The antimicrobial effect of propolis against a range of commonly encountered human pathogens

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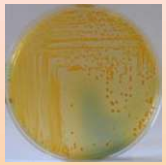
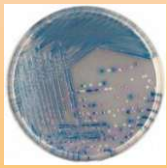
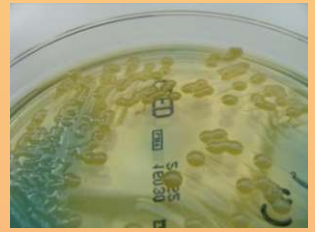






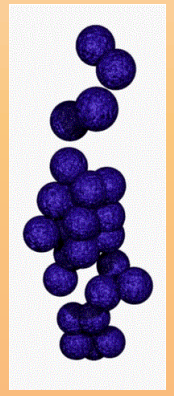
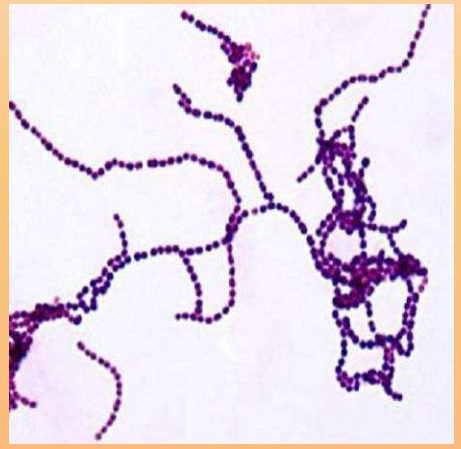
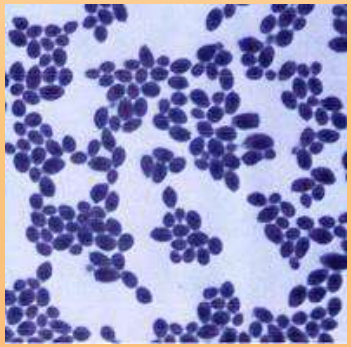
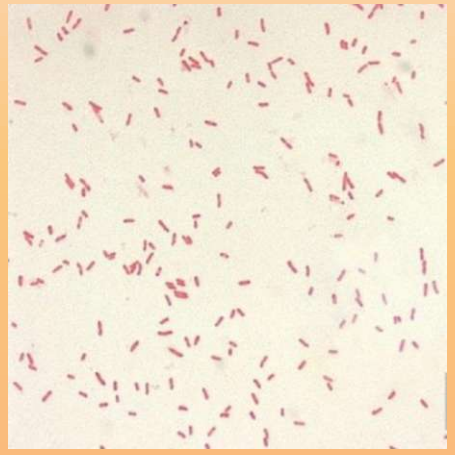
Introduction

The research carried out in this project was to assess the antimicrobial effect of propolis, against commonly isolated human pathogens using the minimum inhibitory concentrations (MIC), minimum bacterial concentration and time-kill curves.

The organisms used were control strains (NCTC) or laboratory isolates from:

- urine samples
- sputum samples
- blood cultures
- groin swabs
- high vaginal swabs

The clinical isolates used included both Gram positive and Gram negative bacteria, and yeasts.


Gram positive organisms used

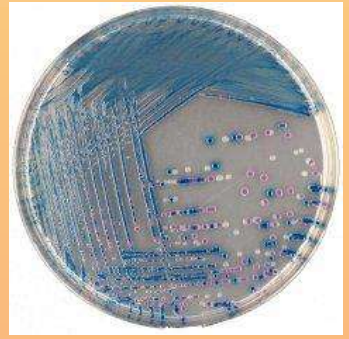
Organism	NCTC No. or isolation site
Oxford *Staphylococcus aureus*	NCTC 6571
Methicillin Resistant *Staphylococcus aureus* (MRSA)	NCTC 10443
Staphylococcus auricularis	Blood culture
Staphylococcus lugdunensis	Blood culture
Vancomycin resistant *Enterococcus faecium*	Groin swab
Methicillin Resistant *Staphylococcus aureus* (MRSA)	Blood culture
Staphylococcus warneri	Blood culture

Gram negative organisms used

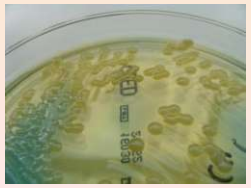
Organism	NCTC No. or isolation site
Eschericia coli	NCTC 10418
Pseudomonas aeruginosa	NCTC 10662
Eschericia coli (sensitive)	Urine
Eschericia coli (resistant)	Urine
Pseudomonas aeruginosa	Urine
Proteus mirabilis (sensitive)	Urine
Proteus mirabilis (resistant)	Urine
Klebsiella oxytoca (Ampicillin sensitive)	Urine
Klebsiella oxytoca (Ampicillin resistant)	Urine
Enterobacter cloacae	Sputum

Yeasts used

Organism	Isolation site
Candida albicans	High vaginal swab
Candida albicans	High vaginal swab
Candida parapsilosis	High vaginal swab
Cryptococcus humicolus	High vaginal swab

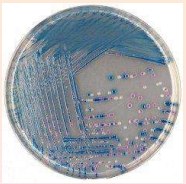


MIC determination – agar dilution method



Bacterial colonies

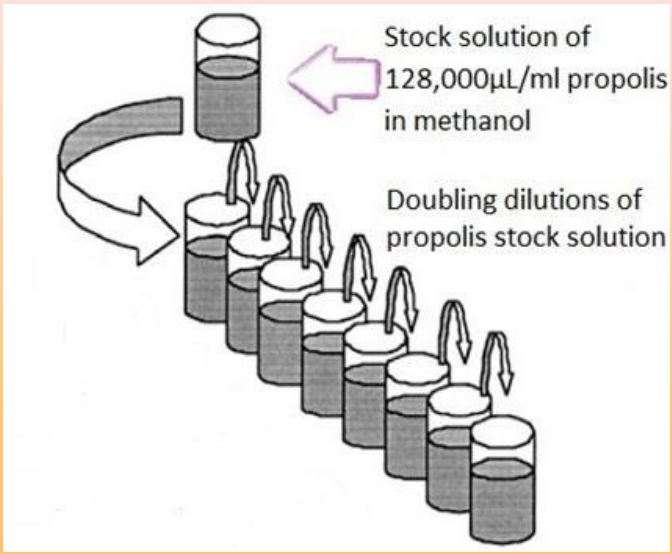
or



Yeast colonies

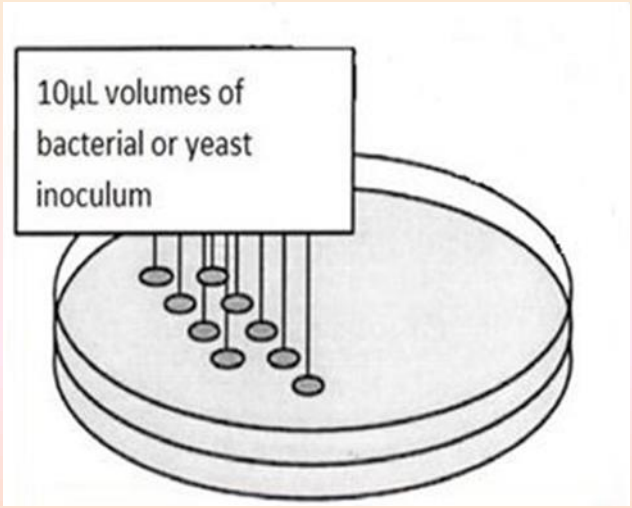
Bacteria or yeast inoculum equivalent to 0.5 MacFarlane (1.5×10^8/ml)

1ml defibrinated horse blood and 1 ml propolis solution added to 19ml isosensitest agar at 65◦C



Final range of concentrations of propolis in agar: 64 mg/ml – 0.125mg/ml

MIC determination – agar dilution method



37◦C + 5% CO_2
for 18 hours

MBC determination

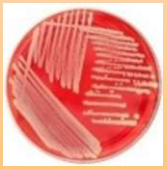
At any given concentration of propolis, any areas showing no growth of bacteria were sub cultured onto Columbia blood agar & incubated for 18 hours to determine the minimum bactericidal concentration (MBC) of propolis for that organism.

