**Combined Antibacterial Effect of Ethanol Extracts of *Psidium guajava* and *Persea americana* Leaves on Methicillin-Resistant *Staphylococcus aureus* (MRSA)**

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**ABSTRACT**

The spread and emergence of increasing resistance to conventional antibiotics, particularly by Methicillin Resistance Staphylococcus aureus strains has prompted this study to be able to opt in for alternative. This study aims to evaluate the combined antibacterial effect of ethanol extracts of Persea americana and Psidium guajava leaves against Methicillin Resistance Staphylococcus aureus isolates.

The plant materials (powdered) were extracted by cold maceration with 80%v/v ethanol. The phytochemical analyses were evaluated using standard procedures. The agar diffusion method was employed for the antibacterial sensitivity test of the extracts.

Ethanol extracts of Persea americana and Psidium guajava were subjected to an antibacterial test separately and combined antibacterial effect against isolates of Methicillin Resistant Staphylococcus aureus, (MRSA), by determining their zones of inhibition using the agar cup diffusion method.

Results show varying zones of inhibition and minimum inhibitory concentration, MIC for Ethanol extracts for Persea americana and Psidium guajava leaves when tested against isolates of MRSA. Zones of inhibition ranging from 4-12mm and MIC ranged from 50mg/ml to 200mg/ml, while that of ethanol extract of Psidium guajava zones of inhibition ranged from 4mm to 12mm and minimum inhibitory concentration, MIC ranged from 12.5mg/ml to 200mg/ml.

The combined antibacterial effect of the ethanol extracts of *Persea americana* and *Psidium guajava* leaves revealed a synergistic effect with the zones of inhibition ranged from 20-30mm. In conclusion, the combined antibacterial effect of both plants showed a synergistic antibacterial effect with a very high antibacterial activity and this could be a good candidate to combat MRSAs and also prevent drug resistance.

**Keywords**: *Persea americana, Psidium guajava,* antibacterial, synergistic, MRSA

**Introduction**

*Psidum guajava* and *Persea americana* are known to be an excellent source of drugs. The incumbent global challenges of the increase in resistance of the infectious agents to the already known synthetic drugs have paved ways for search of newer sources of antibiotics in all institutions.1Every particular plant in nature has medicinal value responsible for its uniqueness. The plant *Psidium guajava*, commonly known as guava is a tropical plant widely grown for fruit. It belongs to the family; Myrtaceae and Class; Magnoliopsida. All parts of *Psidium guajava*are used for therapeutic purposes.2

The leaves of *Psidium guajava* are opposite, oblong, 3 to 7 inches (7.6-18cm) in length, with serrated margins having prominent veins on the lower side. All Guava trees are known to be well adapted to warm subtropical to tropical climatic conditions. The temperature range for its growth and production range from 73F to 83 F.3

*Psidium guajava* is believed to have active components that help to treat and manage various diseases. The aqueous extract of the root bark and leaves has been found to be effective in management of gastrointestinal ulceration, diarrhea, and toothache among others.4 The aqueous extract of the root has also been found to be effective as an indigestion, stomach ache, constipation and antitussives.5 In addition, the extract of the *Psidium guajava* leaves had also been reported to be effective in treatment of pulmonary diseases and in relieving episodes of asthma attacks.6

The *Psidium guajava* extracts has been screened for the presence of bio-active components in the leaves and roots.7 The phytochemical analysis has given an evidence that the aqueous and ethanol leaf extract of *P. guajava* contained different classes of bioactive constituents such as saponins, alkaloids, tannins, sterols cardiac-glycosides, terpenes, and flavonoids. The results showed that saponins, tannins and alkaloids were present in high concentrations, while terpenes, cardiac glycosides, flavonoids and sterols were present in small concentration.8 According to a reported, flavonoids extracted from guava leaves is believed to be responsible for antibacterial activity.9

*Persea americana* also known as ‘avocado pear’ belongs to the family ‘Lauraceae’. To a large extent, they are cultivated amidst the tropics, including the subtropics of the world majorly for edible fruits and most importantly for its therapeutic and prescient uses.10 The aqueous extracts of theleaves, fruits and of course the bark of the avocados have been effective as anti-inflammatory, hypertension, and antibacterial.11-13

