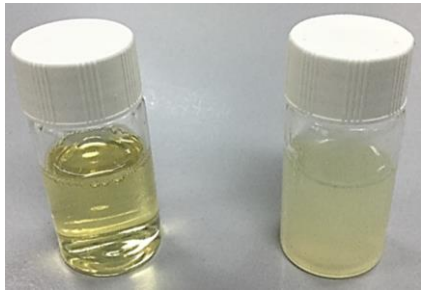
Chitosan-propolis nanoformulation for combating *Enterococcus faecalis* biofilms *in vitro*.

Fabian Davamani*, Ong Teik Hwa*, Ebenezer Chitra*, Srinivasan R*, Rajinikanth P*, Yuen Kah Key# and Stephen Ambu*.

*International Medical University , Kuala Lumpur 57000, Malaysia
#University Sains Malaysia. Penang, Malaysia.







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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

Flavinoids	Isolated for propolis ethanolic extract
Flavones	Chrysin , Apigenin, Luteolin
Flavonols	Rutin, Morin, Quercetin, Myricetin, Kaempferol, Quercitrin, Galangin
Flavanones	Naringin, Naringenin, Hesperetin
Isoflavones	Daidzein, Genistein

Chang CC, Yang MH, Wen HM, Chern JC. 2002. Estimation of Total Flavonoid Content in Propolis by Two Complementary Colorimetric Methods. Journal of Food and Drug Analysis,(10) 3,178-182.)

Propolis extract preparation

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.

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Chitosan

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

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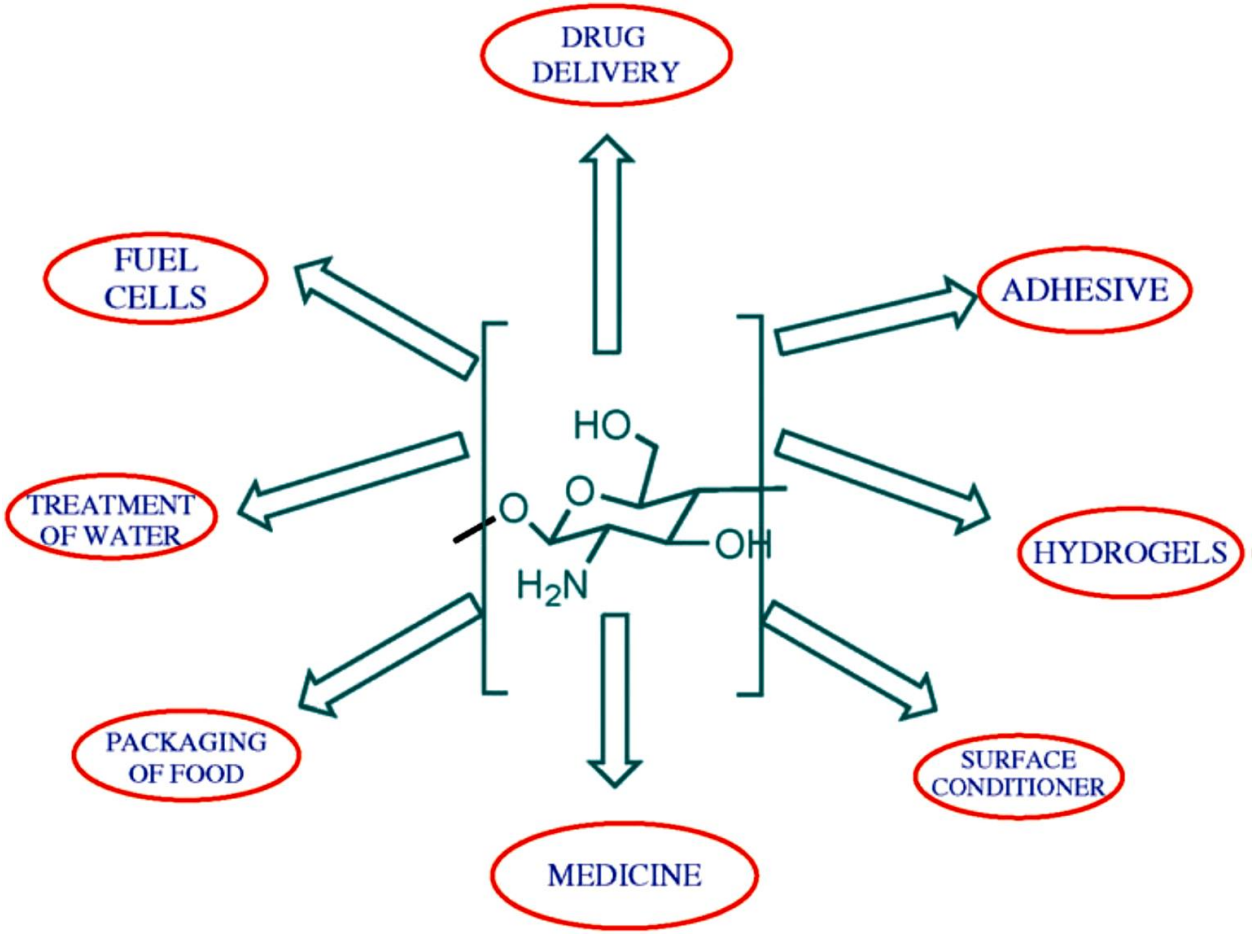
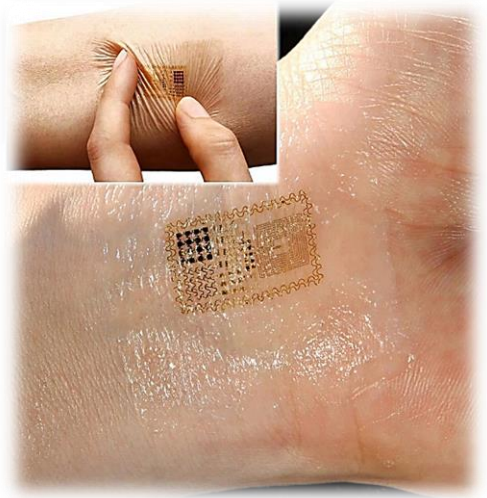
Chitosan application





New J. Chem., 2014, 38, 3181--3186

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Chitosan propolis nanoformulation