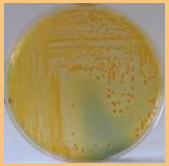
Time kill curves method

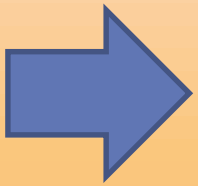
Stock solutions of propolis were made up in concentrations of:

- 128mg/L
- 64mg/L
- 32mg/L
- 16mg/L
- 8mg/L
- 4mg/L
- 2mg/L
- 1mg/L
- 0.5mg/L

in sterile nutrient broth containing dimethylsulphoxide (5% v:v) and Tween 80 (20% v:v). The sterile nutrient broth was also used as a negative control.



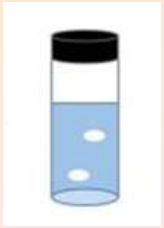




Sterile peptone water containing the bacteria or yeast inoculum equivalent to 0.5 MacFarlane (1.5×10^8/ml)

37◦C + 5% CO_2 for 18 hours

Time kill curves method



Sterile peptone water containing the bacteria or yeast inoculum equivalent to 0.5 MacFarlane (1.5×10^8/ml)

1ml

Propolis solution in nutrient broth with DMS & Tween 80

37◦C + 5% CO_2 for 3, 6, 9, or 12 hours

1ml

3000 rpm
10 minutes

Supernatant removed

Bacterial pellet re-suspended in peptone water for 20 minutes

10µL into sterile distilled water, two 1 in 100 dilutions with sterile distilled water.

10µL

37◦C + 5% CO_2 for 18 hours

Viable count performed

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration results (MBC) for the Gram positive organisms

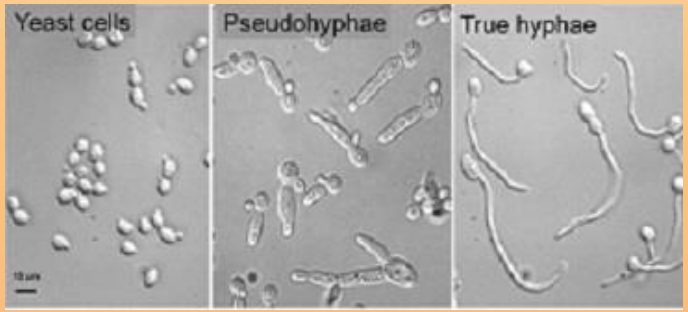
Organism	NCTC No. (if applicable)	MIC	MBC
Oxford *Staphylococcus aureus*	NCTC 6571	0.5mg/L	4mg/L
Methicillin resistant *Staphylococcus aureus* (MRSA)	NCTC 10443	0.5mg/L	4mg/L
Staphylococcous auricularis	n/a	0.25mg/L	2mg/L
Staphylococcus lugdunensis	n/a	8mg/L	64mg/L
Staphylococcus warneri	n/a	1mg/L	8mg/L
Methicillin resistant *Staphylococcus aureus* (MRSA)	n/a	0.5mg/L	4mg/L
Vancomycin resistant *Enterococcus faecium* (VRE)	n/a	2mg/L	8mg/L
Enterococcus faecalis	n/a	2mg/L	8mg/L

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration results (MBC) for the Gram negative organisms

Organism	NCTC No. (if applicable)	MIC	MBC
Eschericica coli	NCTC 10418	64mg/L	128mg/L
Eschericica coli (sensitive)	n/a	64mg/L	128mg/L
Eschericica coli (resistant)	n/a	64mg/L	128mg/L
Pseudomonas aeruginosa	NCTC 10662	64mg/L	128mg/L
Pseudomonas aeruginosa	n/a	64mg/L	128mg/L
Klebsiella oxytoca (Ampicillin sensitive)	n/a	>64mg/L	>64mg/L
Klebsiella oxytoca (Ampicillin resistant)	n/a	>64mg/L	>64mg/L
Proteus mirabilis (sensitive)	n/a	>64mg/L	>64mg/L
Proteus mirabilis (resistant)	n/a	>64mg/L	>64mg/L
Enterobacter cloacae	n/a	>64mg/L	>64mg/L

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration results (MBC) for the yeasts

Organism	MIC	MBC
Candida albicans	8mg/L	16mg/L
Candida albicans	8mg/L	64mg/L
Candida parapsilosis	16mg/L	32mg/L
Cryptococcus humicolus	2mg/L	8mg/L



