Chitosan-propolis nanoformulation for combating *Enterococcus faecalis* biofilms in vitro.

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Propolis

✓ Brown resinous substance gathered by bees from various plants

✓ Antibacterial, antifungal, antiviral, antioxidant and anti-inflammatory activity

✓ The chemical composition of propolis is comprised of flavonoids, steroids, amino acids, terpenes, phenolic and aromatic compounds
## Components isolated from propolis

<table>
<thead>
<tr>
<th>Flavinoids</th>
<th>Isolated for propolis ethanolic extract</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flavones</strong></td>
<td>Chrysin, Apigenin, Luteolin</td>
</tr>
<tr>
<td><strong>Flavonols</strong></td>
<td>Rutin, Morin, Quercetin, Myricetin, Kaempferol, Quercitrin, Galangin</td>
</tr>
<tr>
<td><strong>Flavanones</strong></td>
<td>Naringin, Naringenin, Hesperetin</td>
</tr>
<tr>
<td><strong>Isoflavones</strong></td>
<td>Daidzein, Genistein</td>
</tr>
</tbody>
</table>

Add propolis into ethanol solution

Shake at 37°C for 48 hours

Filter the propolis solution

Concentrate by using a rotary vacuum evaporator

Stored in the dark at 4°C for further analysis.
Chitosan

✓ Biopolymer obtained from crustacean shells
✓ Possesses biocompatibility, biodegradability, nontoxicity and biological properties
✓ Potential application in drug delivery system
Chitosan application

Chitosan propolis nanoformulation

1. Add propolis extract into chitosan solution
2. Sonicate/High pressure the suspension
3. Centrifuge the suspension
4. Purify the nanoformulation
HPLC analysis - standards

Identification of standard flavonoid markers compound (Retention time)
Gradient method

Representative chromatogram of flavonoids at 260nm
### Retention time and linearity - standards

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Retention time (Minutes)</th>
<th>Wavelength: 260nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Regression equation</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>4.6</td>
<td>y=24.086x + 0.0091</td>
</tr>
<tr>
<td>Rutin</td>
<td>9.9</td>
<td>y=35.999x + 5.5071</td>
</tr>
<tr>
<td>Quercetin</td>
<td>12.8</td>
<td>y=81.395x + 5.0073</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>13.3</td>
<td>y=50.948x + 5.7558</td>
</tr>
<tr>
<td>Luteolin</td>
<td>14.1</td>
<td>y=45.031x + 2.3188</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>15.4</td>
<td>y=37.401x + 2.9118</td>
</tr>
<tr>
<td>Apigenin</td>
<td>15.7</td>
<td>y=44.385x − 1.5019</td>
</tr>
<tr>
<td>Pinocembrin</td>
<td>17.6</td>
<td>y=11.522x + 3.3704</td>
</tr>
</tbody>
</table>
Chromatogram – propolis extract
### Identify components in propolis

<table>
<thead>
<tr>
<th>Standard markers</th>
<th>Flavonoid content (ug/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Propolis ethanol extract</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>NA</td>
</tr>
<tr>
<td>Rutin</td>
<td>NA</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1.4348</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>NA</td>
</tr>
<tr>
<td>Luteolin</td>
<td>0.6052</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>5.8832</td>
</tr>
<tr>
<td>Apigenin</td>
<td>1.2224</td>
</tr>
<tr>
<td>Pinocembrin</td>
<td>5.64</td>
</tr>
</tbody>
</table>

Out of 8 standard flavonoids compounds used, we are able to detect 5 of them in Malaysian propolis.
Identification of pinocembrin (Retention time)
Isoocratic method
## Retention time and linearity

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<thead>
<tr>
<th>Compounds</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Regression equation</td>
</tr>
<tr>
<td>Pinocembrin</td>
<td>7.6</td>
<td>$y=9.7579x + 2.572$</td>
</tr>
</tbody>
</table>

Pinocembrin – used as marker compound
Chitosan propolis nano-formulation

- **High performance liquid chromatography analysis**
- **Transmission electron microscopy**
- **Zetasizer**

**Test the presence of markers for the formulation**

**Size, Structure**

**Stability-Zeta potential, Size, Aggregation, No. of particles**
Characterization of nanoparticles

- Particle size distribution
- Surface morphology
- Encapsulation efficiency
- In-vitro release
Physical characterization nanoparticles

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Average particle size (nm)</th>
<th>Polydispersity index (PDI)</th>
<th>Zeta potential (mV)</th>
<th>Encapsulation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan-TPP blank</td>
<td>125.7 ± 0.53</td>
<td>0.438 ± 0.01</td>
<td>35.5 ± 0.91</td>
<td>-</td>
</tr>
<tr>
<td>F1</td>
<td>247.1 ± 1.7</td>
<td>0.225 ± 0.013</td>
<td>45.2 ± 0.26</td>
<td>88.8</td>
</tr>
<tr>
<td>F2</td>
<td>427.1 ± 8.9</td>
<td>0.499 ± 0.012</td>
<td>64 ± 1.89</td>
<td>91.43</td>
</tr>
<tr>
<td>F3</td>
<td>512.3 ± 15.4</td>
<td>0.573 ± 0.07</td>
<td>74.1 ± 2.75</td>
<td>91.11</td>
</tr>
<tr>
<td>F4</td>
<td>198 ± 3</td>
<td>0.453 ± 0.012</td>
<td>48.2 ± 0.85</td>
<td>77.65</td>
</tr>
<tr>
<td>F5</td>
<td>308.3 ± 6.8</td>
<td>0.264 ± 0.001</td>
<td>49 ± 1.37</td>
<td>88.17</td>
</tr>
<tr>
<td>F6</td>
<td>349.9 ± 2.3</td>
<td>0.371 ± 0.053</td>
<td>52.9 ± 3.5</td>
<td>88.2</td>
</tr>
</tbody>
</table>
Particle size and zeta potential of F1
Physical characterization of nanoparticles