Add propolis extract into chitosan solution

Sonicate/High pressure the suspension

Centrifuge the suspension

Purify the nanoformulation

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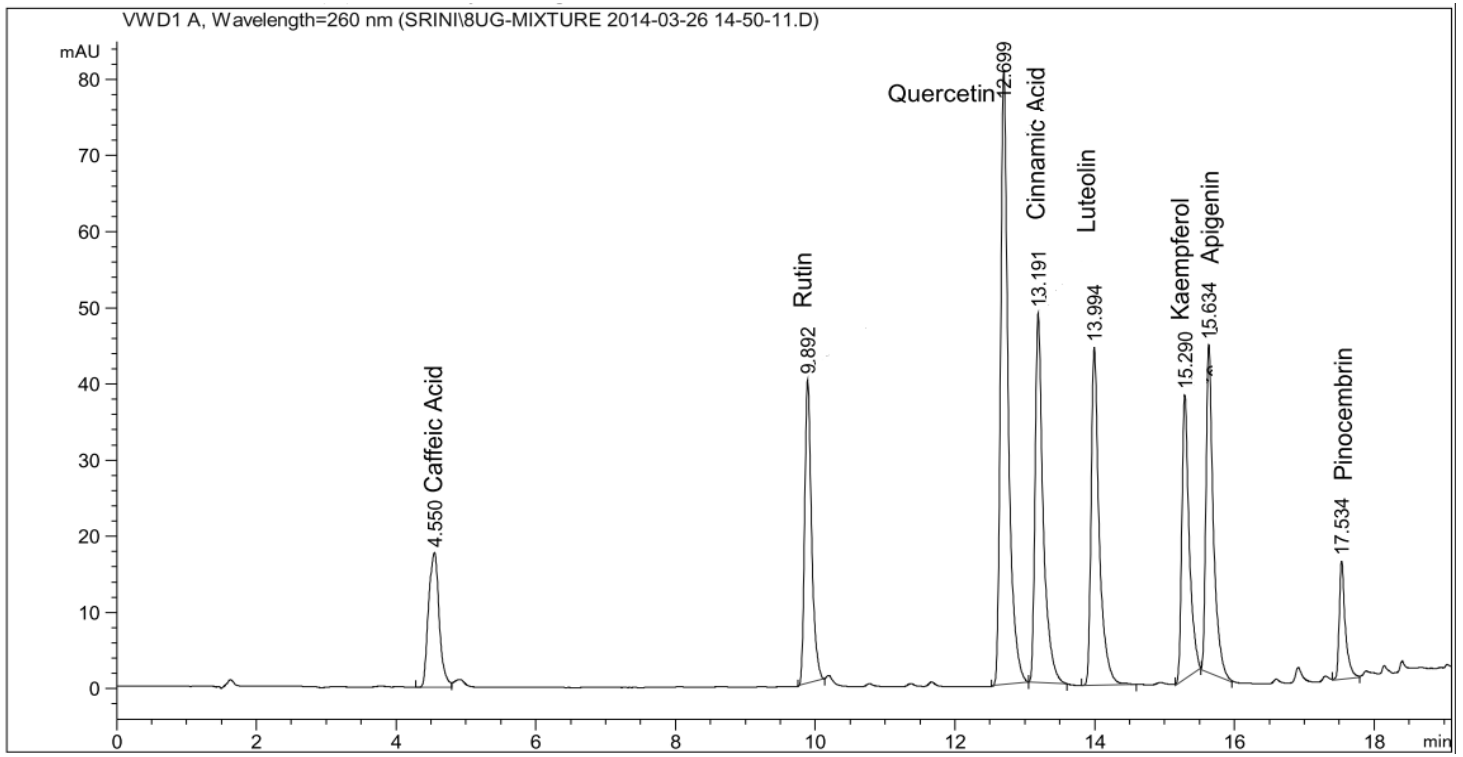
HPLC analysis - standards

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



Representative chromatogram of flavonoids at 260nm

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Retention time and linearity - standards

Compounds	Retention time (Minutes)	Wavelength: 260nm	
		Regression equation	Correlation coefficient (r^2)
Caffeic acid	4.6	y=24.086x + 0.0091	0.9975
Rutin	9.9	y=35.999x + 5.5071	0.9989
Quercetin	12.8	y=81.395x + 5.0073	0.9967
Cinnamic acid	13.3	y=50.948x + 5.7558	0.9989
Luteolin	14.1	y=45.031x + 2.3188	0.9989
Kaempferol	15.4	y=37.401x + 2.9118	0.9982
Apigenin	15.7	y=44.385x – 1.5019	0.9973
Pinocembrin	17.6	y=11.522x + 3.3704	0.9954

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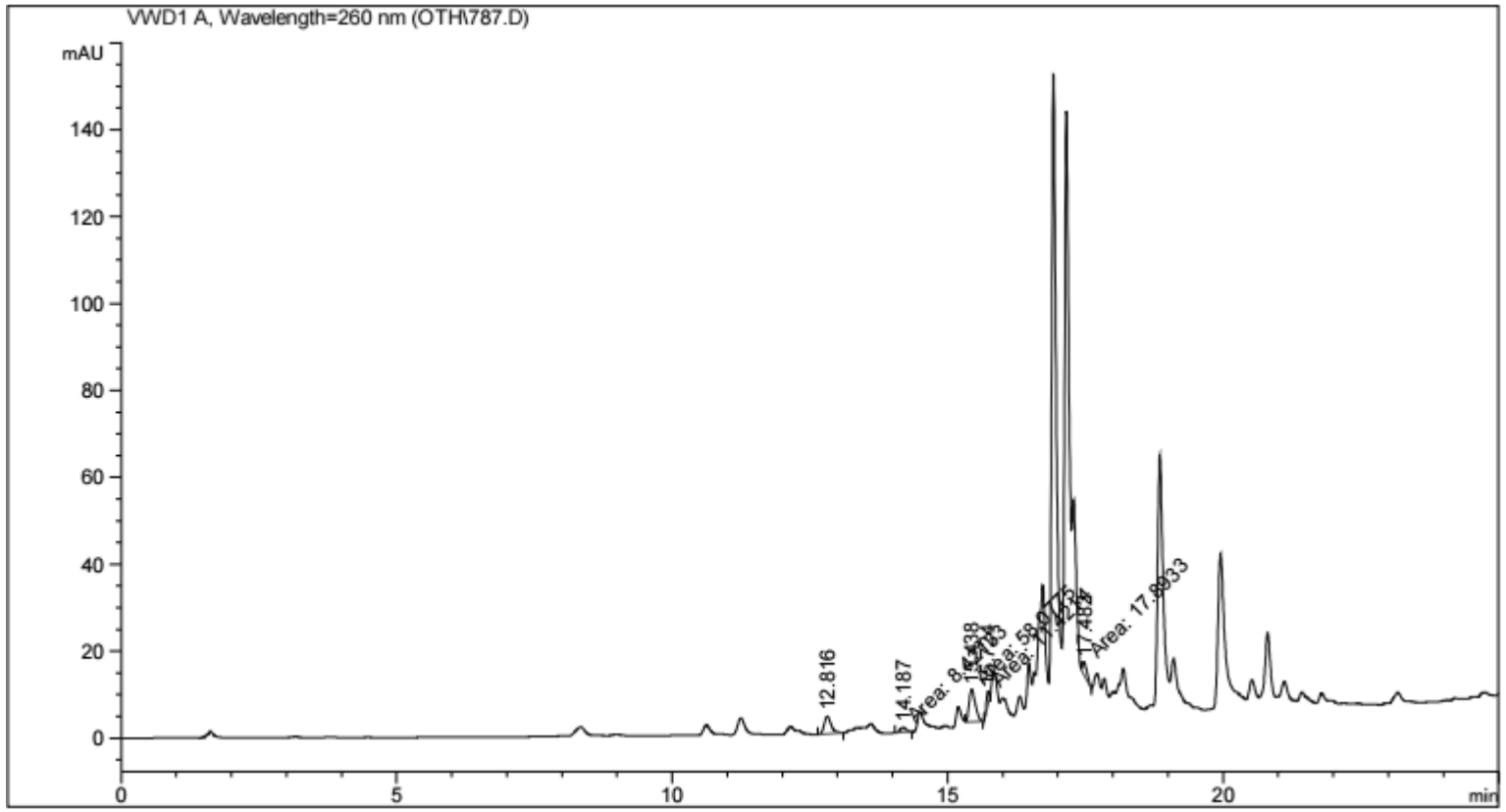
Chromatogram – propolis extract



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Identify components in propolis

Standard markers	Flavonoid content (ug/mL)	
	Propolis ethanol extract	Propolis ethyl acetate extract
Caffeic acid	NA	NA
Rutin	NA	NA
Quercetin	1.4348	1.392
Cinnamic acid	NA	NA
Luteolin	0.6052	0.5096
Kaempferol	5.8832	5.616
Apigenin	1.2224	1.12
Pinocembrin	5.64	4.0612

Out of 8 standard flavonoids compounds used, we are able to detect 5 of them in Malaysian propolis.

