

## Phytochemical Analysis of *Muntingia calabura* Extracts Possessing Anti-Microbial and Anti-Fouling Activities

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### ABSTRACT

The present study was carried out to determine the possible antifouling and anti microbial activity of plant extracts prepared from *Muntingia calabura* and phytochemical screening was demonstrated. The extracts from the following plant parts like stem, leaves, fruits, flower and stems were prepared in different aqueous solvents like ethanol, methanol, acetone, acetonitrile and water. The highest antimicrobial potentials were observed for the methanolic extracts against *K. pneumonia*, *B. subtilis*, *B. megaterium* and *P. aeruginosa*. Its efficacy was comparable to the standard drug, ampicillin. Significant amount of tannins, alkaloids, steroids and flavonoids were found. Interestingly, the present study showed anti fouling effect against pathogenic biofilm forming bacteria. These extracts also contain high hemagglutination activity. The present study provides evidence that solvent extract of *M. calabura* contains medicinally important bioactive compounds and this justifies the use of plant species as traditional medicine for treatment of various diseases.

**Keywords:** Medicinal plants, anti-biofilm activity, antimicrobial activity, phytochemical analysis

### INTRODUCTION

There has been an increased urgency to discover novel antibiotics/anti microbial compounds due to emergence of drug resistant microorganisms. Therefore, an infection caused by antibiotic resistant microbes complicates conventional treatment causing prolonged illness and increases the death risks<sup>1</sup>. Many medical practitioner also employ traditional (Ayurvedic) medicine, in Western and other developing nations like India, in complementary to modern medicines. Today also, as a testament to its persistence, over 80% of people in the world still utilize traditional medicine for primary healthcare. Many underdeveloped countries are rich in biodiversity hotspots like Southeast Asia, Africa, Central and Southern America. Among all the natural resources, plants are the single largest source for traditional medicines<sup>2</sup>, accounting for 25% of new drugs components being tested for clinical trials<sup>3</sup>. In the medical field, around 80% of the drugs tested are derived from natural sources or are modified semi synthetically<sup>4</sup>. The ultimate goal for any discovery is to offer appropriate and efficient antimicrobial drugs for the betterment of the patient.

The acquired knowledge of plant extracts and phytochemicals usage possessing antimicrobial properties can be of great significance in therapeutic treatments. Many plant extracts show their antimicrobial traits, which is due to the presence of compounds synthesized in the secondary metabolism of the plant. These secondary metabolites mainly consists of phenolics including polyphenols, flavonoids, tannins and quinones known for their potent antioxidant, cytotoxic and antimicrobial activities<sup>5-8</sup>; alkaloids are mainly known for their

cytotoxicity<sup>9-11</sup>. These metabolites are secreted in response for plant defensive mechanisms. For example, these metabolites can act directly on the herbivore, whereas others act indirectly via the attraction of organisms from other trophic levels that, in turn, protect the plant by releasing chemicals being toxic, repellent, or antinutritive for herbivores of all types. Examples include cyanogenic glycosides, glucosinolates, alkaloids, and terpenoids; others are macromolecules and comprise latex or proteinase inhibitors. These released compounds act by disrupting the cell membrane, inhibiting transport of nutrient, ions, signal transduction processes, metabolism, or disruption of the hormonal control of physiological processes<sup>12</sup>.

In the present work, we have selected *Muntingia calabura* L. (*M. calabura*), a shrub introduced to Southeast Asia from Tropical America. It is also known locally as Jamaica cherry, belonging to Elaeocarpaceae family<sup>13</sup> It is often seen growing as roadside trees<sup>14</sup>, also used as an air pollution tolerance indicator. It is an annual plant, flowers throughout the year, its leaves are distinctively lanceolate in shape, with margins irregularly serrate and fruits are berries which turn red on maturation and are sweet in taste<sup>15</sup>. In traditional knowledge, its leaves and roots are known to possess various medicinal applications. For example, in Peru, the extract from leaves and bark are used as antiseptics and in South America it is used to reduce gastric ulcers. Few places in Philippines, the flowers are used to treat headache and for relief of incipient colds. The main basis for carrying out the present study is attributed to the presence of several bioactive compounds possessing anti-inflammatory, anti-nociceptive, anti-

Table 1: Phytochemical studies of *M. calabura* extracts prepared from different parts of the plant.