- Propolis loaded chitosan nanoparticle > Chitosan blank
- Chitosan concentration
- Propolis concentration
- Surfactant

Factors that will influence the particle size

Polydispersity index (PDI)

Polydispersity index ranging from 0.225 to 0.573.
Chitosan-TPP nanoparticles are generally characterized by a positive zeta potential.

- Positive zeta potential ranging from +35.5mV to +74.1 mV.
Surface morphology of nanoparticles (SEM)
Nano particle - encapsulation efficiency

Encapsulation efficiency (%) = \left( \frac{\text{Amount of propolis added - free propolis}}{\text{Amount of propolis added}} \right) \times 100\%

Factors that will influence the encapsulation efficiency

- Chitosan concentration
- Propolis concentration
Nano particle - encapsulation efficiency
Nano particle - encapsulation efficiency
In vitro release – encapsulation efficacy

![Graph showing in-vitro propolis release from chitosan nanoparticle in dialysis tubing at 37±0.5°C, mean ± SD, n=3](chart.png)
**In vitro release – encapsulation efficacy**

- Pure propolis solution exhibited a burst release with $39.21\% \pm 3.67\%$ within the first hour and released up to $89.23\% \pm 4.52\%$ within 48 hours.

- Chitosan-propolis nanoparticles demonstrated a controlled and extended release profile up to 48 hours, with a total release of $53.78\% \pm 4.89\%$. 
Enterococcus faecalis

- Gram-positive cocci, normal intestinal flora of humans and animals
- A major cause of nosocomial infections
- Capable of surviving harsh environments
- Urinary tract infection, nosocomial bacteremia and endocarditis
- Biofilm formation
Enterococci

From Commensals to Leading Causes of Drug Resistant Infection

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Massachusetts Eye and Ear Infirmary
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Enterococcal Resistance

Enterococci are gram-positive cocci that grow in chains in broth media and clinical specimens. They are indistinguishable microscopically from streptococci and were originally classified as group D streptococci under the old Lancefield classification. However, enterococci are genetically quite different from true streptococci and, for that reason, have been classified as a separate genus (the genus enterococcus). This genus now contains more than a dozen species but only a relatively small number of these are important as human pathogens. A recent study of bloodstream isolates of enterococci in the United States (US) confirms that E. faecalis are still the most frequent cause of enterococcal infections in man, followed by E. faecium (Table 1). The data in Table 1 document a clear-cut decrease in the overall occurrence of enterococcal resistance compared to previous reports. The rise of vancomycin-resistant enterococci (VRE) and the emergence of trimethoprim-sulfamethoxazole-resistant enterococci (TSR) is a matter of concern. The selection of suitable antimicrobial agents against enterococci is greatly influenced by their biological characteristics and resistance patterns.
Antibacterial efficacy of propolis against *E. faecalis*
Antibacterial efficacy of nano-propolis against *E. faecalis*
0.5 McFarland of *E. faecalis* bacteria with propolis

Incubation for 24 hours @ 37ºC

Remove the planktonic bacteria

Stain with CV

Rinse the 96 well plate

Solubilize the CV with absolute ethanol

Measure absorbance at 600nm
Crystal violet assay

- Quantification of static biofilm

Lower intensity = Lesser biofilm mass

Higher intensity = More biofilm mass
## Percentage reduction of viable bacteria in biofilms

<table>
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<tr>
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<th>Propolis ethanol extract</th>
<th>Chitosan-propolis nanoparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td>50µg/ml</td>
<td>23.08%</td>
<td>22.73%</td>
</tr>
<tr>
<td>100µg/ml</td>
<td>47.31%</td>
<td>54.55%</td>
</tr>
<tr>
<td>125µg/ml</td>
<td>68.08%</td>
<td>68.18%</td>
</tr>
<tr>
<td>200µg/ml</td>
<td>79.23%</td>
<td>81.36%</td>
</tr>
</tbody>
</table>
Nano Propolis against *E. faecalis* biofilms

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<th>Propolis ethanol extract</th>
<th>Chitosan - propolis nanoparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td>50µg/ml</td>
<td>26.92%</td>
<td>36.84%</td>
</tr>
<tr>
<td>100µg/ml</td>
<td>34.62%</td>
<td>47.37%</td>
</tr>
<tr>
<td>125µg/ml</td>
<td>42.31%</td>
<td>55.79%</td>
</tr>
<tr>
<td>200µg/ml</td>
<td>53.85%</td>
<td>58.95%</td>
</tr>
</tbody>
</table>
Scanning Electron Microscopy

Control

Propolis

Nano-propolis
Thank You

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