*Staphylococcus aureus,* a Gram-positive bacterium, non-motile, found in nasal mucosa of human in commensalism relationship.14The disruption in the cutaneous and the mucosa layer, as found in soft tissue infection could allow the penetration of the *Staphylococcus aureus* into the bloodstream to cause infection. Patients with compromised immune system are more vulnerable.15

Penicillin discovery by Alexander flemming was seen as a huge effort to wage war against infection caused by *Staphylococcus aureus*. However, due to the survival ability of this pathogen that paved ways to the emergence of strain that are resistant to penicillins, there were exertions that lead to the development of newer Beta lactam (the anti-staphylococcus class), which include the Methicillin, flucloxacillin, Oxacillin and dicloxacillin.16 In the early 1960s the emergence of the MRSA was documented.17The strain Methicillin Resistant Staphyloccocus aureus,(MRSA), has posed a greatest challenge in healthcare system and community settings. This in few cases leading to prolonged infection, increase in direct and indirect costs, increase in numbers of stays in the hospital and in many cases increased in mortality.

The resistant of MRSA to anti-staphylococcal penicillin subclass of the beta-lactam class of antibiotics and emergence of accumulating resistance to obtainable synthetic antibacterial antibiotic has implications in current and future treatment options of this particular pathogen. This has called for the investigation into plants as source for novel drugs compounds. Plants as known provide a very reliable source of lead compounds which are effective in mitigating the spread of infection globally. These plants can as well be improved through modifications to render them less toxic. Consequently, the objective of this research is to evaluate the combined effect of the ethanol extract of *Psidium guajava leaves* and the ethanol extract of *Persea americana leaves* on Methicillin Resistant Staphylococcus aureus, (MRSA).

**MATERIALS AND METHODS**

**Collection and Identification of Plant Materials**

The leaves of *Psidium guajava* and *Persea Americana* were collected from Umuno in Abraka, Delta State, Nigeria in December 2022. The identification and authentication of the plants were done by Dr. Emmanuel Ikpefan, a botanist in the department of Pharmacognosy and traditional medicine, Faculty of Pharmacy, Delta State University, Abraka, Nigeria. A sample was kept at the herbarium, department of Pharmacognosy &Traditional medicine, Delta State University, Abraka, Nigeria. The plants samples collected were air-dried and comminuted, and then both powders were stored at room temperature.

**Extraction of Plant Materials**

About 420g of the powdered sample of *Persea americana* leaves and 280g of the powdered sample of *Persea americana* were collected. A 420g of the powdered sample of *Persea americana* was divided into three portions in a separate bucket. A 280g of the powdered sample of *Psidium guajava* was divided into two portions. Each portion containing 140g of each powdered plant material. They were macerated with 80% of ethanol, with each 140g of the different plant material soaked in 560mls of ethanol separately in an airtight bucket for 72hours. The extracts of the three portions of *Psidium guajava* were filtered using the muslin cloth. Also the extracts of the three portions of *Persea americana* were filtered using muslin cloth. The filtrates were collected in separate beakers and were concentrated to dryness in a water bath. The resulting brown concentrate was then reconstituted using distilled water for a final weight per volume of 100mg/ml, and stored in a refrigerator at 4c until when it was required for use in the experiment.

**Phytochemical Screening**

Secondary metabolites such as terpenes, alkaloids, flavonoids, tannins, and saponins were screened for using the standard approaches according to Evans and Trease (2005).18

**Cell Cultures**

Stock cultures of 30 *Staphylococcus aureus* previously isolated were kept on Nutrient agar slants at room temperature in the Pharmaceutical Microbiology laboratory, Delta State University, Abraka for further use in this study. The stock cultures were further sub-cultured on Mannitol Salt agar and incubated at 37°C for duration 24h for re-identification. Those that produced yellow color indicated *Staphylococcus aureus* and these were the ones further subjected for Methicillin resistant test. A single colony of these bacteria isolates were picked and streaked on a fresh nutrient agar plate and incubated overnight and they were then stored at 4C for further studies.