Results – Control Organism

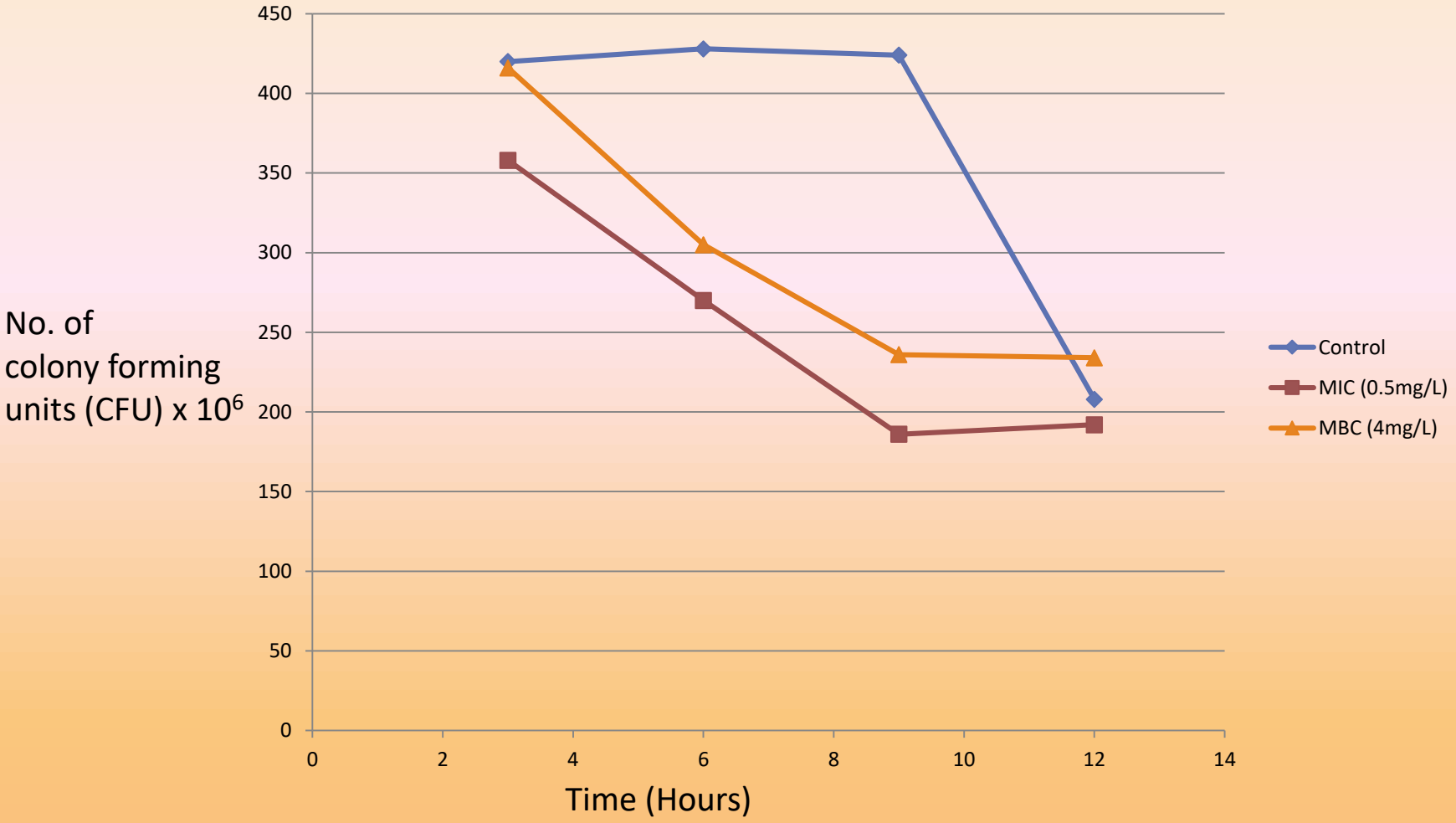


Figure 1: Time kill curves for Oxford *Staphylococcus aureus* (NCTC 6571) at concentrations of propolis equivalent to the minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC)

Results – Control organism

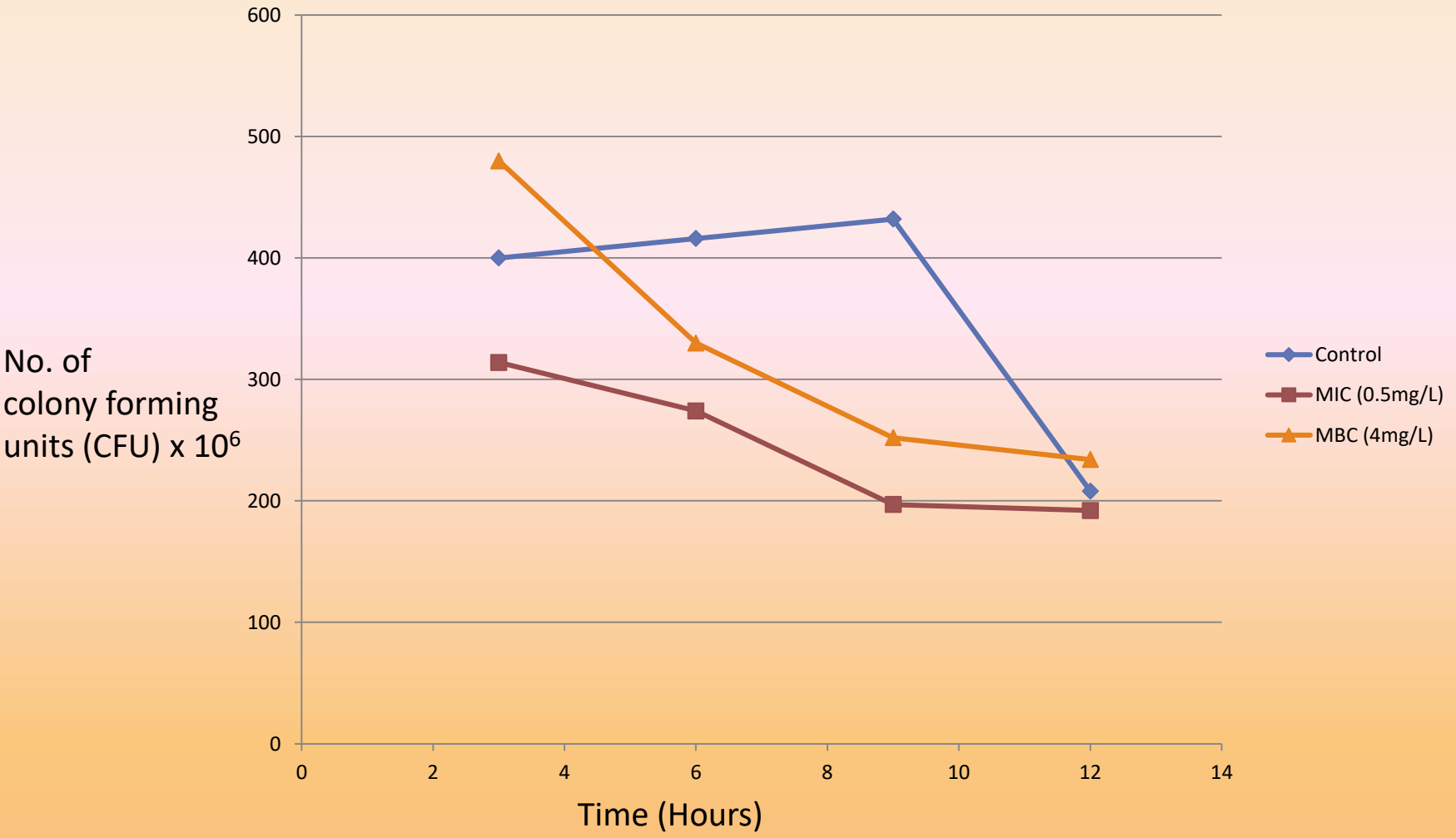


Figure 2: Time kill curves for Methicillin Resistant *Staphylococcus aureus* (NCTC 10443) at concentrations of propolis equivalent to the minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC)