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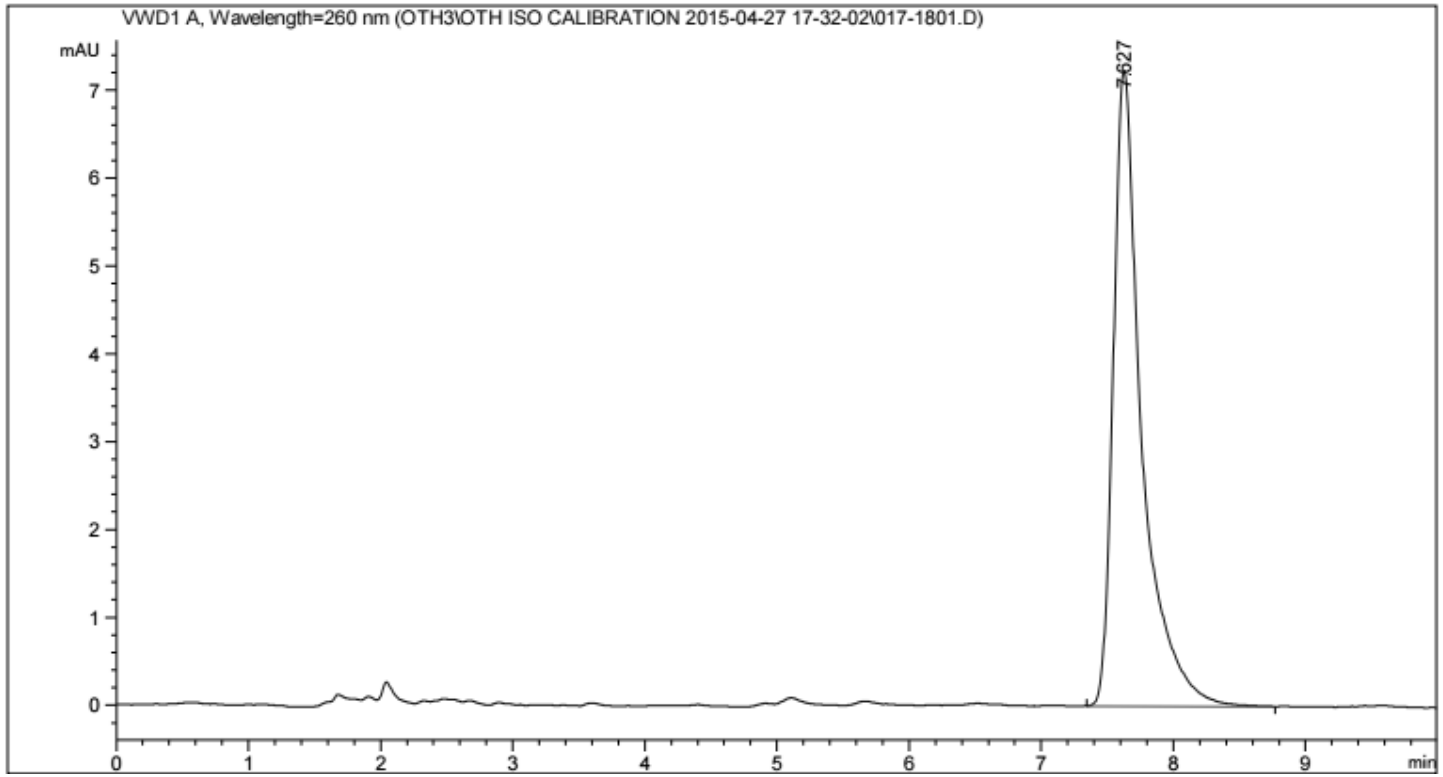
Identification of pinocembrin (Retention time) Isocratic method



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Retention time and linearity

Compounds	Retention time (Minutes)	Wavelength: 260nm	
		Regression equation	Correlation coefficient (r^2)
Pinocembrin	7.6	y=9.7579x + 2.572	0.9989

Pinocembrin – used as marker compound

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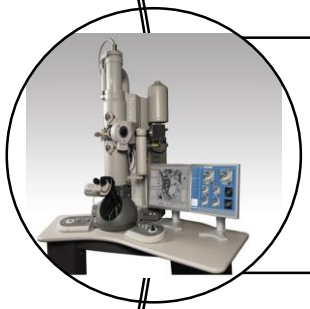
Chitosan propolis nano-formulation

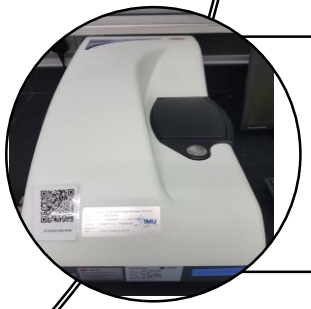
High performance liquid chromatography analysis

Test the presence of markers for the formulation



Transmission electron microscopy

Size, Structure



Zetasizer

Stability-Zeta potential, Size, Aggregation, No. of particles

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Characterization of nanoparticles

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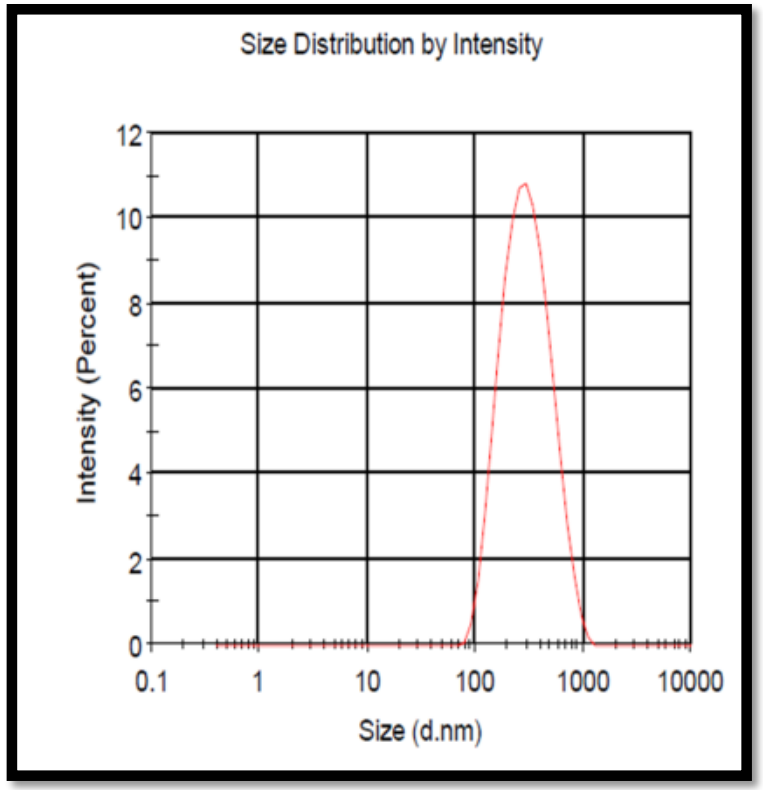
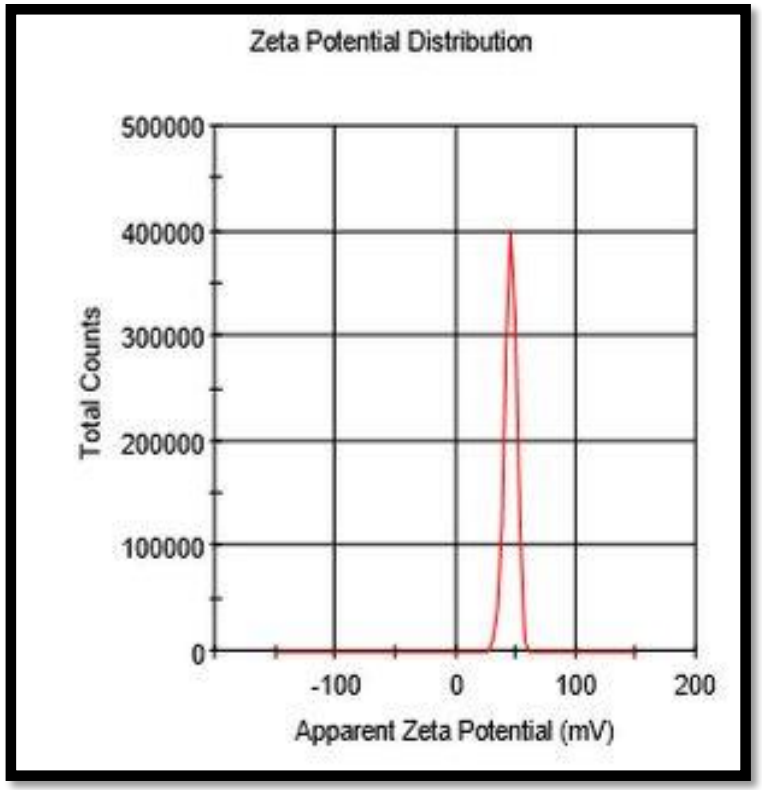
Physical characterization nanoparticles

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

Particle size and zeta potential of F1





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Physical characterization nanoparticles

- Propolis loaded chitosan nanoparticle > Chitosan blank

- Chitosan concentration
- Propolis concentration
- Surfactant

} Factors that will influence the particle size

PDI (POLYDISPERSITY INDEX)

Polydispersity index ranging from 0.225 to 0.573.