**Test for Methicillin Resistance**

The agar diffusion approaches were used for the evaluation of the 30 *Staphylococcus aureus* isolates for Methicillin resistance.19 Flucloxacillin cap. (500mg) (Ernest Chemists Limited, Accra, Ghana) was used for this study. A pure colony of each *Staphylococus aureus* isolate was picked using a wire loop and then was inoculated into already sterilized nutrient broth covered with an aluminum foil and left overnight. The turbidity of each nutrient broth culture was doctored to suit McFarland turbidity standards. Each of the adjusted broth culture was then used to swap the surface of the already solidified 30 Mueller Hinton Agar plates carefully labeled. The left over nutrient broths were discarded into a disinfectant jar, and then the surface of each inoculated Mueller hinton agar plate was given a room to dry. Using sterile surgical blade, the antibiotic discs were placed aseptically in duplicate on each plate rightly on the surface of the already dried inoculated Mueller Hinton agar plates. The plates were incubated at 37°C for duration of 24h. After the incubation, all plates were carefully examined for inhibition zone around the two paper discs on each plate. The zones of inhibitions on each plate were measured using a meter rule in diameters and they were recorded accordingly. Means of inhibition zone diameter were calculated and recorded to a nearest whole millimeter. Thereafter each of the organism isolate was classified as Methicillin Resistant Staphylococcus aureus strain, (MRSA), or not using a guideline given by the CLSI (2022).

**Antimicrobial Testing**

**Determination of the Zones of inhibition of Ethanol extract of *Persea americana* and *Psidium guajava* leaves*.***

Sensitivity: Agar well diffusion method

Ethanol extracts of *Persea americana* leaves was screened for its effect on Methicillin Resistant Staphylococcus aureus (MRSA).

Mueller Hinton agar was prepared for 15 petri dishes according to the manufacturer specifications and autoclaved at 121C for 15minutes. The media was allowed to cool before pouring 20ml into each petri dish and they were allowed to solidify. The petri dish was each labeled according to the numbered strain of MRSA previously identified. Each of the petri dish was also labeled accordingly with different concentration (200, 100, 50, 25, 12.5, 6.25)mg/ml of the ethanol plant extract previously prepared and ciprofloxacin used as a positive control. The agar plates were swabbed with the test organisms as labeled aseptically. Using a 6mm cork borer, a duplicate well were pouched in the agar plates. Two drops of each concentration of the ethanol extract of *Persea americana* leaves was placed into the corresponding well using a Pasteur pipette. Ciprofloxacin which served as the positive control of the experiment was placed in the well at the centre of the agar plate using a 2ml syringe.

Ethanol extract of *Psidium guajava* leaves was also evaluated using the same procedures as above. The plates were incubated for 24hrs at 37C. After incubation, zones of inhibition were examined using a hand lens for proper magnifications and zones measured. A metric rule was placed across zones of inhibition, and measured from one edge of the zone to the other edge. The plates were observed for inhibition zones around the wells. The diameters of the zones were measured with meter ruler to the nearest whole millimeter. Each test was carried out three times and the mean IZD recorded to the nearest whole millimeter.

**Determination of Minimum Inhibitory Concentration (MIC) Of Plant extracts**

TheMIC was evaluated using agar dilution method as specified in the procedures of CLSI (2022). Mueller Hinton agar was prepared according to the manufacturer instruction. 19ml of molten nutrient agar was mixed with 1ml of the dilution extract of *Persea americana*, mixed thoroughly and was poured into a sterile petri dish and allowed to solidify. Each petri dish contained different concentrations of the dilution of ethanol extract of *Persea amaricana leaves*, (200, 100, 50, 25, 12.5, 6.5 mg/ml). The agar plates were divided into 15 parts and labeled for each test strain of MRSA. The plates were kept in the incubator overnight to check for their sterility. Using a sterile wire loop, an overnight broth culture of each of the test organism was streaked on the surface of the agar plate on the part of the plate labeled for the highest concentration of the dilution of ethanol extract of *Persea americana* leaves (200mg/ml). Same procedure was repeated for the other five different concentrations. A nutrient agar without an extract was as well streaked and this served as a negative controls. The plates were then incubated for 24hours at 37C and they were observed for any visible growth of each MRSA. The least concentrations that inhibited the growth of the test organisms were selected as the MIC.

