

## Nano-Green Betel Leaf Extracts (*Piper betle* L.) Inhibits the Growth of *Streptococcus mutans* and *Staphylococcus aureus*

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**Abstract:** *Streptococcus mutans* is a type of bacterium that initiates plaque formation on the tooth surface causing tooth decay, meanwhile, *Staphylococcus aureus* causes pyogenic infections such as abscesses to necrosis. Green betel leaf (*Piper betle* L.) contains secondary meta-bolites that have the potential as antibacterial. This study aimed to investigate the effectiveness of green betel leaf extract (*Piper betle* L.) in micro and nano sizes against *Streptococcus mutans* and *Staphylococcus aureus*. This was an experimental and laboratory study with a post-test-only design. The results showed that nano-green betel leaf extracts had antibacterial activity against *Streptococcus mutans* and *Staphylococcus aureus*. Green betel leaf extract had a much larger inhibition zone against *Staphylococcus aureus* rather than against *Streptococcus mutans* in all groups ( $p < 0.05$ ), with inhibitory diameters of  $13,883 \pm 1.1496$  mm (micro 10%),  $16,767 \pm 1.8779$  mm (micro 30%), and  $18,667 \pm 3.148$  mm (nano), respectively. A stable increase in antibacterial activity was derived from micro-green betel leaf extracts (*Piper betle* L.) concentrations of 10%, 30%, and nanoparticle size. In conclusion, nano-green betel leaf extract (*Piper betle* L.) showed better antibacterial effectiveness than micro-sizes in inhibiting *Streptococcus mutans* and *Staphylococcus aureus* bacteria.

**Keywords:** *Piper betle* L.; nanoparticles; antibacterial; *Streptococcus mutans*; *Staphylococcus aureus*

### INTRODUCTION

Caries is a multifactorial infectious disease that interacts with each other.<sup>1</sup> Several factors that can trigger the occurrence of caries include host, substrate, time, and bacteria.<sup>2</sup> Bacteria that play a significant role in caries formation are *Streptococcus mutans*.<sup>3</sup> *S. mutans* is a normal flora often found in the oral cavity but causes tooth decay by forming plaque on the tooth surface.<sup>4</sup> Apart from dental caries, pyogenic infections in the oral cavity are also of concern, impacting patient productivity.<sup>5</sup> Pyogenic infections are characterized by *Staphylococcus aureus*'s local inflammation, necrosis, and abscess formation.<sup>6,7</sup> As one of the normal microflora in the oral cavity, *S aureus*

could turn into a pathogen when its quantity increases and the host's immune system decreases.<sup>8</sup>

Green betel leaf (*Piper betle* L.) is a plant in the *Piperaceae* family, genus *Piper*,<sup>9</sup> with the characteristic of yellowish-green to dark green leaves and a glossy upper surface.<sup>10</sup> Green betel leaves are oval-shaped, pointed, have a stalk, has a length of 5–18 cm with a width of 3–12 cm.<sup>11</sup> Green betel leaf contains essential oils, giving it an aroma that varies from sweet to spicy.<sup>12</sup> In addition, essential oils produce a distinctive aroma with thick and slightly oily characteristics.<sup>13</sup> Betel plants are often used for the treatment of infectious diseases.<sup>14</sup> In dentistry, betel leaf has been studied for its effectiveness in

strengthening gums, controlling halitosis, and preventing tooth decay.<sup>15</sup> Previous research has shown that green betel leaf contains alkaloids, flavonoids, tannins, saponins, phenolics, and terpenoid compounds that inhibit bacterial growth.<sup>16</sup> These phytochemical compounds are also found to possess anti-inflammatory properties in other medicinal plants, namely: *Aloe vera*, *Citrus amblycarpa*, and *Passiflora edulis*.<sup>17-19</sup>

Nanoparticles' sizes range from 10-1000 nm, which can form reactive oxygen species in the bacterial cell wall and cause damage to the wall.<sup>20</sup> These nanoparticles have better antibacterial and anti-inflammatory efficacy by increasing their ability to penetrate cell walls.<sup>21</sup> Nanoparticles in natural leaf extracts were found to reduce the treatment dose, increase the compound's solubility, and increase absorption.<sup>22</sup> This study intended to investigate the role of particle sizes on the antibacterial effectiveness of green betel leaf extract (*Piper betle* L.) by comparing microparticles with nanoparticles. We hypothesized that nano-sizes have better anti-bacterial capacity than micro-sizes.