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Physical characterization nanoparticles

- Chitosan-TPP nanoparticles are generally characterized by a positive zeta potential

- Positive zeta potential ranging from +35.5mV to +74.1 mV.

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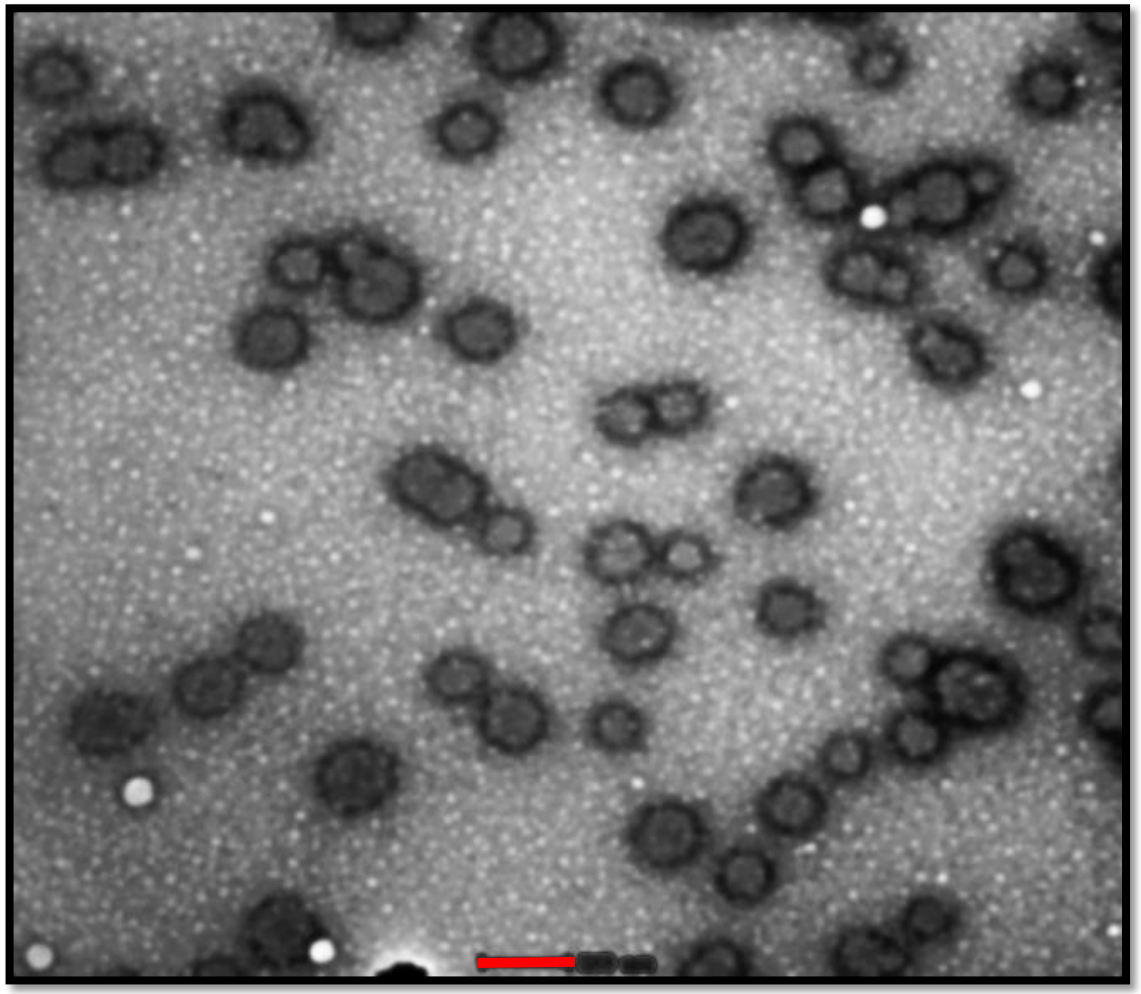
Surface morphology of nanoparticles (SEM)



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Nano particle - encapsulation efficiency

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

Chitosan concentration

Propolis concentration

} Factors that will influence the encapsulation efficiency

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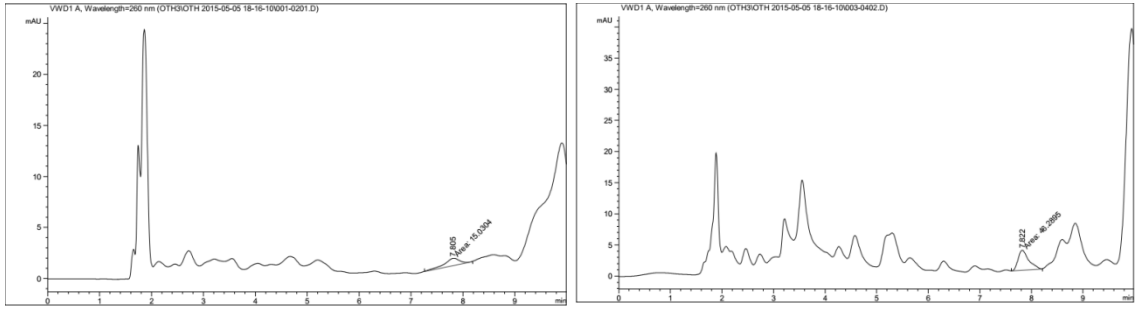
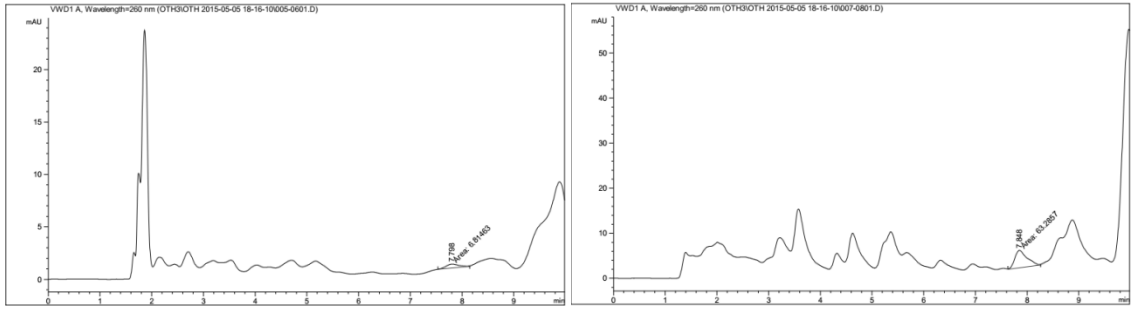
Nano particle - encapsulation efficiency