The same procedures above were repeated to determine the minimum inhibitory concentrations of the different concentrations of the dilution of ethanol extract of *Psidium guajava* leaves*,* (200, 100, 50, 25, 12.5, 6.5 mg/ml). The least concentrations that inhibited the growth of the test organisms were selected as the MIC.

**Determination of Combined Zone of Inhibitions of Ethanol Extract of *Persea americana* and *Psidium guajava* leaves.**

Agar plates with a subculture colony of Methicillin Resistant Staphylococcus aureus labeled MRSA 1, MRSA 4, MRSA 6, MRSA 7, MRSA 10 were selected to prepare overnight broth.

Mueller Hinton agar was prepared according to the manufacturer specification and autoclaved at 121C for 15minutes. The media was allowed to cool before pouring 20ml into each petri dish and they were allowed to solidify. The agar plate surface was swabbed using a swab stick with the first test organism (standardized overnight nutrient broth of MRSA 1). The petri dishes were labeled according to the test organisms being used.

Using a 6mm cork borer, two wells were pouched close to each other with a distance of about 3mm in each agar plate. A 1ml of each concentration with the least minimum inhibitory concentration, MIC on both plant extracts of this test organism was individually placed into the corresponding well with the aid of Pasteur pipette..

This was also done for other test organisms (MRSA 4, MRSA 6, MRSA 7, MRSA 10) using both extracts as well.

The plates were incubated for 24hours at 37C. The combined antibacterial assay was evaluated in duplicate. After incubated, the combined zones of inhibition were carefully examined using a hand lens for proper magnification and the zones measured. A metric rule was placed across combined zones of inhibitions and measured from one edge of the zone to the other edge, both vertically and horizontally and was averaged. The combined Inhibition zones diameter (IZD) were reported in millimeters.

**RESULTS AND DISCUSSION**

The use of combination of these extracts could be efficient in treatment of complicated infections as compared to a single plant extracts. It would as well reduce resistance, reduce high cost of drugs, increases effectiveness and reduces toxicity. However, the process could be tedious and clinical trials could be required to ascertain safety and efficacy.

*Persea americana* has previously been documented to possess many antimicrobial activities.13 Research has also been previously carried out and reveals *P. guajava* Linn. to have a promising medicinal properties in combating and management of resistant bacteria like MRSA.20

In this study, a combined antibacterial effect of ethanol extract of *Psidium guajava* and *Persea americana* leaves on Methicillin Resistant Staphylococcus aureus was done.

The preparative phytochemical constituents of ethanol extract of *Psidium guajava* and *Persea americana* leaves are presented in Table 1 and Table 2 respectively. Table 1 indicates that ethanol extract of *Psidium guajava* leaves contained alkaloid, saponins and tannins in high concentration while terpenes, flavonoids and cardiac glycosides in moderate concentration. Table 2 indicates that ethanol extract of *Persea americana* leaves contained Alkaloids in high concentration while Saponins, terpenes, flavonoids and tannins in moderate concentration. Tannins had previously been reported to be responsible for the antibacterial actions against *Staphylococcus aureus*.21

The results from this study revealed that the combined antibacterial effect of ethanol extract of *Psidium guajava and Persea americana* produced a synergistic antibacterial effect against Methicillin Resistant Staphylococcus aureus isolates, (MRSA). The antibacterial activity of ethanol extract of *Psidium guajava* leaves was evaluated by comparing the zone of inhibition of each MRSA isolate with that of the standard antibiotic (control) Ciprofloxacin using the agar well diffusion method. The agar well diffusion test was carried out on 10 clinical isolates of MRSA and the results of the screening test of the leaf extract of *Persea americana* against MRSA are presented in Table 3, similarly, that of screening test of the leaf extract of *Psidium guajava* against MRSA isolates are presented in Table 4. The Minimum Inhibitory concentrations were also carried out. The MIC of tested MRSA isolates varied in their sensitivities to different concentrations of the extracts.

The Ethanol extract of *Persea americana* leaves was active against the ten MRSA isolates tested with a mean inhibition zone diameter between the range from 4 to 13 mm.

The Ethanol extract of *Psidium guajava* leaves was active against the ten MRSA isolate tested with a mean inhibition zone diameter within the range of 4 to 12 mm. The cleared zones that appeared around the well after incubation showed the degree of inhibition/antibacterial effect possessed by each concentration of the individual plant extracts against the test MRSA isolates, while those with cloudy appearance around the wells with no clear zones indicated that isolates were not inhibited by the extracts or they were resistant to the extracts.