## METHODS

This was an experimental laboratory study with a post-test-only design. In six experimental groups, we investigated green betel leaf extract power against two common oral bacteria. Each group was replicated six (6) times, with a total study size of 36 samples. Samples used in this study were micro (10% and 30%) and nanoparticles of green betel leaf extract, and pure cultures of *S. mutans* and *S. aureus* bacteria. The green betel leaf extracts used in the study were 10% of microparticles, 30% of microparticles, and nanoparticles. The extraction process was carried out by the maceration method. Fresh green betel leaves (5 kgs) were washed and oven-dried for a few days. Then 125 grams dried samples were pulverized into powder, 350 ml of 70% ethanol were added, covered with aluminum foil, and shaken for 24 hours before filtering using a vacuum pump. One gram of filtrate was evaporated using a rotary evaporator for 4-5 hours at 40°C. Then two concentrations of 10% and 30% were made

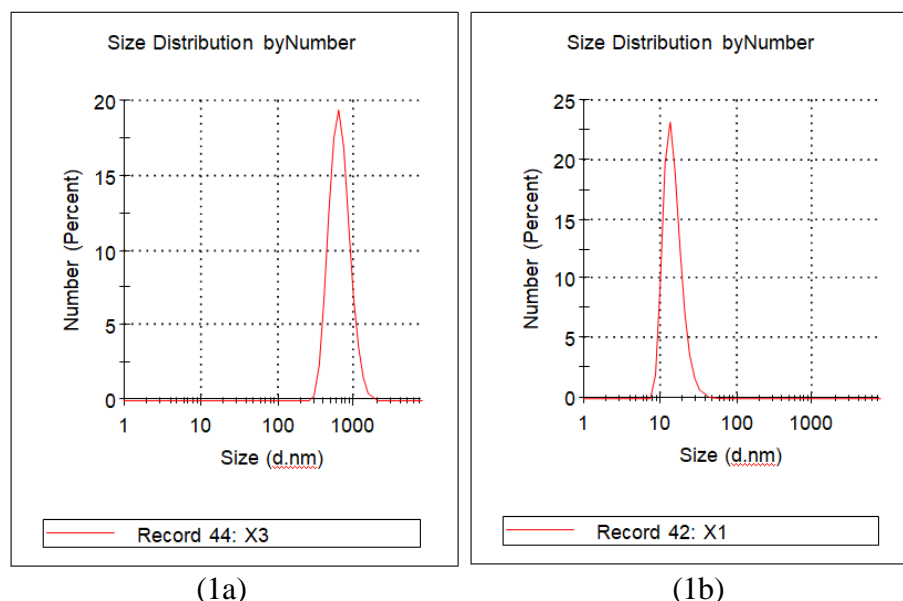
in microparticle size by adding DMSO solvent. Nanoparticles were formulated by mixing 1.5 grams of filtrate of extracted green betel leaf with 10 ml of propylene glycol and 5 ml of isopropyl myristate, and stirred for 15 minutes to form an oil phase. A new beaker glass was prepared and added 35 ml of PEG and 5 ml of tween 80 into it and stirred for 10 minutes. Then the two solutions were mixed using an ultrasonication stirrer for 15 minutes. The micro and nano-green betel leaf extracts were analyzed to confirm their particle sizes. Phytochemical screening used Gas Chromatography-Mass (GC-MS) to examine the chemical compounds in the extracts in both particle sizes.<sup>23</sup>

*S. mutans* and *S. aureus* were cultured by placing one ose (10 µl) of bacterial culture in a test tube containing 1 ml of 0.9% NaCl solution, then were stirred until homogeneous and incubated at 37°C for 24 hours. The antibacterial activity test was carried out by the disc diffusion method. Medium Nutrient Agar (NA) was poured into a sterile petri dish and allowed to solidify. Then the bacterial suspension was scratched using a loop on the surface of the NA medium. Paper disc dripped with green betel leaf extracts were placed in the Petri dishes. Then the Petri dish was aerobically incubated at 37°C for 24 hours, observed as an inhibition zone. The clear zone formed around the disc was measured using a caliper expressed in millimeters.