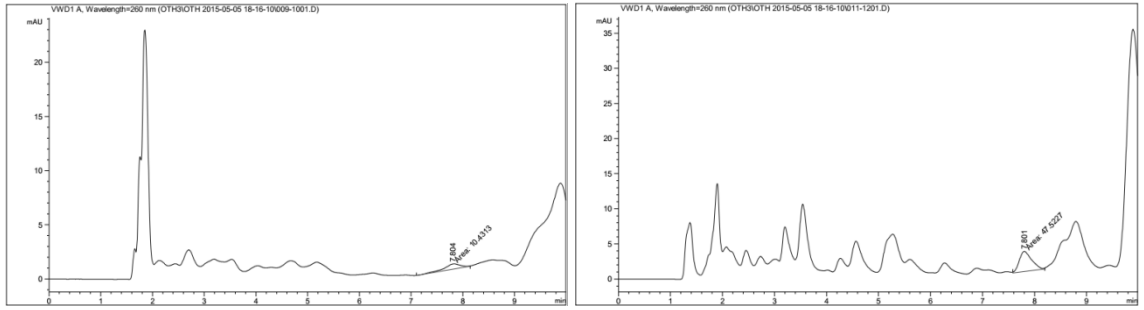
F1



F2



F3



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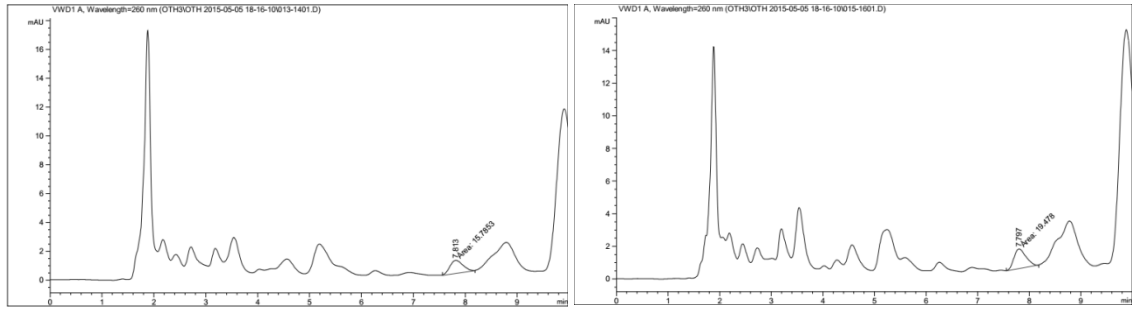
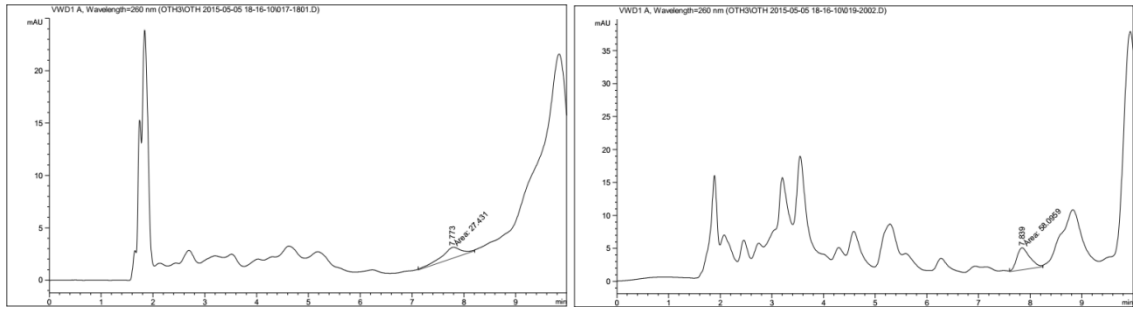
Nano particle - encapsulation efficiency

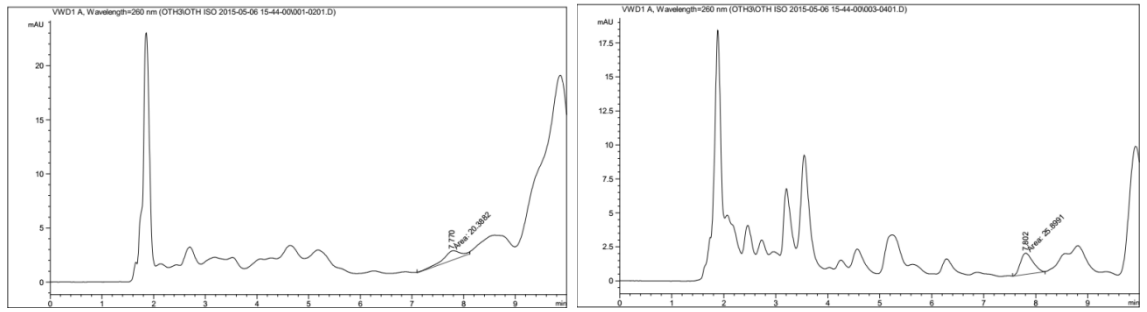
F4



F5



F6



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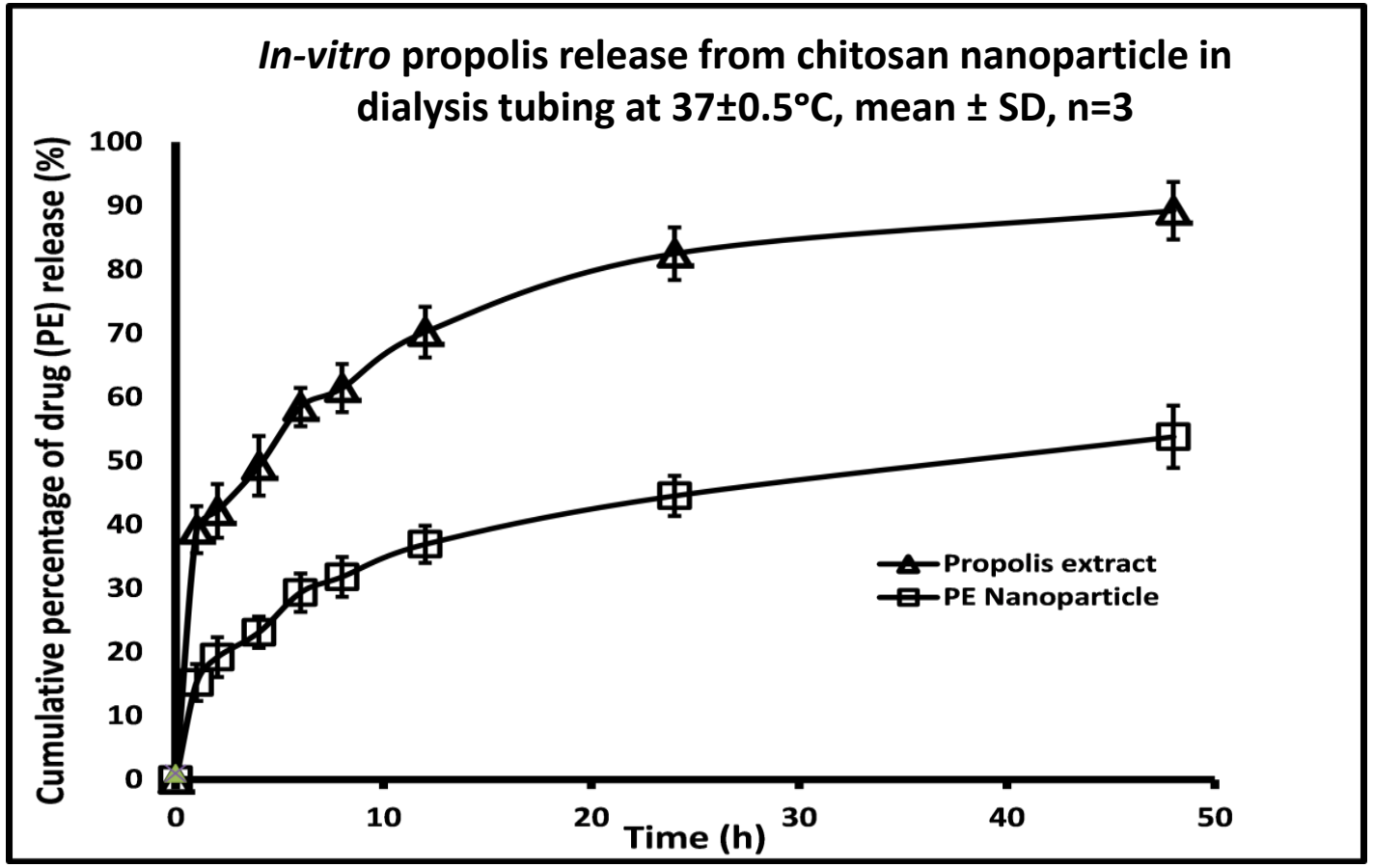
In vitro release – encapsulation efficacy



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In vitro release – encapsulation efficacy

- Pure propolis solution exhibited a burst release with 39.21% ± 3.67% within the first hour and released up to 89.23% ± 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% ± 4.89%.

