curve (AUC) for percentage increase against time was computed from 0 to 5 hours using GraphPad Prism version 5. The percentage of inhibition of total oedema formation was calculated with the formula below

$$\% \text{ inhibition} = [(AUC \text{ of control} - AUC \text{ of treatment}) / AUC \text{ of control}] \times 100$$

2.9 Anti-inflammatory Effect by Membrane Stabilizing Property Assay

In vitro anti-inflammatory effect by membrane stabilizing activity as previously described by Sadique et al. [24] using 10% red blood cell suspension. Blood was collected into alsever solution by cardiac puncture from ether anaesthetized male rats. The Red blood cells was obtained by repeated washing in isotonic phosphate buffer solution (154 mM NaCl in 10 mM Sodium phosphate buffer, pH 7.4) The RBC was re-suspended to 10% (v/v) in the isotonic buffer. MEMm (10, 5 and 2.5 mg/mL) and indomethacin (2.5 mg/ml) were prepared in isotonic solution (154 mM NaCl). The assay mixture consisted of 2 mL hyposaline (50 mM NaCl in 10 mM Sodium phosphate buffer, pH 7.4), 1 mL of MEMm (10, 5 and 2.5 mg/mL in isotonic buffer) and Indomethacin (2.5 mg/mL), 0.3 mL RBC suspension (10% v/v) and made up to 5 mL with isotonic buffer. The reaction mixture (final concentration, 2, 1 and 0.5 mg/mL) was incubated at 56°C for 30 minutes in a water bath. Absorbance of supernatant was read at 560 nm using a UV/VIS Spectrophotometer (INESA). The percentage membrane stability was estimated using the expression:

$$\% \text{ Membrane Stability} = C - T / C * 100$$

Where, C - Absorbance of Control, T - Absorbance of Test Sample

2.10 Data Analysis

Data were reported as mean \pm standard error of the mean, and statistical significance was taken for $p < 0.05$. Statistical analysis was done using one-way analysis of variance (ANOVA), significant main effects were further analyzed by *post hoc* test using Bonferroni's multiple comparison test to compare the treatment groups (for 1-way ANOVA). Graphs and statistical analysis were done using Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA) and Graph pad prism (version 5).

3. RESULTS AND DISCUSSION

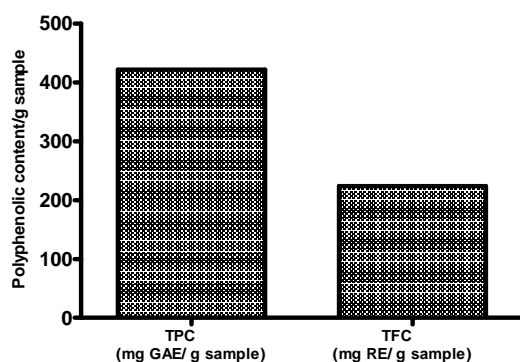
Preliminary screening for the presence of secondary metabolites in plant extract is an important step for quality control and standardization. Preliminary phytochemical screening result showed the presence of tannins, flavonoids, saponins, alkaloids, cardiac glycosides and anthraquinones but absence of cyanogenetic glycoside and sterols (Table 1). The phytochemical profile of *Morus mesogygia* stem bark is similar with what has been reported in *Morus* species [25,26]. Kapche et al. [13] has reported the isolation of prenylated arylbenzofurans derivatives and flavonoids in the methanol extract. Flavonoids, and tannins present in the extract may be responsible for some of the anti-inflammatory activities demonstrated by the extract.

Spectrophotometric estimation of the total phenolic and total flavonoid content revealed that the methanol extract of *M. mesozygia* stem bark contain total phenolic (422.0 mgGAE/g sample) and flavonoid content (224.0 mgRE/ g sample). The flavonoid to phenolic ratio is 0.53, implying about half of the total phenol are flavonoids. This ratio showed the suitability of extraction with methanol, it is more efficient in extraction of lower molecular weights polyphenols [27,28]. Dzoyem and Eloff [14] had earlier reported flavonoid/phenolic ratio of 0.07 in an acetone extract. Plant polyphenols are important secondary metabolites that are generally involved in defense against ultraviolet radiation or aggression by pathogens. They have drawn increasing attention due to their potent antioxidant properties, owing to their redox potential in absorbing and neutralizing free radicals. They are considered most important chemopreventive agents in prevention of various oxidative stress-associated diseases such as cancer, rheumatism, neurodegenerative diseases [27].