Results – Clinical isolate

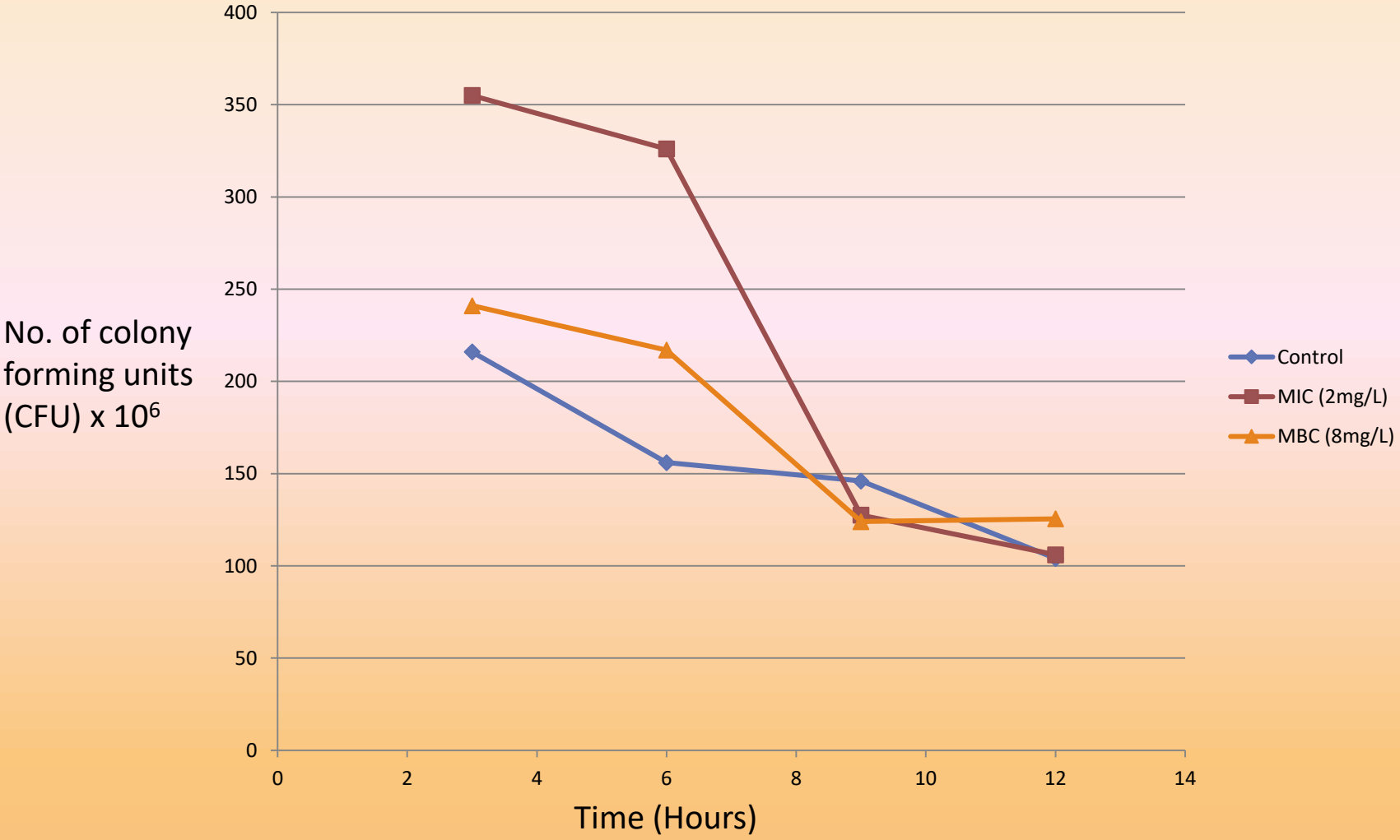


Figure 3: Time kill curves for Vancomycin resistant *Entercoccus faecium* (VRE) at concentrations of propolis equivalent to the minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC)

Results – Clinical isolate

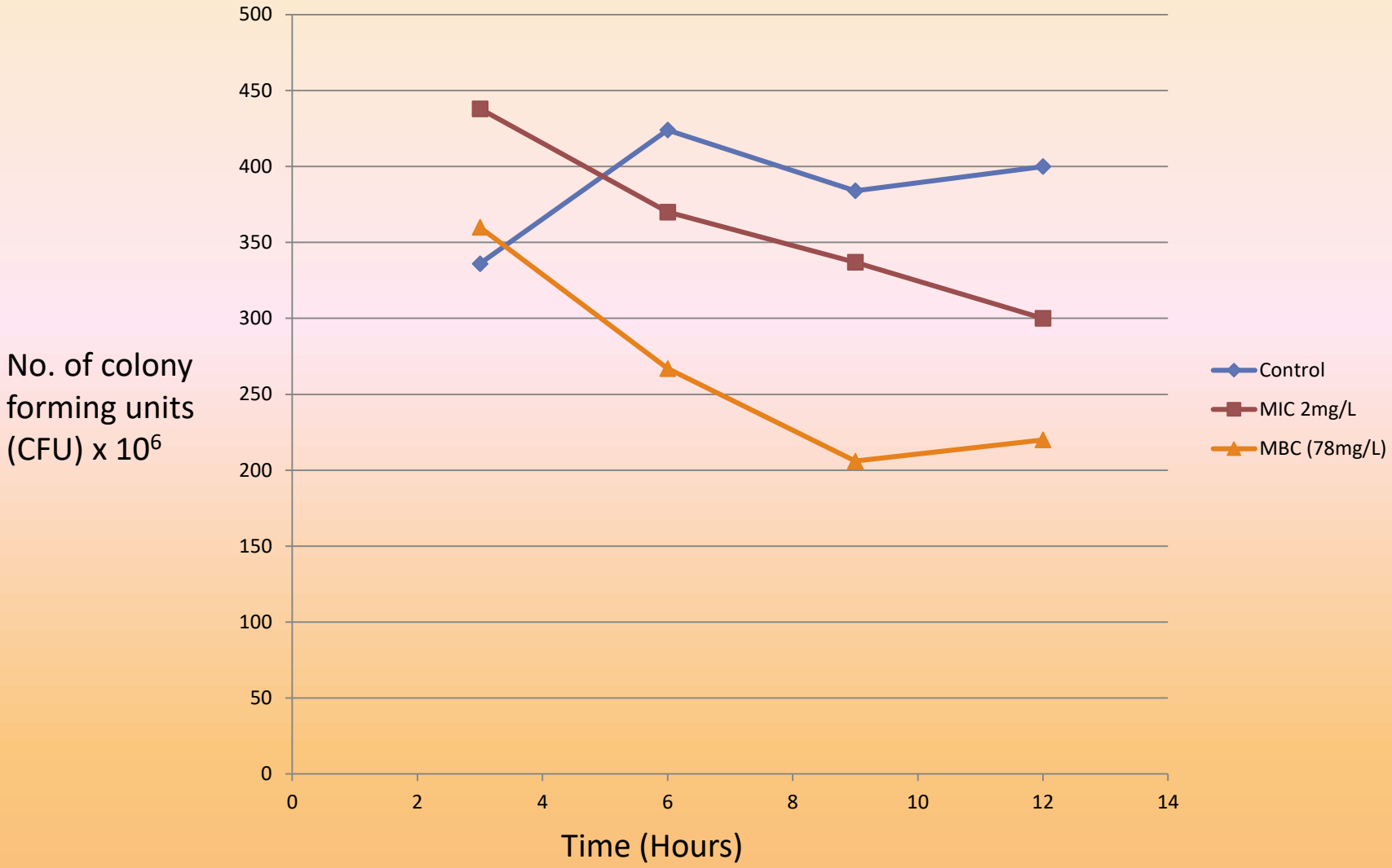


Figure 4: Time kill curves for *Entercoccus faecalis* at concentrations of propolis equivalent to the minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC)