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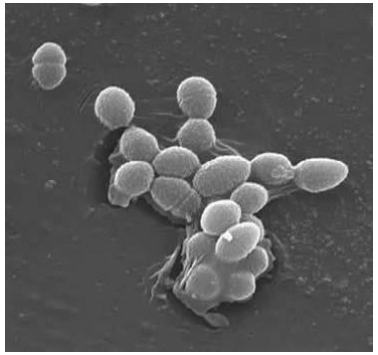
Enterococcus faecalis

- ✓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



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Enterococcus faecalis - drug resistance

Enterococci

From Commensals to Leading Causes of Drug Resistant Infection

Michael S Gilmore, Editor-in-chief
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Yasuyoshi Ike, Editor
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Massachusetts Eye and Ear Infirmary
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National Foundation for Infectious Diseases

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Supported by an unrestricted educational grant from **Glaxo Wellcome Inc**

Volume IV, Issue 3 - April 1998

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, 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

Original Article

DOI: 10.7860/JCDR/2016/17552.7201

Detection of Vancomycin Resistance among *Enterococcus faecalis* and *Staphylococcus aureus*

Microbiology Section

Journal of Clinical and Diagnostic Research. 2016 Feb, Vol-10(2): DC04-DC06

RAMYA RENGARAJ[1], SHANTHI MARIAPPAN[2], UMA SEKAR[3], ARUNAGIRI KAMALANADHAN[4]

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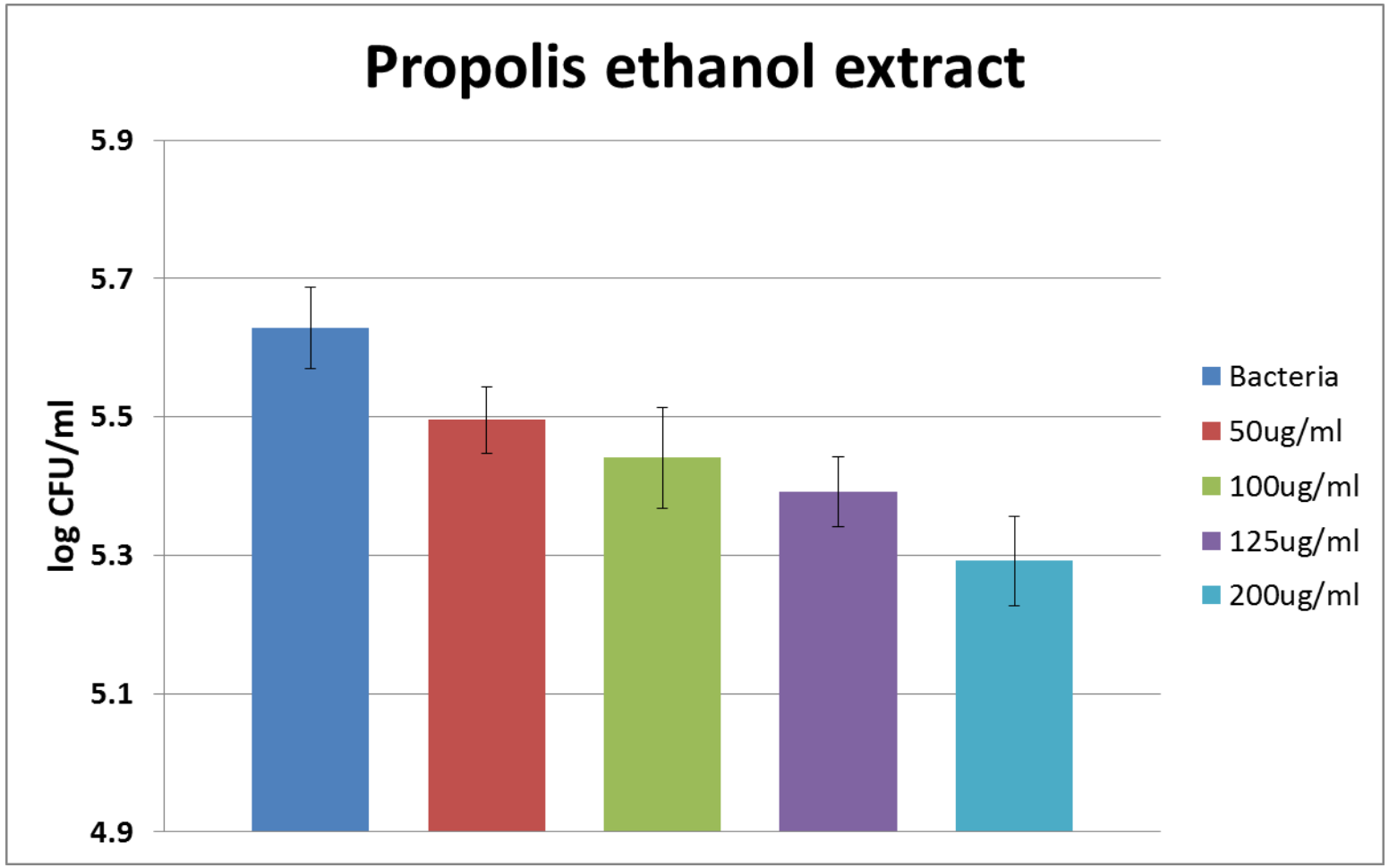
Antibacterial efficacy of propolis against *E. faecalis*



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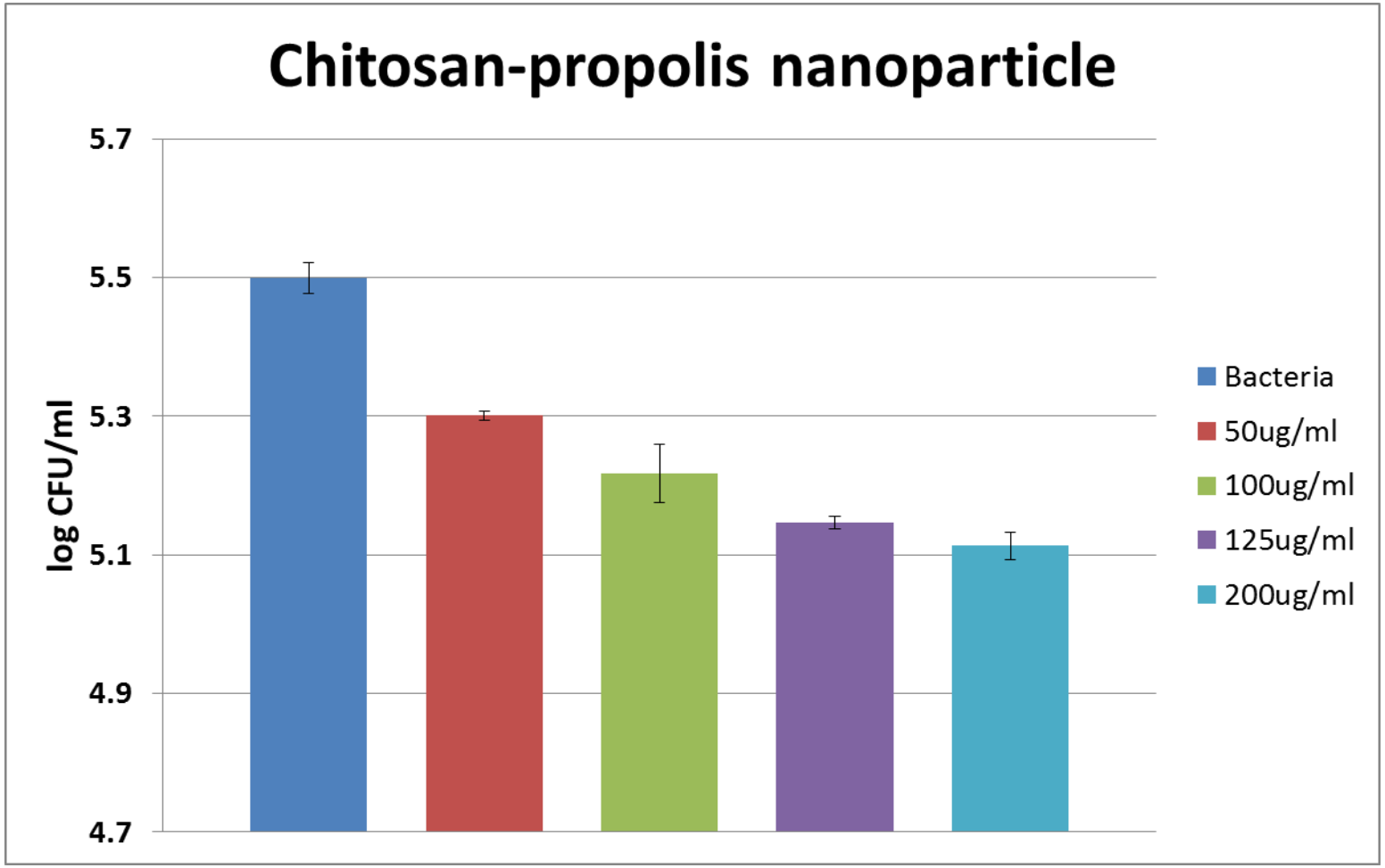
Antibacterial efficacy of nano-propolis against *E. faecalis*