Plant sample	Phlobatannins	Reducing sugar	Terpenoids	Flavonoids	Alkaloids	Steroids
Flower	-	+	+++	+++++	+++++	+++++
Fruit (ripen)	+++	+++++	+++	++	+++	-
Fruit(unripe)	+++++	+++	+++++	++	++++	+
Leaf	++	++++	+++++	++	+++	+++
Stem	++++	+++	+++	++	++	++

‘+’ - indicates presence

‘-’ - indicates absence

Table 2: Antimicrobial activity profile of different extracts of *M. calabura*.

Micro organisms	Leaf extract					Flower extract					Fruit (unripe) extract				
	Dist water	EtOH	MeOH	Ace	ACN	Dist water	EtOH	MeOH	Ace	ACN	Dist water	EtOH	MeOH	Ace	ACN
<i>P. aeruginosa</i>	-	7	-	-	12	6	-	16	-	15	-	-	-	6	8
<i>E.coli</i>	10	-	4	7	7	-	10	18	12	6	7	-	13	13	8
<i>A.faecalis</i>	-	-	6	-	-	-	-	-	6	-	12	-	9	-	10
<i>B.megartarium</i>	-	-	11	5	-	-	7	21	7	-	-	9	11	10	-
<i>B.subtilis</i>	-	9	-	-	-	14	-	11	6	9	-	8	14	6	-
<i>K.pneumoniae</i>	9	-	-	-	7	17	-	16	-	10	10	-	11	7	6
<i>S.aureus</i>	-	-	-	7	8	12	-	-	9	-	9	7	5	11	11
<i>S.typhimurium</i>	8	-	5	11	-	5	-	-	10	-	7	-	7	10	-

Micro organisms	Stem extract					Fruit (ripe) extract				
	Dist water	EtOH	MeOH	Ace	ACN	Dist water	EtOH	MeOH	Ace	ACN
<i>P. aeruginosa</i>	-	-	-	-	9	4	-	-	6	-
<i>E.coli</i>	5	10	-	13	12	5	-	5	10	14
<i>A.faecalis</i>	-	7	13	9	9	-	-	7	8	13
<i>B.megartarium</i>	-	14	12	9	8	-	-	12	7	11
<i>B.subtilis</i>	-	10	3	10	8	-	-	13	7	9
<i>K.pneumoniae</i>	8	6	23	-	10	-	-	-	7	9
<i>S.aureus</i>	-	10	8	5	-	-	-	5	9	7
<i>S.typhimurium</i>	5	8	-	12	-	4	-	3	-	-

Table 3: Inhibition of biofilm formation by the methanolic extract of flower of *M. calabura*.

Sl.No.	Pathogen Names	Sample	Percentage of inhibition (%)
1.	<i>Pseudomonas aeruginosa</i>	Control	0
		MeOH Flower Extract	81.3
2.	<i>Escherichia coli</i>	Control	0
		MeOH Flower Extract	75.6
3.	<i>Bacillus megaterium</i>	Control	0
		MeOH Flower Extract	82.6
4.	<i>Klebsiella pneumoniae</i>	Control	0
		MeOH Flower Extract	92.0

oxidant activity<sup>16</sup> with few reports on antimicrobial activity of the leaves<sup>17</sup>. Keeping all the biomedical application potential of *M. calabura*, we have presented the antimicrobial and antifouling activity of the crude extracts prepared in different solvents from the leaves, flower, roots, fruits and stems of *M. calabura* against the selected Gram positive and Gram negative bacteria: *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus megaterium*, *Klebsiella pneumoniae* and

*Alcaligenes faecalis*. The phytochemical analysis was also carried out being responsible for the bioactive properties of the extracts.