Results – Clinical isolate

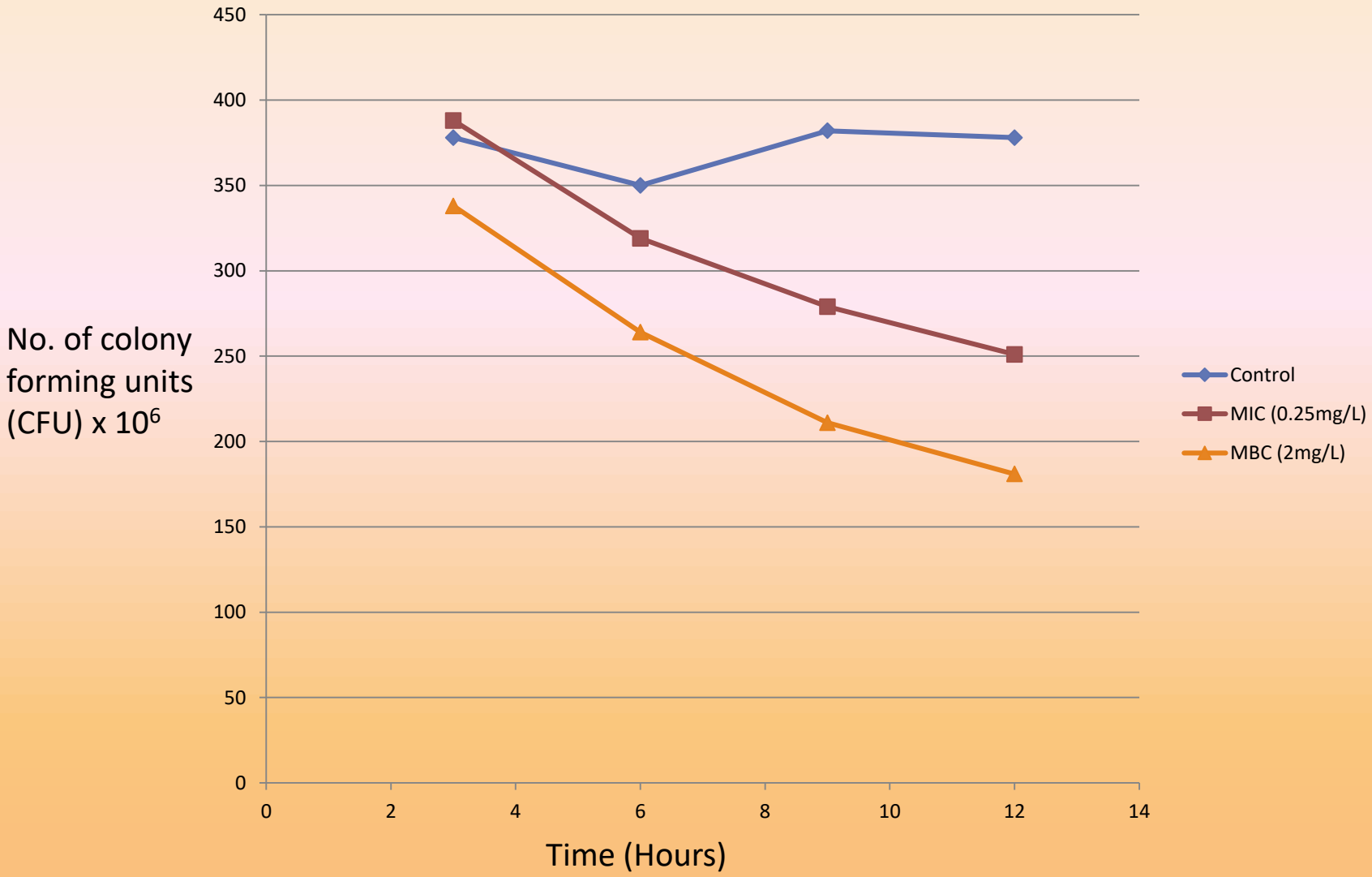


Figure 6: Time kill curves for *Staphylococcus auricularis* at concentrations of propolis equivalent to the minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC)

Results – Control organism

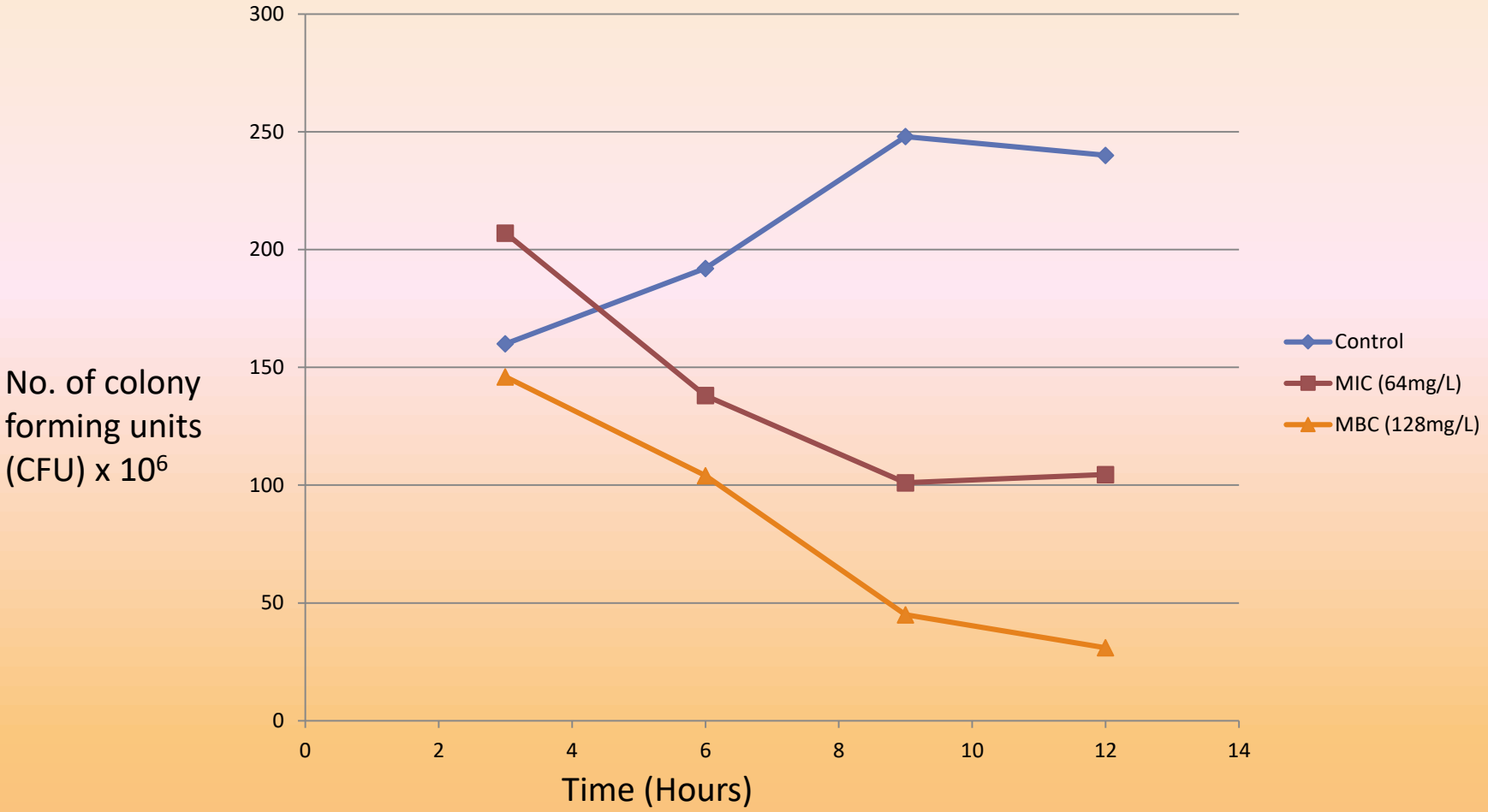


Figure 5: Time kill curves for *Eschericia coli* (NCTC 10418) at concentrations of propolis equivalent to the minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC)