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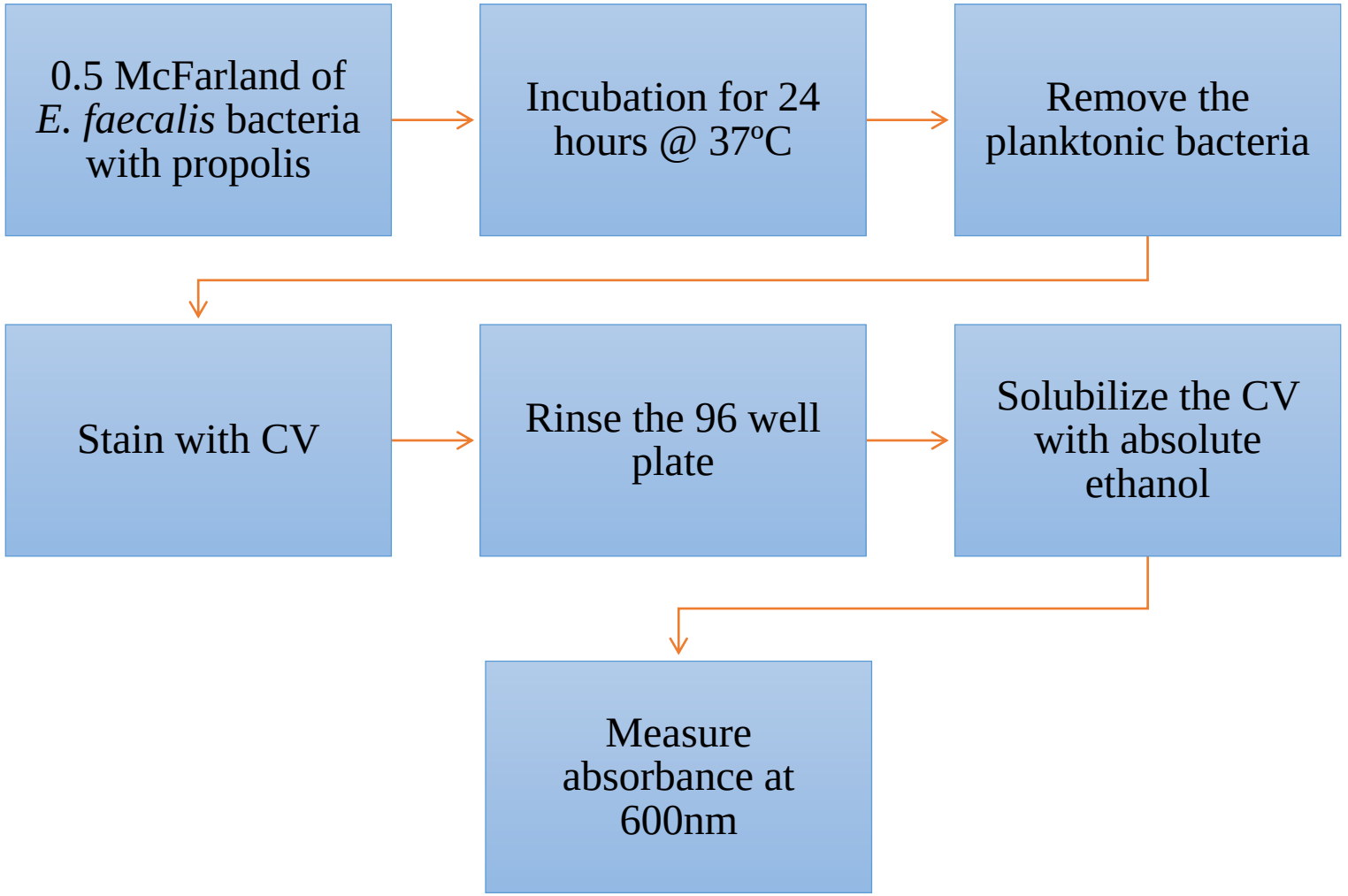
Crystal violet assay



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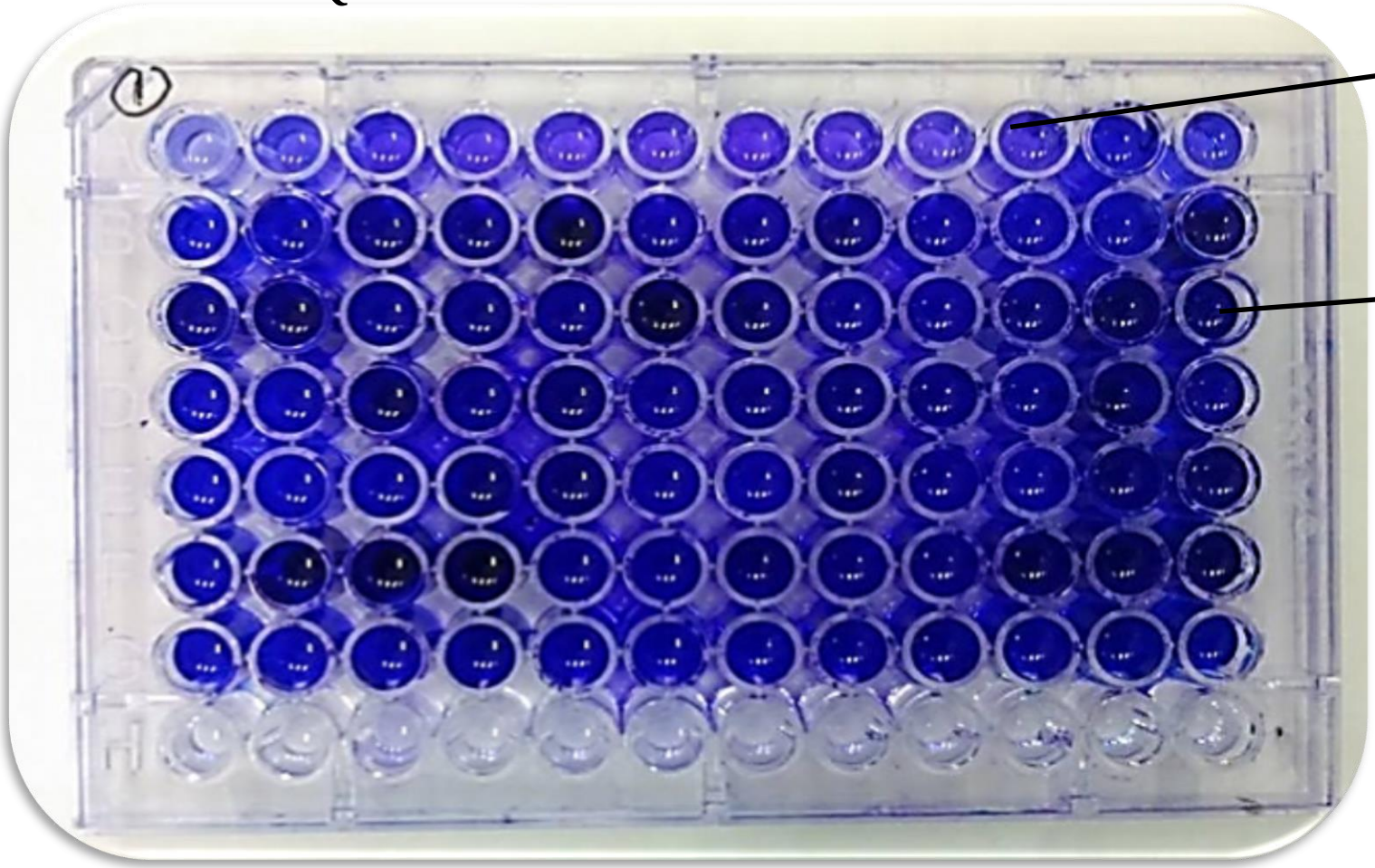
Crystal violet assay