## RESULTS

The sample used in this study was identified as *Piper betle* Linn (identification number 6795/MEDA/2022). The diameter of microparticle green betel leaf extract analyzed was 656.1 nm, while of nano-particles were figured out at 15.21 nm (Figure 1). Phytochemical testing using the GC-MS showed the compounds contained by microparticles and nanoparticles of green betel leaf extracts (*Piper betle* L.) (Table 1a and 1b).

There was a consistent trend for *S. aureus*, in which the inhibition strength for 30% and nanoparticles were intensified, i.e., 16.767±1.8779 and 18.667± 3.1948, consecutively.



**Figure 1.** Particle size of green betel leaf extracts (1a) microparticles, and (1b) nanoparticles

**Table 1a.** GC-MS phytochemical analysis of micro-green betel leaf extracts

RT	Area %	Name	DB Formula
6.468	41.75	3-Allyl-6-methoxy phenol	C <sub>10</sub> H <sub>12</sub> O <sub>2</sub>
5.415	26.13	chavicol	C <sub>9</sub> H <sub>10</sub> O
7.373	11.86	4-Chromanol	C <sub>9</sub> H <sub>10</sub> O <sub>2</sub>
11.991	2.36	1-Octadecanol	C <sub>18</sub> H <sub>38</sub> O
12.194	2.13	Ethyl(9E)-9-Octadecenoate	C <sub>20</sub> H <sub>38</sub> O <sub>2</sub>
9.829	1.83	4-Hydroxy-3,5,5-trimethyl-4-(3-oxo-1butenyl)-2-cyclohexen-1-one	C <sub>13</sub> H <sub>18</sub> O <sub>3</sub>
10.845	0.72	Dodecylpalmitate	C <sub>28</sub> H <sub>56</sub> O <sub>2</sub>
12.545	0.48	Phytol Acetate	C <sub>22</sub> H <sub>42</sub> O <sub>2</sub>

**Table 1b.** GC-MS phytochemical analysis of nano-green betel leaf extracts

RT	Area %	Name	DB Formula
12.009	40.46	cis-13-Octadecenoic acid	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>
11.751	10.93	9-Octadecenoic acid (Z)-, methyl ester	C <sub>19</sub> H <sub>36</sub> O <sub>2</sub>
9.922	7.89	Isopropyl myristate	C <sub>17</sub> H <sub>34</sub> O <sub>2</sub>
10.845	7.67	n-Hexadecanoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>
6.468	6.8	Phenol,2-methoxy-4-(2-propenyl)-	C <sub>10</sub> H <sub>12</sub> O <sub>2</sub>
5.433	4.98	Chavicol	C <sub>9</sub> H <sub>10</sub> O
12.194	4.86	Ethyl (9E)-9-Octadecenoate	C <sub>20</sub> H <sub>38</sub> O <sub>2</sub>
7.945	4.22	Dodecanamide,N,N-bis(2-hydroxyethyl)	C <sub>16</sub> H <sub>33</sub> NO <sub>3</sub>
6.32	1.54	Decanoic acid	C <sub>10</sub> H <sub>20</sub> O <sub>2</sub>
10.605	1.18	Hexadecanoic acid, methyl ester	C <sub>17</sub> H <sub>34</sub> O <sub>2</sub>

The statistical analysis using SPSS v.25 showed a non-significant difference within the *S. mutans* group ( $p > 0.05$ ). In contrast, within *S. aureus* groups, significant differences were

noted ( $p < 0.05$ ). There was also a significant difference between *S. mutans* and *S. aureus* in nanoparticles groups ( $p < 0.05$ ) (Table 2).

The inhibition zone of green betel leaf

extracts against *S. mutans* and *S. aureus* demonstrated an increasing trend in micro-particle groups, in which 30% concentration had higher power than the 10%. The nanoparticle betel leaf extracts showed higher capability in preventing *S. mutans* and *S. aureus* from cultivating than the microparticle groups (Figure 2).