Table 1. Preliminary phytochemical screening of the stem bark of *Morus mesozygia*

Compound classes	Test	Observation	Inference
Tannins	Filtrate + 0.1% ferric chloride	Emerald-green coloration	+++
	Filtrate + few drops of lead acetate	Yellow precipitate	+++
Saponins	Frothing test	Frothing head formed	++
Anthraquinone	Anthraquinone	Rose-pink coloration in the aqueous layer	++
Alkaloids	Meyer's reagent	A cream coloration	++
	Dragendorff's reagent	Reddish-brown coloration	++
Cardiac glycosides	Keller-killani test	Reddish-brown ring at the interface and green coloration in the acetic acid layer	+
Flavonoids	Filtrate + dilute ammonia solution + concentrated sulphuric acid	yellow color which disappears on standing	+++
	Filtrate + 1% aluminum chloride solution	yellow coloration	+++
	Shinoda's test	Orange coloration	+++
Sterols/Steroids	Lieberman-burchard test	No green coloration	-
	Salkowski's test	No yellow coloration	-
Cyanogenetic glycoside	Plant sample + little water + sodium picrate paper (boil)	Yellow picrate paper faded and became colorless	-

- Absent; + Present, ++ Moderately present, +++ Abundantly present

**Fig. 1. Polyphenolic contents in MEMm**

Keeping this in view, we also evaluated the antioxidant activity of MEMm. Antioxidant activity was assessed by determining percentage inhibition of DPPH free radical. Ascorbic acid was used as standard antioxidant. MEMm showed a significant antioxidant activity that is comparable with ascorbic acid (Fig. 2). Kapche et al. [13,29] had reported the antioxidant and hepatoprotective activity of the methanol extract

and isolated arylbenzofurans and flavonoids from the stem bark. The antioxidant activity in stem bark of *M. mesozygia* is linked to the presence of the flavonoids and arylbenzofurans.

Acute toxicity testing of MEMm revealed that at the first phase, and at the different concentration of 10 mg/kg, 100 mg/kg, 1000 mg/kg, the mice were active and grooming and no mortality was recorded within 24 hours observation. In the second phase with higher dose (1600, 2900, and 5000 mg/kg), it was observed that the mice did not show any pharmacotoxic signs or mortality within the 24 hours observation and subsequently for 14 days. This observation suggests that methanol extract of *M. mesozygia* stem bark at the highest dose (5000 mg/kg) tested was non-lethal. Acute toxicity test provides preliminary information on the toxic nature of a material for which no other toxicological information is available [30]. In the absence of observable toxic signs, we can conclude that the no-observed-effect-level (NOAEL) of *M. mesozygia* is 5000 mg/kg body weight. The high safety margin of the extract may partly explain

the historical use of stem bark decoction or infusion in the traditional management of rheumatism, malnutrition, stomach disorders, and infectious diseases [9,31].

The *in vivo* anti-inflammatory activity was investigated using the classical carrageenan – induced rat paw oedema [23]. In this experiment, injection of 0.1 mL carrageenan (1%) produced a progressive increase in paw volume that peaked at the fourth hour. For the control group, percentage increase in rat paw oedema increased progressively to a maximum volume at 4 h after carrageenan injection, while groups pretreated with MEMm (200 and 400 mg/kg) and Indomethacin (10 mg/kg) showed a reduction in percentage increase in rat paw oedema as compared with the control group (Fig. 3). Furthermore, total oedema produced by each treatment is expressed in arbitrary units as area under the curve (AUC) of the percentage increase in rat paw oedema versus time curves (Fig. 4). AUC during the five hour duration is as follows; control group (274.80 ± 5.21), MEMm 200 mg/kg (170.60 ± 5.70), MEMm 400 mg/kg (147.8 ± 4.70) and Indomethacin (86.26 ± 2.23). The AUC of MEMm (200 and 400 mg/kg) and indomethacin (10 mg/kg) were significantly ($p < 0.001$) smaller compared to the control group.

MEMm (200 and 400 mg/kg) reduced rat paw oedema with percentages of inhibition of 37.9 and 46.2% respectively (Fig. 4). Similarly percentage of inhibition produced by Indomethacin (10 mg/kg) was 68.6%. Carrageenan - induced paw oedema has been increasingly used to test new anti-inflammatory drugs as well as to study the mechanisms involved in inflammation [32]. Carrageenan-induced rat paw oedema is largely associated with the production of several inflammatory mediators such as histamine, prostaglandins, kinins, nitric oxide (NO) and cytokines. Histamine, serotonin and bradykinin are the first detectable mediators in the early phase of carrageenan-induced inflammation; prostaglandins (PGs) are involved in the increased vascular permeability and are detectable in the late phase of inflammation. Pro-inflammatory cytokine TNF- α also plays a critical role in the late phase oedema formation as well as recruitment of leukocytes following injection of carrageenan [33]. The extract showed inhibition at both phase of the oedema, with significant reduction from the 3rd to the 5th hour post carrageenan injection. Agents that suppressed carrageenan - induced paw oedema particularly in the second phase are good candidates for further anti-inflammatory screening.