The Minimum Inhibitory Concentration, MIC results reported in Table 5 shows that the ten MRSA isolates tested were inhibited by the Ethanol extracts of *Persea americana* leaves with activities ranging from 50 to 200 mg/ml while the nine (9) isolates inhibited by the ethanol extract of *Psidium guajava* leaves varied between 12.5 and 200 mg/ml. The Result of the MIC of *Psidium gujava* plant extract confirmed the antibacterial actions on Methicillin Resistant Staphylococcus aureus as previously published by other co-workers.22 Also the result of the MIC of *Persea americana* plant extract ascertained the antibacterial action on Methicillin Resistant Staphylococcus aureus as previously published by other co-workers.13

The combined antibacterial activity of ethanol extract of *Psidium guajava and Persea americana* leaves was evaluated by comparing the combined Inhibition zone diameter, (CIZD), of each MRSA isolate with least minimum inhibitory concentration,(MIC), combined on both extracts. Also, the CIZD was compared with that of the standard antibiotic (control) Ciprofloxacin using the agar well diffusion method. The agar well diffusion test was carried out on 5 clinical isolates of MRSA and the results of the screening test of the extracts against the tested MRSA isolates are further presented in Table 6.

MRSA 1 with the least MIC of 50mg/ml and 25mg/ml for the ethanol extract of *Persea americana* and *Psidium guajava* leaves respectively, gave a mean combined inhibition zone diameter of 23mm. MRSA 4 with the least MIC of 50mg/ml and 25mg/ml for the ethanol extract of *Persea americana* and *Psidium guajava* leaves respectively, gave a mean combined inhibition zone diameter of 22.25mm. MRSA 6 with the least MIC of 50mg/ml and 25mg/ml for the ethanol extract of *Persea americana* and *Psidium guajava* leaves respectively, gave a mean combined inhibition zone diameter of 20mm. MRSA 7 with the least MIC of 100mg/ml and 12.5mg/ml for the ethanol extract of *Persea americana* and *Psidium guajava* leaf respectively, gave a mean combined inhibition zone diameter of 20.75mm. MRSA 8 with the least MIC of 100mg/ml and 12.5mg/ml for the ethanol extract of *Persea americana and Psidium guajava* leaves respectively, gave a mean combined inhibition zone diameter of 29.5mm.

The mean of combined inhibition zone diameter varied between 20 to 30mm. This result signifies a synergism in the antibacterial effects of both plant extracts against Methicillin resistant Staphylococcus aureus. The zone of inhibition of the test Isolate MRSA 1 when conducted as a single extract using *Persea americana* produced 8mm with the least MIC (50mg/ml), while 7mm was observed with the least MIC (25mg/ml) using *Psidium guajava*. The combined zone of inhibition using this same isolate with the same MICs when conducted gave 23mm which signifies a synergistic effect. Same observation was carefully noted with significant improvement on other isolates. In addition, this combined effect showed a significant improvement in the antibacterial action, when compared with the antibacterial effect of a sole plant extract and in addition with the standard antibiotic. Thus, the interesting synergistic effect of ethanol extract of *Persea americana* and *Psidium guajava* leaves extracts will be of good alternative to combat multidrug resistance organisms and to the best of my knowledge and literature review, this is the first report on the combined effects of *Persea americana* and *Psidium guajava* leaves on Methicillin Resistant Staphylococus aureus, (MRSA). This could be of significance in health care as it could be used as alternative to conventional drugs in the treatment of diseases caused by Methicillin Resistant Staphylococus aureus, (MRSA). This could also be used In case of blind treatment where the case of infection is not known. Since time immemorial, early man has been said to use plants in the treatment of various ailments. Herbal medicine is still practiced in many parts of the world for the treatment and prevention of diseases especially local regions with variety of vegetation.