- Quantification of static biofilm



Lower intensity = Lesser biofilm mass

Higher intensity = More biofilm mass

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Percentage reduction of viable bacteria in biofilms

	Propolis ethanol extract	Chitosan-propolis nanoparticles
50µg/ml	23.08%	22.73%
100µg/ml	47.31%	54.55%
125µg/ml	68.08%	68.18%
200µg/ml	79.23%	81.36%

Nano Propolis against *E. faecalis* biofilms

	Propolis ethanol extract	Chitosan - propolis nanoparticles
50µg/ml	26.92%	36.84%
100µg/ml	34.62%	47.37%
125µg/ml	42.31%	55.79%
200µg/ml	53.85%	58.95%

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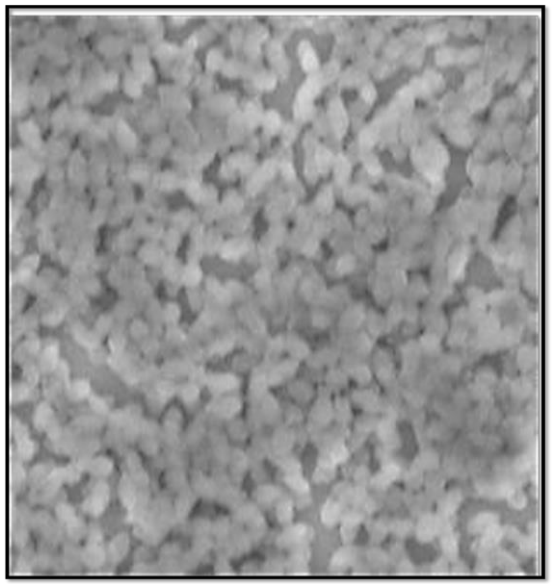
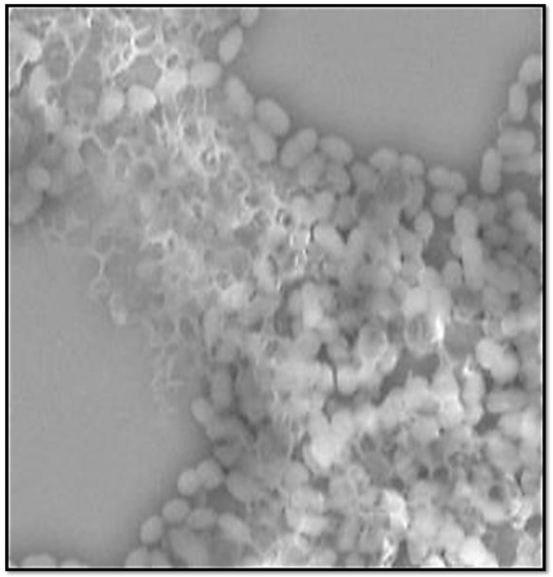
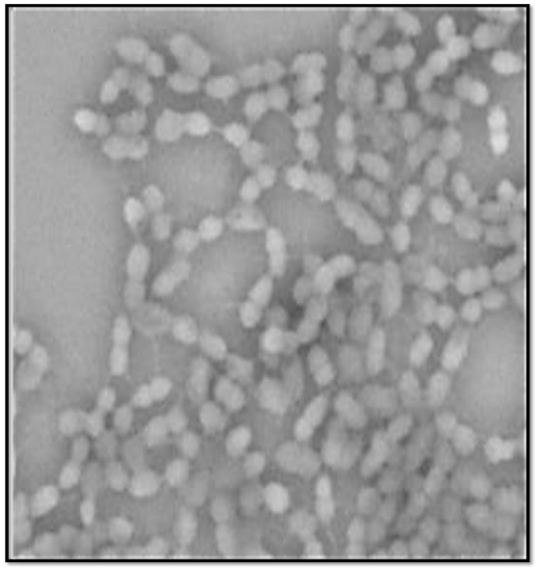
Scanning Electron Microscopy



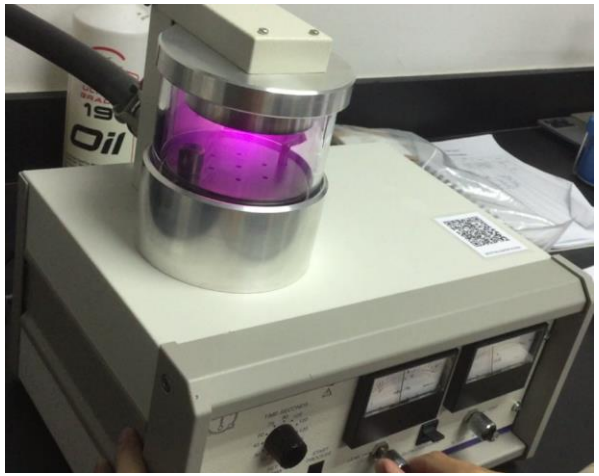
Control

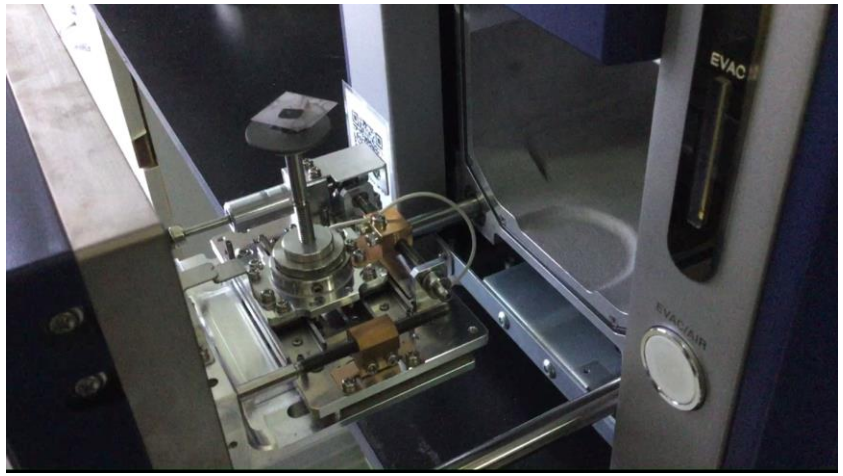


Propolis



Nano-propolis





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Dr. Fabian Davamani
Principle investigator
(Molecular and Microbiology)

Dr. Srinivasan Ramamurthy
Co- investigator
(Analytical Chemistry- Pharmacy)

Dr. Rajinikanth Siddalingam
Co- investigator
(Pharmaceutical Technology)

Dr. Ebenezer Chitra
Co- investigator
(Cell and Molecular biology)

Ong Teik Hwa
Ph.D Student

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