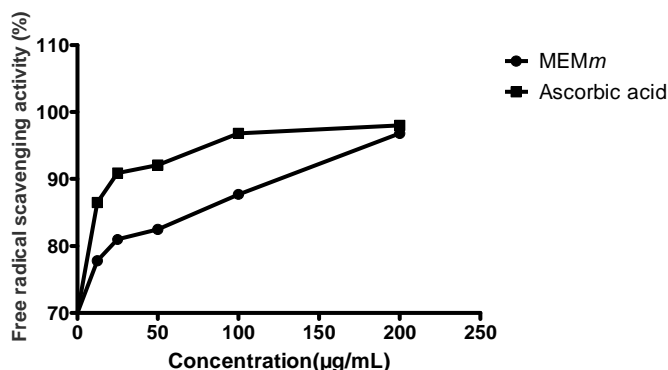


Fig. 2. *In vitro* antioxidant effect of MEMm in DPPH free radical scavenging activity assay

Table 2. Membrane stability effect of MEMm on rat erythrocytes

Treatment	Conc. (mg/mL)	Mean absorbance ± SEM	Percentage membrane stability
Control	-	0.476 ± 0.008	-
MEMm	0.5	0.319 ± 0.008	33.0***
MEMm	1	0.192 ± 0.004	59.7***
MEMm	2	0.082 ± 0.009	82.1***
Indomethacin	0.5	0.391 ± 0.016	17.9**

*** $p < 0.001$ and $p < 0.01$ are significant compared with control using the one-way ANOVA followed by Bonferroni post hoc test

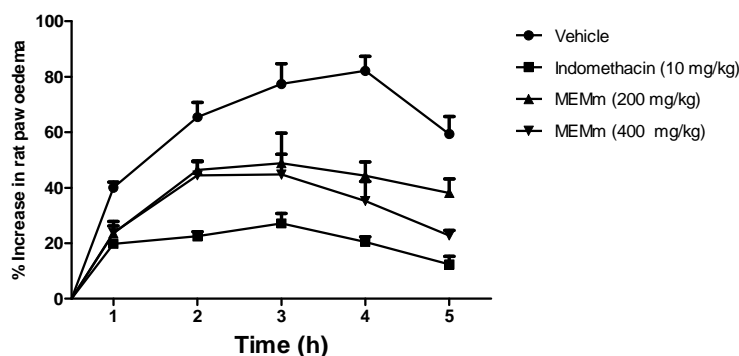


Fig. 3. Effect of MEMm (200, 400 mg/kg) on carrageenan –induced rat paw oedema
Data represent Mean \pm SEM of five rats

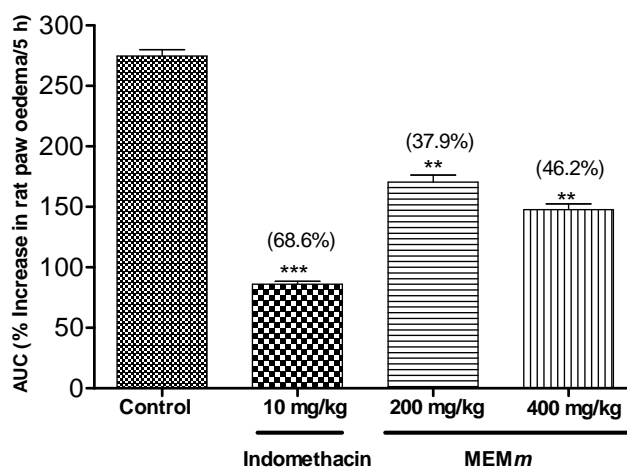


Fig. 4. Area under the curve of percentage increase in rat paw oedema
Data represent Mean \pm SEM of five rats, values in parenthesis are percentages of inhibition. ** $p < 0.01$, *** $p < 0.001$ by 1-way ANOVA followed by post hoc Bonferroni's test compared to carrageenan control group (pretreated orally with 1% tween 80)

Erythrocyte membrane stabilization has been an *in vitro* method used to assess anti-inflammatory activity because erythrocyte membrane is analogous to the lysosomal membrane [34]. Lysosomal stabilization is an important step in limiting the inflammatory response. Activated neutrophil release lysosomal proteases which causes further tissues inflammation and damage of extracellular structures. Non steroidal anti-inflammatory drugs (NSAIDs) mediate their anti-inflammatory effects partly by inhibiting these lysosomal enzymes of stabilizing the lysosomal membrane [35]. MEMm (0.5, 1 and 2 mg/mL) showed a significant ($p < 0.001$), dose dependent inhibition of erythrocytes lysis by 33.0, 59.7, 82.1 respectively. The protective effect against erythrocytes membrane lysis is considered to be a biochemical index of anti-inflammatory activity of test compounds [36]. The rat erythrocytes membrane stabilization effect of MEMm implies

that the extract may well stabilize lysosomal membranes. The membrane stabilization effect of MEMm may contribute to its anti-inflammatory mechanism, preventing the leakage of serum protein and fluids into tissues during a period of increased permeability caused by inflammatory mediators [37]. Phenolics and Flavonoids in extracts have the ability to bind cations and other biomolecules and as such have protective effect on membrane proteins [35]. Therefore the membrane stabilizing activity in MEMm may be as a result of the abundance of polyphenols in the extract.

4. CONCLUSION

The experimental observations in this experiment suggest the presence of bioactive phytochemicals especially the polyphenols. The demonstrated anti-inflammatory activity in the

methanol stem bark extract of *Morus mesozygia* seems to support the use of this plant in managing rheumatism. However, further study is needed for elucidating the mechanism of anti-inflammatory activity and characterizing the active principles and standardization of the extract.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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