## MATERIALS AND METHODS

### Microbial samples

Microorganisms tested in this study were: *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus*

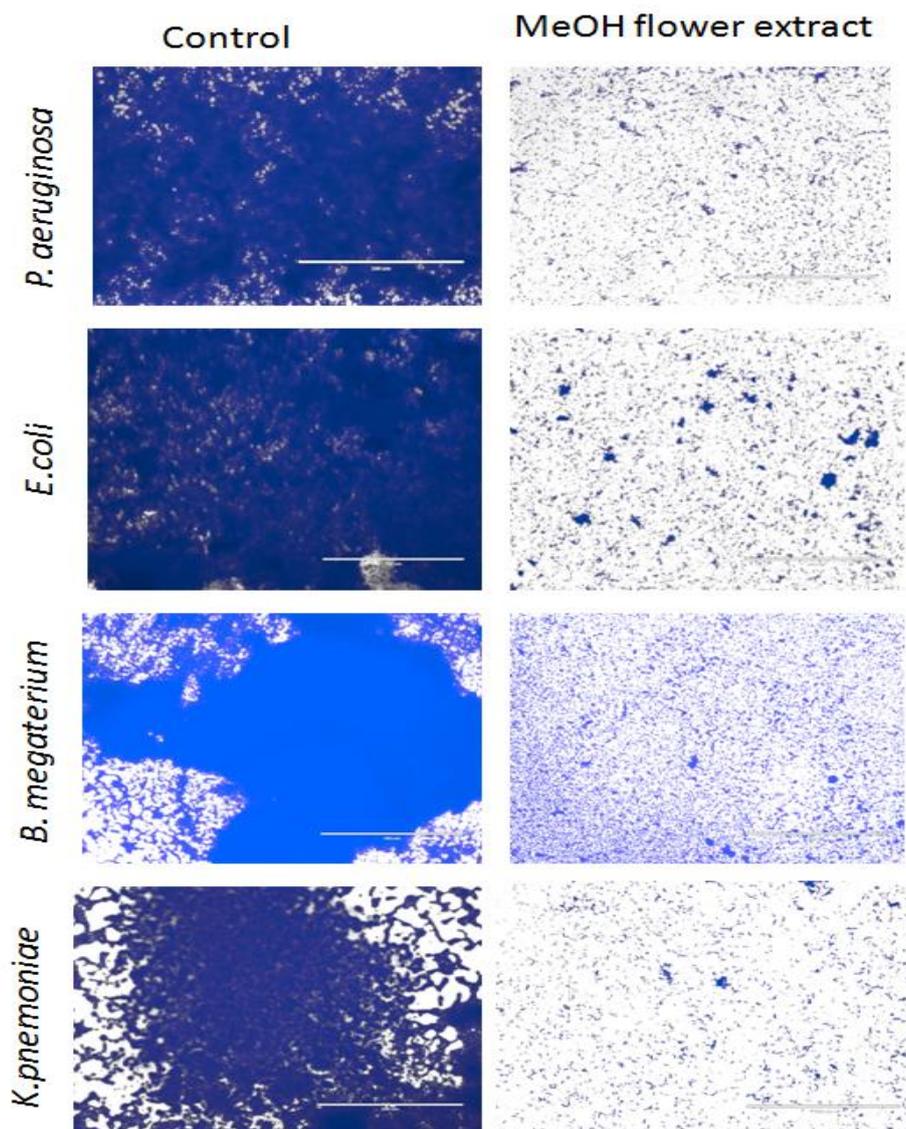


Figure 1: Biofilm inhibition shown by methanolic extract of *M. calabura* flower against various pathogenic organisms. The assay was carried out for 72 hr.

*megaterium*, *Klebsiella pneumoniae* and *Alcaligenes faecalis*. These organisms were stored on Nutrient agar slants and antibacterial assays were carried at Department of Biotechnology, Modern College of Arts, Science and Commerce, Ganeshkhind, Pune, India.

#### Chemicals

Antibiotics like Ampicillin and tetracycline (SRL Diagnostics) were used as positive control. Nutrient Broth, Agar, solvents and other chemicals were procured from Himedia.

#### Plant material

Different parts of *M. calabura* were collected from the same tree in the month of Dec 2015. The collected samples were cleaned, surface sterilized and stored.

#### Preparation of crude extracts

Different parts of *M. calabura* like leaves, flower, fruit and stems weighing 1 g each were crushed and extracts were prepared in aqueous, methanol (MeOH), ethanol (EtOH), acetone and acetonitrile (ACN) in the ratio of 1:10 (w/v). The mixtures were then decanted and filtered. The final

extract was centrifuged to remove all the cell debris at 10,000 x rpm for 20 mins and used for further bioassays.