Results - Control organism

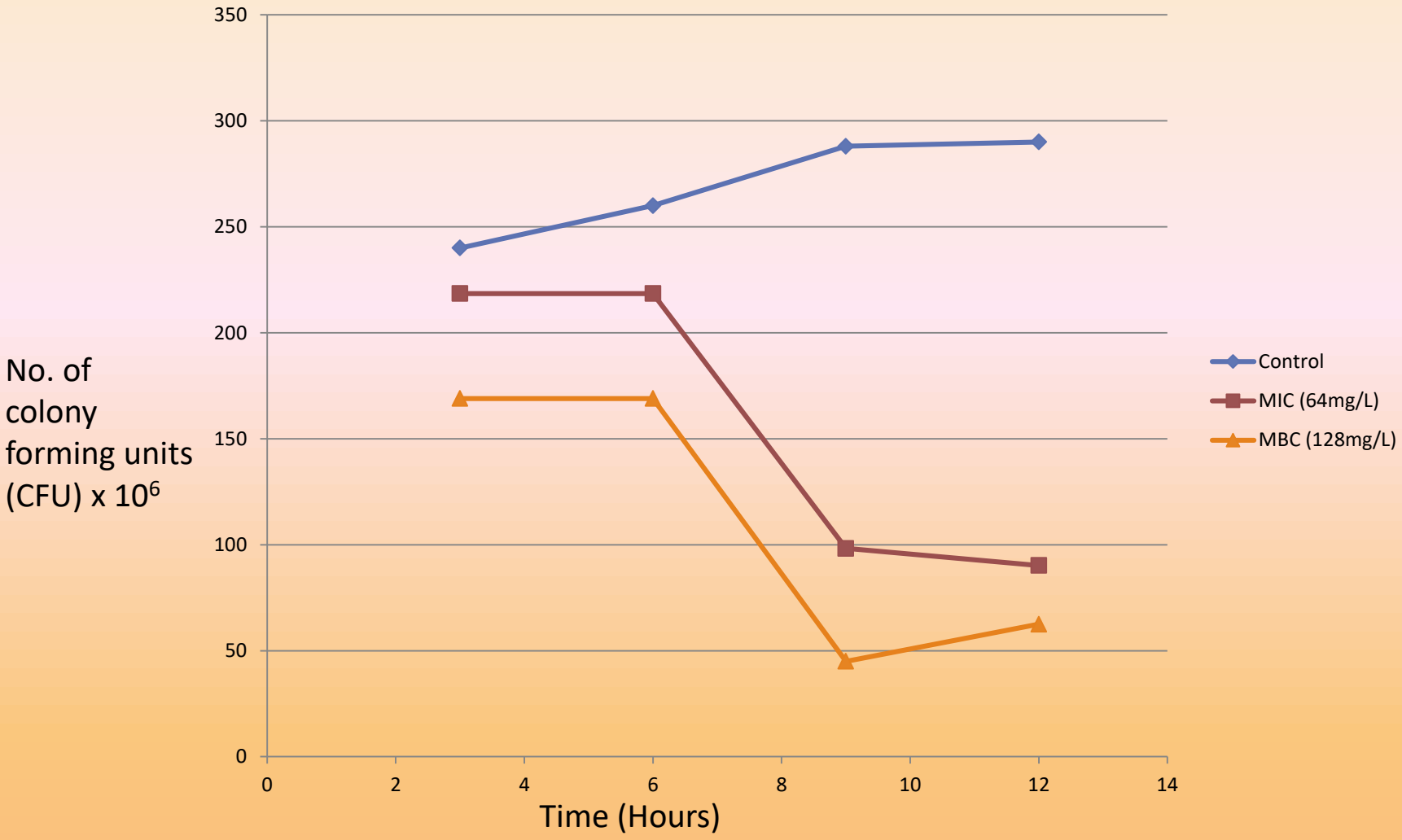


Figure 6: Time kill curves for *Pseudomonas aeruginosa* (NCTC 10662) at concentrations of propolis equivalent to the minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC)

Results – Clinical isolate

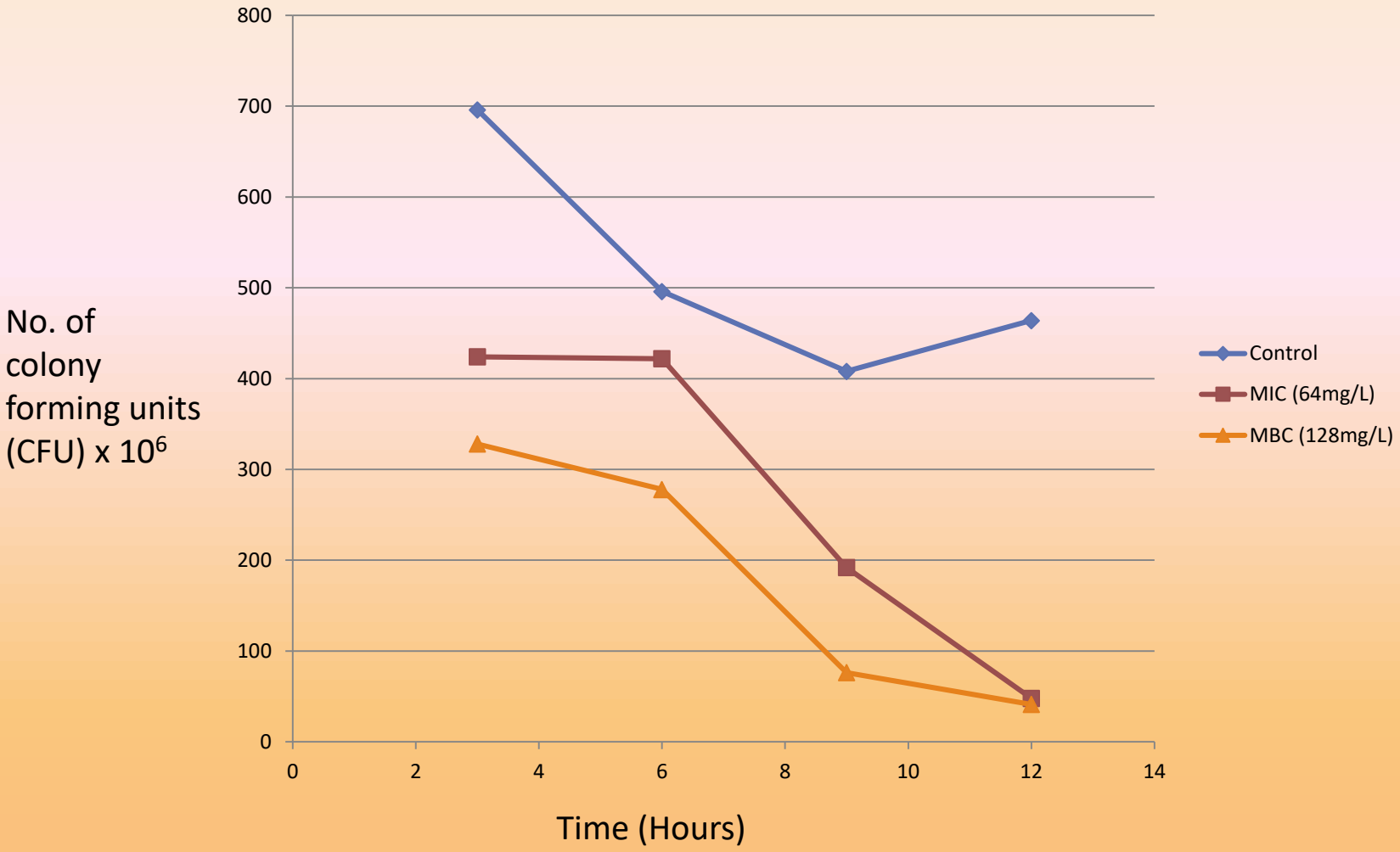


Figure 7: Time kill curves for *Eschericia coli* at concentrations of propolis equivalent to the minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC)