**DISCUSSION**

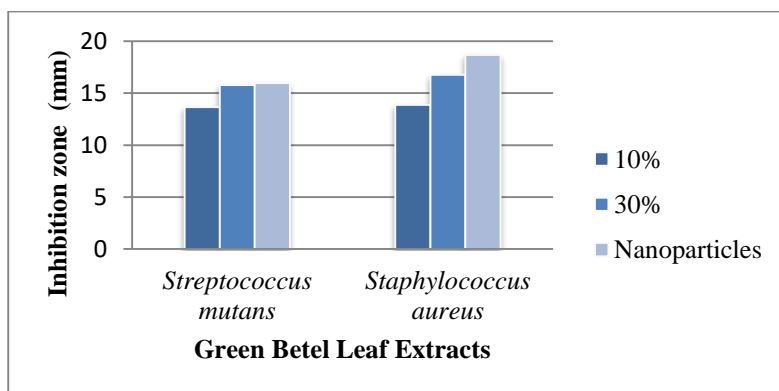
The size of green betel leaf extracts was recorded at 656.1 nm for microparticles and 15.21 nm for nanoparticles. The particles size impacted their antibacterial capacity, where nano-green betel leaf extracts raise the inhibition diameter against *S. mutans* by 0.4 mm, and 3.2 mm against *S. aureus* compared to micro-particles size. In line with our findings, Rahmat et al<sup>25</sup> concluded that micro-pineapple extracts had moderate efficacy (17.3125 mm), whereas nano-pineapple extracts exhibited more substantial potential (23.025 mm) against *S. aureus*. The increment in antibacterial power was also seen in a study explored by Saputra and Susanty<sup>26</sup> using

gambir against Streptococcus mutans from 15.33 mm (gambir) to 27.0 mm (nanogambir). These studies supported the hypothesis that particle size affected the antibacterial potential of natural products.

As shown in Figure 2, the nanoparticle betel leaf extracts showed higher capability in preventing *S. mutans* and *S. aureus* from cultivating than the microparticle groups. Research by Novita<sup>27</sup> on 0.3125 mg/ml, 0.625 mg/ml, 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml, and 10 mg/ml of betel leaf extracts (*Piper betle* L.) disclosed an increase steadily in the inhibition section from 0.60 mm up to 16.00 mm. These findings also followed a study conducted by Bustanussalam et al<sup>28</sup> who tested green betel leaf extract against *S. aureus* with concentrations of 5%, 10%, 15%, 20%, and 25%. The bacteria were eliminated at approximately 1.07 mm, 1.29 mm, 1.31 mm, 1.52 mm, and 1.66 mm, respectively. These studies showed a proportional relationship between increasing concentration and amplifying the clear zone formed.<sup>29</sup>

**Table 2.** Inhibition zone of green betel leaf against *S. mutans* and *S. aureus*

Green betel leaf concentration	Inhibition zone (mm)	
	<i>S. mutans</i>	<i>S. aureus</i>
10% Microparticles	13.667±0.7659	13.883±1.1496
30% Microparticles	15.783±2.2013	16.767±1.8779
Nanoparticles	15.967±2.4022	18.667±3.1948
p-value	0.105	0.007



**Figure 2.** Inhibition zone of green betel leaf extracts in various sizes against *S. mutans* and *S. aureus*

Phytochemical testing using the GC-MS method on green betel leaf (*Piper betle* L.) in micro-particle size showed that 87.26% contained eight secondary metabolites that function as antibacterial (Table 1a). The significant component of eugenol, 3-Allyl-6-methoxy phenol (C<sub>10</sub>H<sub>12</sub>O<sub>2</sub>), was found 41.75% of the total compounds, with antifungal properties and could inhibit aflatoxins.<sup>30</sup> The hydrophobicity of oil in eugenol has the potential to partition lipids and disrupt the outer membrane of Gram-positive and Gram-negative bacteria, thereby increasing the effectiveness of eugenol.<sup>31</sup> Eugenol is often found in phytochemical reactions and has functioned as an anti-inflammatory, antioxidant, anticancer, and insecticide.<sup>32</sup> Chavicol with the formula (C<sub>9</sub>H<sub>10</sub>O) (26.13%) is a derivative of phenolic compounds, which have many benefits including antioxidant, anticancer, anti-inflammatory effects, and can inhibit and kill bacteria.<sup>33</sup> Phenolic compounds work by blocking the entrance of toxins to the target and destroying the lipid membrane of bacteria.<sup>34</sup> Another active compound with the potential as antibacterial and antibiofilm is 4-Chromanol with the formula (C<sub>9</sub>H<sub>10</sub>O<sub>2</sub>), which accounted for 11.86%,<sup>35</sup> and was effective as an antifungal.<sup>36</sup> The ability of the 4-Chromanol inhibition zone against pathogens in the oral cavity has the potential to overcome membrane-related resistance of Gram-negative bacteria.<sup>37</sup>