#### Preparation of Microorganism culture for Bioassay

The before mentioned bacteria were inoculated into nutrient broth and incubated for 24 hr at 37 °C. The nutrient agar (NA) plates were prepared under sterilized condition. Using spread plate technique, 0.1 ml of bacteria culture ( $10^6$  bacteria per ml) was uniformly spread on the NA plates. Sterilized discs were loaded with antibiotics and plant extracts and then positioned on the solid agar medium by pressing gently. The plates were then kept in incubator for 24 hr at 37 °C. Ampicillin was used as positive control and solvents in which the extract was prepared was taken as negative control. The plates were incubated at 37 °C for 24 hr. The diameter of zone of inhibition (mean of triplicates  $\pm$  SD) as indicated by clear area which was devoid of microbial growth was measured and tabulated.

#### Anti biofilm assay

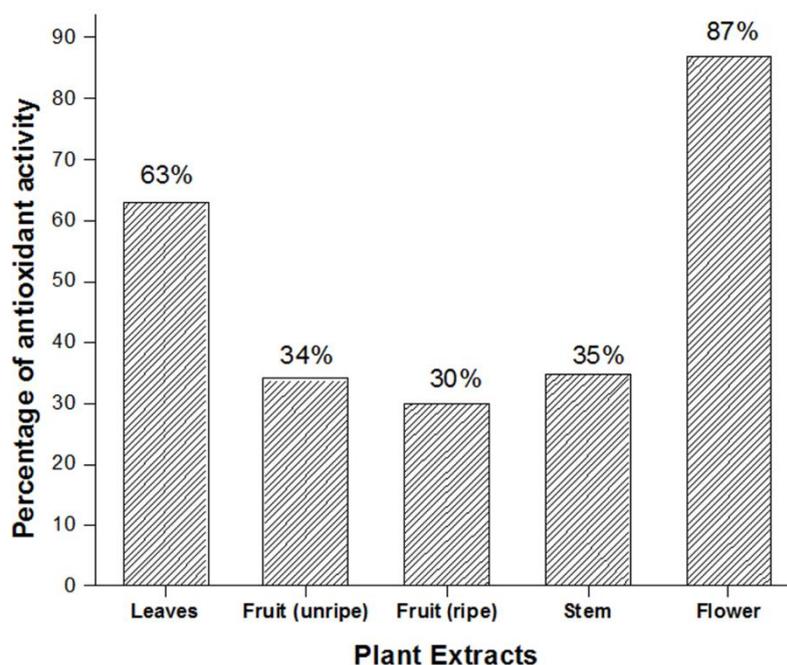


Figure 2: Anti oxidant activity of plant extracts of *M. calabura*.

For biofilm inhibition assay, different strains were grown overnight at 37 °C and grown for 16 hr on a rotary shaker at 180 rpm. On next day, the inoculum was transferred into 3 ml sterilized nutrient broth. Biofilm formation was assessed in sterile flat-bottomed 96-well polystyrene microtiter plates<sup>18</sup>. The wells were then filled with 100 µl of the bacterial culture and the plates were incubated at 37 °C without agitation. Growth was monitored by the optical density (OD) at 600 nm with a microplate reader (VersaMax Molecular Devices, Sunnyvale, USA). After 72 hr incubation, the medium was discarded and the wells were gently washed three times with 200 µl of sterile Phosphate buffer saline, pH 7.4 (PBS). The staining of biofilm formed was performed with 125 µl of 0.1 % crystal violet for 30 mins. Each well was re-washed three times with 200 µl of sterile distilled water prior to the addition of 125 µl of 30 % acetic acid in water. The absorbance was measured at 550 nm. Biofilms without any addition of drugs were used as controls. The assay was repeated three times. The compounds were added into the wells after 24 hr incubation and further incubated to complete 72 hr incubation. The biofilm disruption was then evaluated and percentage of inhibition was calculated according to the formula given as

$$\text{Percentage of Biofilm reduction} = \frac{\text{Abs of (Control- Test)}}{\text{Abs of control}} \times 100$$

#### Estimation of Protein content

Protein concentration of different extracts prepared in sterilized water was determined by Lowry method with BSA used as standard protein<sup>19</sup>.