Results – Clinical isolate

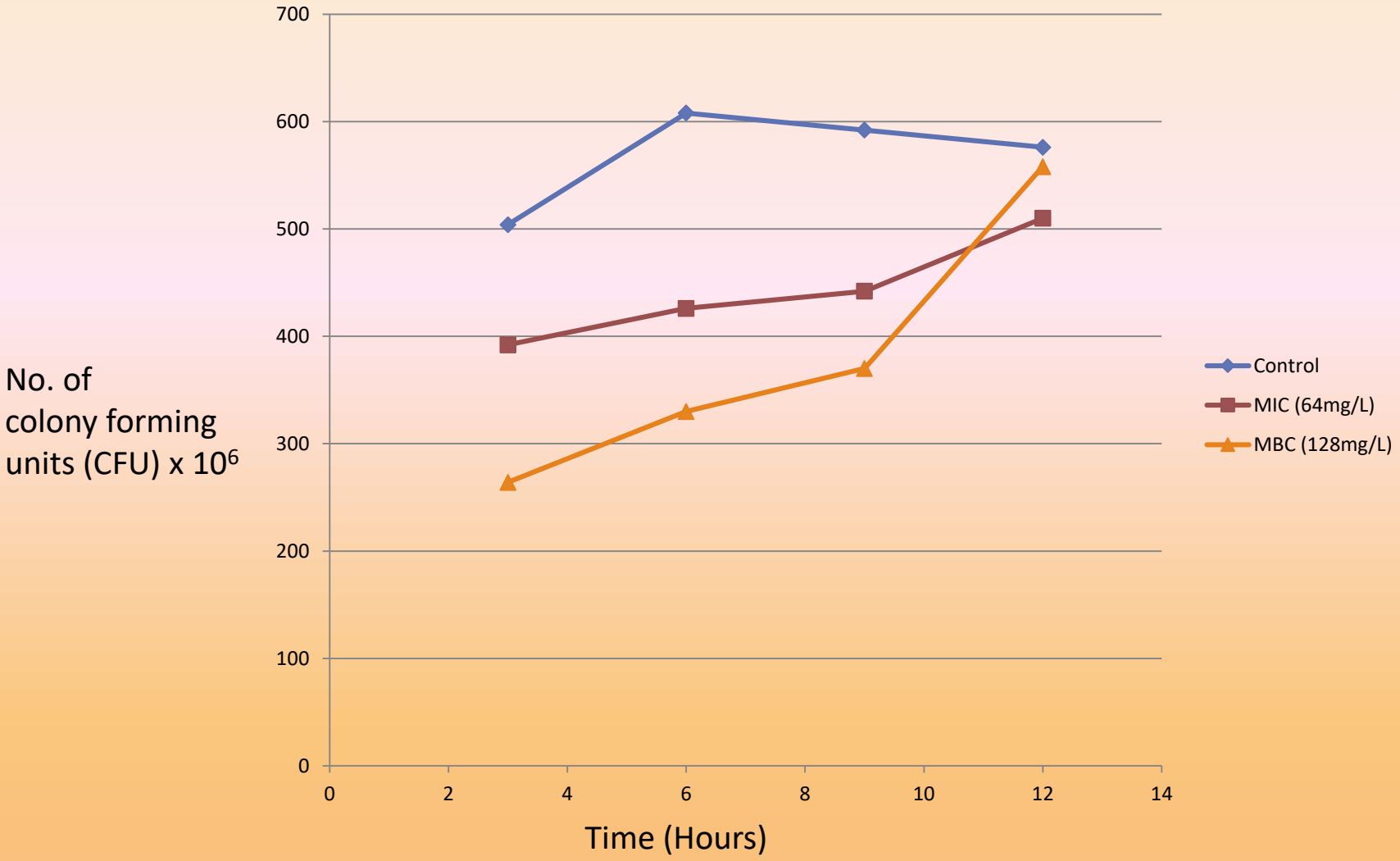
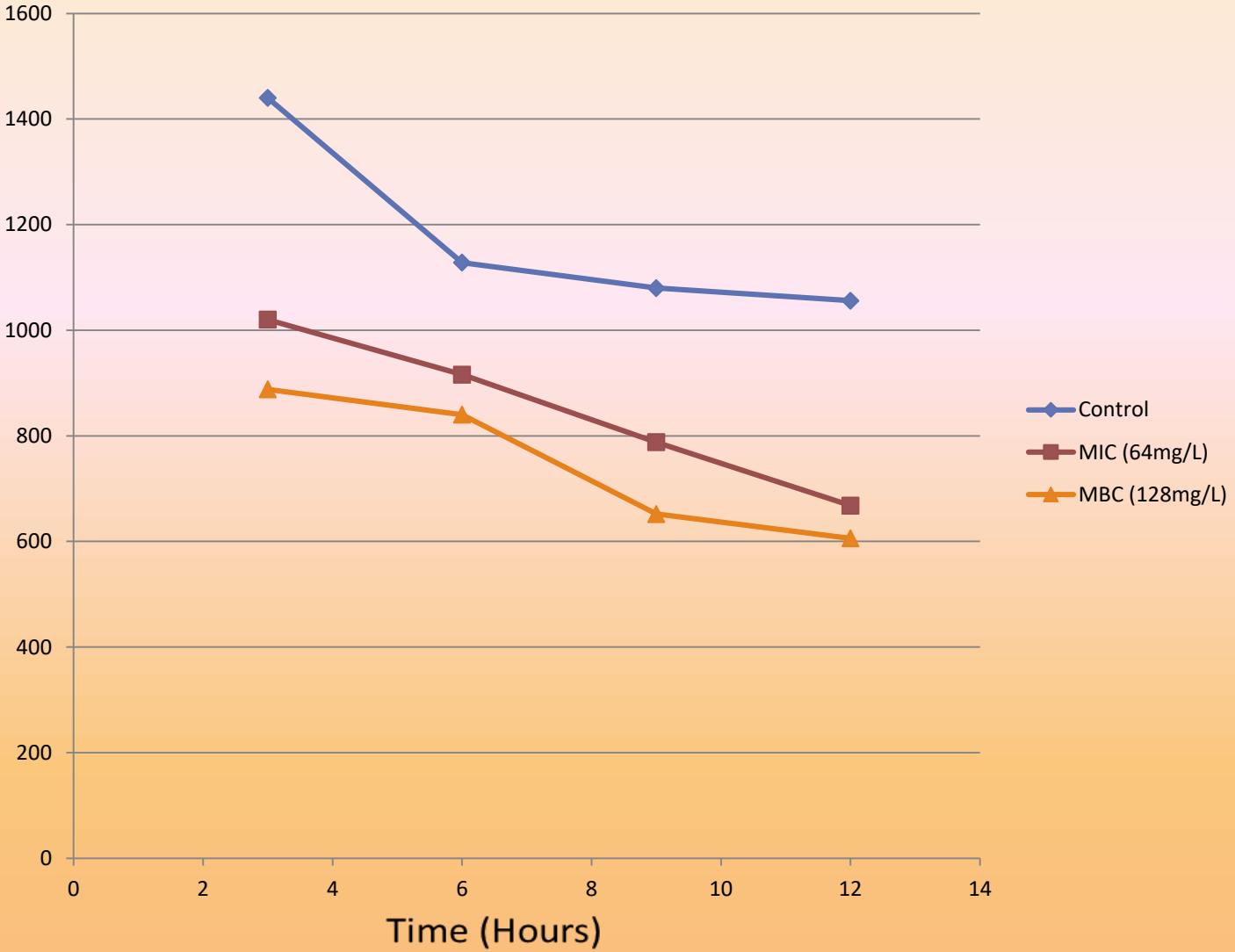


Figure 8: Time kill curves for *Klebsiella oxytoca* at concentrations of propolis equivalent to the minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC)

Results – Clinical isolate



No. of colony forming units (CFU) x 10^6

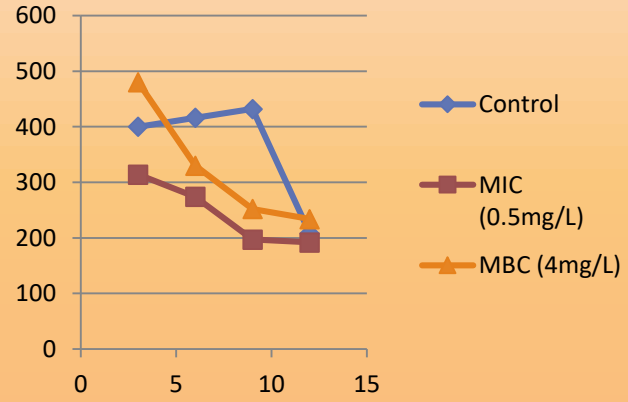
Figure 9: Time kill curves for *Enterobacter cloacae* at concentrations of propolis equivalent to the minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC)

Summary of results

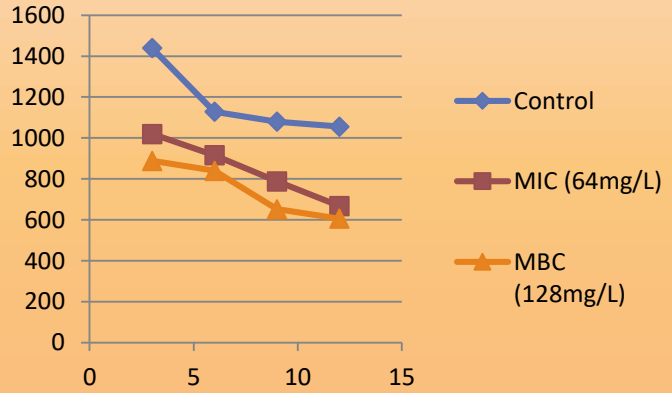
- The MIC and MBC investigations show that propolis has both antimicrobial and anti-fungal activity.
- Particularly effective against Gram positive organisms, such as staphylococci and streptococci, and yeasts.
- Less effective against Gram negative organisms.