Meanwhile, nano-green betel leaf (*Piper betle* L.) has 90.35% secondary metabolites with ten characteristics, as shown in Table 1b. The primary chemical compound was cis-13-Octadecenoic acid (C<sub>18</sub>H<sub>34</sub>O<sub>2</sub>) which was a compound of elaidic acid<sup>38</sup> that functioned as antibacterial and antifertility.<sup>39</sup> In addition, cis-13-Octadecenoic acid is also an anti-inflammatory and cancer prevention compound.<sup>40</sup> Moreover, 9-Octadecenoic acid (Z)-, a methyl ester with the formula (C<sub>19</sub>H<sub>36</sub>O<sub>2</sub>), is a derivative of fatty acid methyl ester, which has antimicrobial, anti-oxidant, and anticancer abilities.<sup>41</sup> The fatty acid methyl ester is known to have an antifungal function.<sup>42</sup> Isopropyl myristate with the formula (C<sub>17</sub>H<sub>34</sub>O<sub>2</sub>) is an ester derivative of isopropanol and myristic acid,<sup>43</sup> that can be an anti-

bacterial and antioxidant.<sup>44</sup> n-Hexadecanoic acid with the formula (C<sub>16</sub>H<sub>32</sub>O<sub>2</sub>) (7.67%) has functioned as antimicrobial, antioxidant,<sup>45</sup> antibacterial, and antifungal.<sup>46</sup> In addition, n-Hexadecanoic acid is also found to have anti-inflammatory properties.<sup>47</sup> Phenol,2-methoxy-4-(2-propenyl) with the formula (C<sub>10</sub>H<sub>12</sub>O<sub>2</sub>) (6.8%) is a derivative of eugenol compounds<sup>48</sup> and has the ability as antibacterial, antifungal, antioxidant, and anti-inflammatory.<sup>49</sup> Dodecanamide, N, N-bis(2-hydroxyethyl) with the formula (C<sub>16</sub>H<sub>33</sub>NO<sub>3</sub>) is a derivative of amide compounds in the form of fatty acids<sup>50</sup> with function as an antiviral.<sup>51</sup> Decanoic acid (capric acid) with the formula (C<sub>10</sub>H<sub>20</sub>O<sub>2</sub>) (1.54%) is a fatty acid that has antibacterial properties that are effective against Gram-positive bacteria.<sup>52</sup> Fatty acids work by entering into bacterial cell membranes and causing intracellular acidification, therefore, they can inhibit bacterial growth.<sup>53</sup> Hexadecanoic acid, a methyl ester with the formula (C<sub>17</sub>H<sub>34</sub>O<sub>2</sub>) (1.18%), is an unsaturated fatty acid<sup>54</sup> that functions as antibacterial, anti-allergic,<sup>55</sup> anti-oxidant, antimicrobial, anti-inflammatory, and has the ability of cytotoxic activity.<sup>56</sup>

## CONCLUSION

Green betel leaf extract (*Piper betle* L.) has antibacterial effectiveness against *Streptococcus mutans* and *Staphylococcus aureus*. In the antibacterial test using the diffusion method, the extract of green betel leaf (*Piper betle* L.) with microparticle size in a concentration of 30% showed an increased efficacy than a concentration of 10%. Green betel leaf extract (*Piper betle* L.) in nanoparticle size has a better antibacterial value than in microparticles in inhibiting *Streptococcus mutans* and *Staphylococcus aureus* bacteria.

## Conflict of Interest

The authors declare no conflicts of interest.

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