#### Detection of lectin/ hemagglutination activity

Lectin activity was determined by serial dilution of compound using 3 % of washed rabbit erythrocytes in 20 mM potassium phosphate buffer (PBS) (v/v). To carry out the assay, 50 µl of sample was serially diluted in PBS, 50 µl of erythrocytes suspension was added and agglutination

activity was observed after 1 hr of incubation at room temperature<sup>20</sup>.

#### Anti oxidant activity

The antioxidant activity of the extracts were determined using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay<sup>21</sup> with some modifications. 100 µl of *M. calabura* extracts were taken and 5 ml 0.004% (w/v) solution of DPPH was added. The obtained mixture was vortexed and incubated for 30 min at room temperature under dark conditions. The absorbance was measured using spectrophotometer at 520 nm. The blank was 80% (v/v) methanol. Ascorbic acid (Vitamin C) was used as standard anti-oxidant for comparison. Measurements were taken in triplicate. DPPH scavenging effect was calculated using the following equation:  

$$\text{DPPH scavenging effect (100\%)} = \frac{(A_0 - A)}{A_0} \times 100$$
 where  $A_0$  is the absorbance of negative control (0.004% DPPH solution) and  $A$  is the absorbance in presence of extract.

#### Phytochemical analysis (Qualitative approach)

The different extracts prepared were subjected to phytochemical screening for the presence of various secondary metabolites like alkaloids, tannins, flavonoids, sterols etc.

#### Test for Alkaloids

Mayer's Test – 3 ml of ammonia was added to plant sample and allowed to stand for few minutes. 3 ml of chloroform was added and allowed to stand in water bath till chloroform evaporates. Cream coloured precipitate observed on addition of Mayer's reagent indicates a positive test.

#### Test for Steroids and Triterpenoids

Liebermann Burchard test - Crude plant extract was mixed with few drops of acetic anhydride, boiled and cooled. To the sample, concentrated sulphuric acid was then added from the sides of the test tube and observed for the formation of a brown ring at the junction of two layers.

Green color formation at the upper layer and the formation of deep red color in the lower layer would indicate a positive test for steroids and triterpenoids respectively.

#### Test for Phlobatannins

1 ml of plant sample was taken in a test tube and few drops of concentrated HCl was added. It was allowed to stand in water bath for few minutes. Red coloured precipitates indicate the presence of phlobatannins.

#### Test for Reducing Sugar

To 2 ml of plant extract, 1 ml each of ethanol and Fehling solution A and B was added and boiled for few minutes. Presence of red colour precipitates indicates the presence of reducing sugars.

#### Test for Flavanoids

To test the presence of flavanoids in the plant sample, 5 ml of ammonia was added and few drops of concentrated sulphuric acid. Yellow colouration indicates a positive result.

## RESULTS AND DISCUSSIONS

### Phytochemical analysis

Freshly prepared extracts were subjected to a preliminary phytochemical screening for various constituents. The results of the phytochemical analysis of the different extracts have shown a remarkable variation, as tabulated in Table 1. Extract prepared from flower showed maximum presence of alkaloids, terpenoids and steroids with absence of tannins. Leaf and stem extracts showed presence of high quantity of terpenoids and phlobatannins, respectively. On the other hand, extract prepared from fruit showed the maximum quantity of reducing sugars, tannins and terpenoids. The present study regarding the qualitative analysis of the selected medicinal plants is in agreement with the previous findings by the various researchers.

### Antibacterial activity of the plant extracts

The antibacterial activities of the plant extracts prepared in different solvents are depicted in Table 2. The results indicated that the plants extracts showed antibacterial activities at variable degrees against pathogenic strains selected. The methanolic extracts of *M. calabura* flowers displayed the most important spectrum of activity, its inhibitory effects being observed against most of the bacterial strains selected, followed by the extracts prepared in acetone and acetonitrile. The water and ethanolic extracts showed less inhibitory effect against the bacteria. The methanolic flower extract showed maximum inhibitory effect against *B. megaterium* with a zone of inhibition measured about 21 mm in diameter, followed by *E.coli* and *P. aeruginosa* at 18 mm and 16 mm, respectively. The highest inhibitory activity was shown by the methanolic stem extract against *K. pneumonia* with 23 mm in diameter as zone of inhibition as measured.