<u>Time-kill curves:</u>

- At lower concentrations propolis is bacteriostatic against Gram positive organisms.
- At higher concentrations, propolis is bactericidal against both Gram negative and Gram positive organisms.



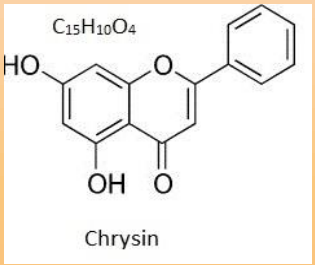
S. aureus

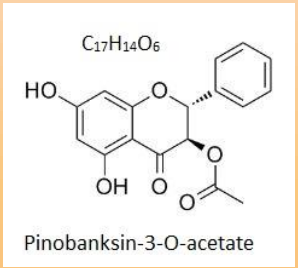


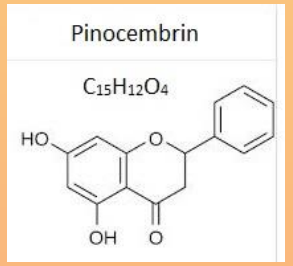
E. coli

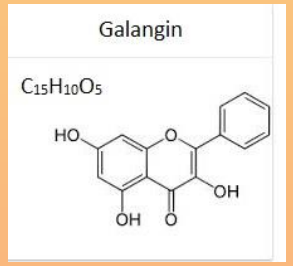
Flavinoids in propolis and their activity

Organisms	Active flavinoid	Reference
S. aureus	Phenolic compounds (i.e. flavinoids)	Ristivojević *et al* (2016) PLoS ONE 11 (16): e0157097 Doi: 10.1371/journal.pone.0157097
S.aureus, *E. coli* & *C. albicans*	Pinocembrin	Rasul *et al* (2013) BioMed Research International, Article ID 379850
MRSA & *S. aureus*	Pinocembrin, Pinobankin-3-O-acetate & Chrysin	Darwish *et al* (2010) African Journal of Biotechnology Vol. 9 (36): 5966-5974
E. faecalis, S. aureus & *E. coli*	Pinobankin	Uzel *et al* (2005) Microbiological Research 189-195
MRSA, *Enterococcus spp.*, *P. aeruginosa*	Galangin	S. Pepeljnjak & I. Kosalec (2004) FEMS Microbiology letters 240: 111-116







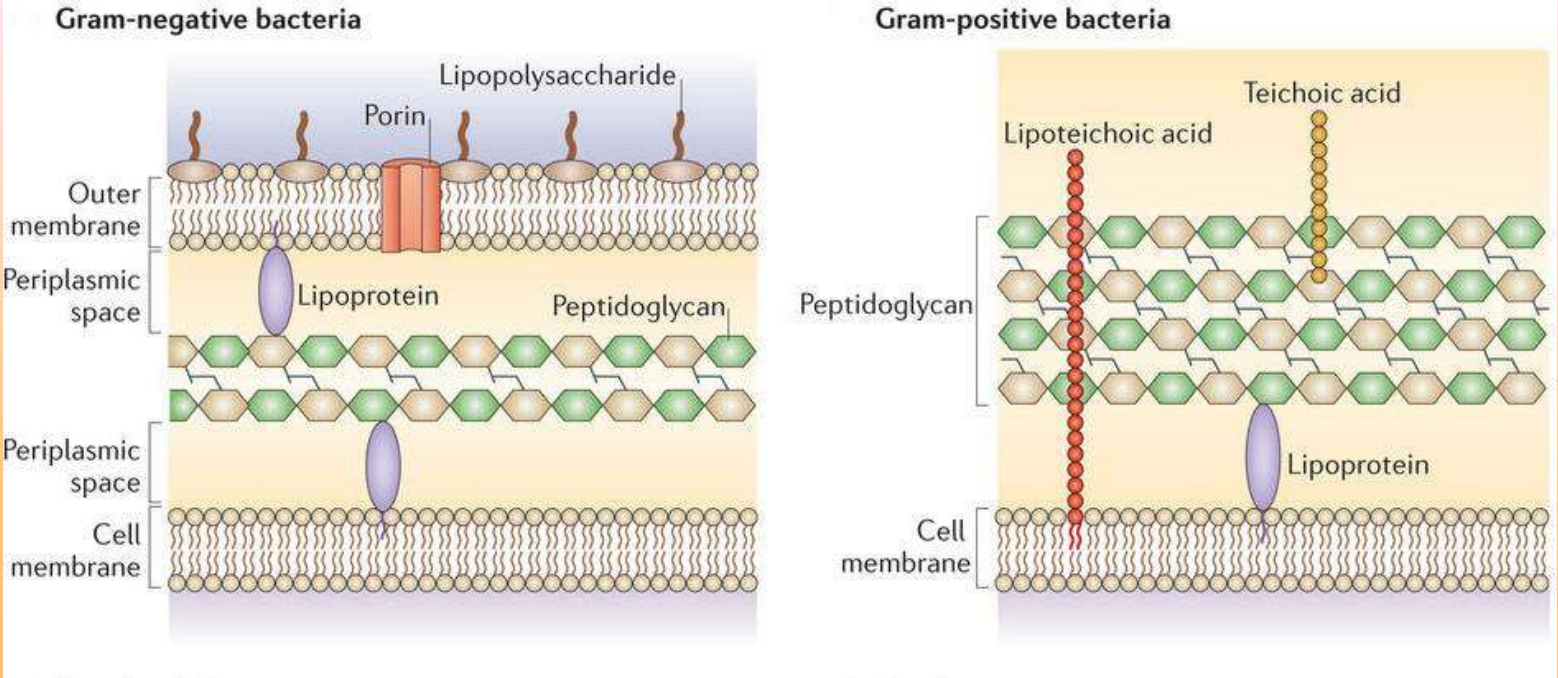


Propolis - batch analysis details:

- Chrysin (5,7-dihydroxyflavone) 3.16% w/w
- Galangin (3,5,7-trihydroxyflavone) 2.39% w/w
- Pinocembrin (5,7-dihydroxyflavone) 2.44% w/w
- Lead 0.5 mg/kg

Conclusion

- The double membrane layer of Gram negative organisms, reduces the ability of propolis to access it's site of action – the cytoplasm.
- Gram positive organisms only have a single membrane, whereas Gram negative organisms have a double membrane.
- A mucoid layer around the bacterial cells in some Gram negative organisms, such as *E. cloacae* & *K. oxytoca,* also hinders the action of propolis.



Overall, both the antimicrobial activity of propolis, and it's possible clinical applications merits further investigation.

Any questions?

Thank you:

Research kindly sponsored by:

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Propolis

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