**Table 1:** Phytochemical constituents of *Psidium guajava* Leaves

**S/N TEST PLANTS COMPOUNDS RESULT**

1. Alkaloid +++
2. Terpenes ++
3. Flavonoids ++
4. Saponins +++
5. Tannins +++
6. Cardiac glycosides ++

 ++: moderate concentration +++: high concentration

**Table 2:** Phytochemical constituents of *Persea americana* Leaves

**S/N TEST PLANTS COMPOUNDS RESULT**

1. Flavonoids **++**
2. Terpenes ++
3. Alkaloids +++
4. Saponins ++
5. Tannins ++

++: moderate concentration +++: high concentration

**Table 3:** Sensitivity of MRSA Isolates to Ethanol Extract of *Persea americana* Leaves

|  |
| --- |
| **MRSA isolates diameters of the inhibitory zones (in mm)** |
| Extract concentration in mg/ml | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 200mg/ml | 11 | 13 | 10.5 | 7.5 | 7 | 8.5 | 8.5 | 8.5 | 8..5 | 10.5 |
| 100mg/ml | 9 | 11 | 9 | 7 | 6 | 8 | 7 | 7 | 7 | 10 |
| 50mg/ml | 8 | 8 | 8 | 6 | 6 | 7 | 6 | 6 | 7 | 9 |
| 25mg/ml | 6 | 7 | 7 | 5 | 5 | 6 | 6 | 6 | 6 | 9 |
| 12.5mg/ml | 6 | 5 | 5 | 5 | 5 | 6 | 5 | 5 | 5 | 7 |
| 6.25mg/ml | 5 | 5 | 5 | 4 | 4 | 5 | 5 | 5 | 4 | 7 |

**Table 4:** Sensitivity of MRSA Isolates to Ethanol Extract of *Psidium guajava* Leaves

|  |
| --- |
| **MRSA isolates diameters of the inhibitory zones (in mm)** |
| Extract concentration in mg/ml | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 200mg/ml | 12 | 11 | 11 | 11 | 10 | 10 | 9 | 10 | 11 | 11 |
| 100mg/ml | 9 | 9 | 8 | 8 | 8 | 7 | 7 | 8 | 8 | 7 |
| 50mg/ml | 8 | 7 | 7 | 6 | 7 | 6 | 6 | 7 | 6 | 6 |
| 25mg/ml | 7 | 6 | 7 | 6 | 6 | 5 | 6 | 5 | 5 | 6 |
| 12.5mg/ml | 6 | 5 | 5 | 6 | 6 | 6 | 5 | 6 | 5 | 5 |
| 6.25mg/ml | 5 | 4 | 4 | 5 | 5 | 6 | 5 | 5 | 5 | 4 |

**Table 5:** Minimum Inhibitory Concentration for the MRSA Isolates

**MRSA Isolates Ethanol Extract of Persea americana leaves Ethanol Extract of Psidium guajava leaves**

 MIC (mg/ml) MIC(mg/ml)

1 50 25

2 50 12.5

3 50 25

4 50 25

5 100 12.5

6 50 25

7 100 12.5

8 100 12.5

9 50 200

10` 200 -

**Table 6:** Combined Zone of Inhibitions of Selected MRSA Isolates with Minimum Inhibitory Concentration for Both Plant Extracts.

|  |
| --- |
|  **MRSA isolates diameters of the combined inhibitory zones (in mm)** |
| Combined MIC on Both Extracts | 1 | 4 | 6 | 7 | 8 |
| 50mg/ml + 25mg/ml | 23 |  |  |  |  |
| 50mg/ml +25mg/ml |  | 22.5 |  |  |  |
| 50mg/ml + 25mg/ml |  |  | 20 |  |  |
| 100mg/ml +12.5mg/ml |  |  |  | 20.75 |  |
| 100mg/ml + 12.5mg/ml |  |  |  |  | 29 |

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**Figure 1:***Combined zones of inhibition of Ethanolic extract of Persea americana and Psidium guajava leaves.*

**Conclusion**

The results of this research indicated that the combined antibacterial effect of both plants extracts have a significant antibacterial activity in isolation and a significant synergistic effect against Methicillin Resistant Staphylococcus aureus, (MRSA), when combined. And subsequently from the results, it could serve as a good candidate to those strains which have developed resistance or as an alternative to conventional drugs in the treatment of these strains of *Staphylococcus aureus*. This combined effect would not only produced a synergistic effect but reduce drug toxicity and also reduce emergence of drug resistance.

**Conflict of Interest**

No conflict of interest.

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