On contrary, the water and ethanolic extract of ripened fruit did not show antibacterial activity against the majority of the bacteria tested. The microorganisms of the species *P. aeruginosa*, known for their multi-resistance to drugs, were almost resistant to all the plant extracts tested in this work except methanolic extract of flower. The methanolic extract exerted greater antibacterial activity than the other solvents extracts at the same concentration. These

observations (Table 2) may be attributed to two reasons; firstly, the nature of biologically active components (Alkaloids, terpenes, steroids, flavanoids and tannins) which could be enhanced in presence of methanol. It has been documented that these components are well known for anti-microbial activity<sup>22</sup>. Secondly, the stronger extraction capacity of methanol could have produced greater active constituents responsible for anti-microbial activity.

### Antibiofilm activity of *M. calabura* extracts

Biofilm formation inhibition results of plant extracts against *P. aeruginosa*, *E. coli*, *B. megaterium* and *K. pneumonia* indicated that the obtained effect was time-dependent. The assay was carried out for 72 hr and observed as shown in Fig 1. The best biofilm reduction was observed against the above mentioned strains and their percentage inhibition was tabulated in Table 3. The presence of methanolic flower extracts inhibited the biofilm formation. The maximum activity was observed against *K. pneumonia* with 92 % of reduction, followed by *P. aeruginosa* (81%) and *B. megaterium* (82%). As observed in the Fig.1, there is a difference in biofilm thickness. This could be due to different reasons such as differences in isolates capacity to adhere and form biofilm, the quantity of autoinducers released from each isolate may vary playing an essential as well as important role in biofilm formation<sup>23</sup>. Reports from Issac has also shown similar results, where the methanolic caper extract significantly inhibited biofilm formation and EPS production in *E. coli*, *P. aeruginosa*, *S. marcescens*, and *Proteus mirabilis*<sup>24</sup>. Inhibition of biofilm formation can be explained by the presence of flavonoids. From earlier reports by Vikram et al, compounds like quercetin, kaempferol, narngenin, and apigenin are capable of reducing biofilm synthesis because they can suppress the activity of the autoinducer-2 responsible for cell-to-cell communication<sup>25</sup>.

### Anti oxidant activity

The DPPH radical scavenging activity results are shown in Fig 2. The activities of the extracts were compared with known antioxidant Vitamin C. From the analysis of Fig 2, we can conclude that the scavenging effects of leaves and flower were excellent as compared to other extracts. The extracts of fruit (ripen and unripe) revealed a very poor antioxidant activity. The high percentage of antioxidant activity of the methanolic flower extracts (87 %) from *M. calabura* could be attributed to the presence of flavonoid constituents, as was evidenced by the phytochemical screening Table 1. As reported earlier, these flavonoids acts as glycosides in plants. Phenols and polyphenols exert their protective effects through diverse mechanism such as blocking, interfering or suppressing the activities of enzymes involved in reactive oxygen species generation, quenching free radicals, chelating transition metals to render inactive species<sup>26</sup>.

### Lectin activity

Protein estimation showed that leaf extract has more protein content of about 1 mg/ml followed by fruits (0.8 mg/ml) and flower (0.6 mg/ml) extracts. Very less quantity was observed in fruit extracts of *M. calabura*. The

carbohydrate binding activity was also determined using rabbit's erythrocytes. Surprisingly, it was observed that, the flower extract showed maximum lectin activity followed by leaf extract. Many lectins are also known to possess antimicrobial and anti cancer activities<sup>27,28</sup>.

## CONCLUSIONS

The present work on different extracts prepared from *M. calabura* offer a scientific basis for traditional use of both water and methanol extracts as potent antimicrobial and antifouling agents. Phytochemical screening of *M. calabura* extracts revealed the presence of sterols, flavonoids, alkaloids, and tannins. The maximum antimicrobial activity was observed against 4 species. This activity could be enhanced if active components are purified and adequate dosages are determined using in vivo studies for proper administration. The work also concluded the presence of good amount of lectin activity, if purified can be used for drug targeted therapy depending on its carbohydrate specificity.

## CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

## ACKNOWLEDGMENTS

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