MASTER OF MEDICINE (OPHTHALMOLOGY)

A COMPARISON OF THE CLINICAL AND ANTIBACTERIAL EFFECTS BETWEEN TUALANG HONEY AND MANUKA HONEY AS ADJUNCTIVE TREATMENT IN *PSEUDOMONAS* KERATITIS IN RABBIT EYES

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DISCLAIMER

I hereby certify that the work in this dissertation is my own except for the quotations and summaries which have been duly acknowledged.

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ABSTRAK

PENGENALAN

Madu dikenali sebagai rawatan alternatif untuk pelbagai jenis penyakit. Ubat titis mata antibakteria madu Manuka telah dipasarkan untuk penyakit jangkitan mata. Kami berhasrat untuk meneroka potensi madu Tualang untuk dikomersialkan dalam penggunaan oftalmologi. Oleh itu, kami telah menjalankan satu kajian eksperimen untuk membandingkan kesan klinikal dan antibakteria madu Tualang dan Manuka sebagai rawatan tambahan dalam *Pseudomonas* keratitis.

OBJEKTIF

Beberapa kajian in-vitro telah menunjukkan bahawa madu Tualang dan Manuka mempunyai kesan antibakteria terhadap *Pseudomonas aeruginosa*. Kajian ini dilakukan untuk membandingkan min skor Slit Lamp Examination (SLE) dan min kiraan Colony Forming Unit (CFU) atas *Pseudomonas* keratitis pada mata arnab.

KAEDAH KAJIAN

Kajian eksperimen haiwan ini terdiri daripada 20 ekor arnab yang dijangkiti dengan *Pseudomonas* keratitis dan dibahagikan secara rawak dan sama rata kepada dua kumpulan. Kumpulan A telah dirawat dengan gabungan madu Tualang 30% dan gentamicin 0.3% lapan kali sehari, manakala kumpulan B telah dirawat dengan gabungan madu Manuka dan gentamicin 0.3% lapan kali setiap hari. Pada masa yang sama, satu kajian selari dengan kaedah yang sama telah dijalankan ke atas sepuluh ekor arnab yang telah dirawat dengan gentamicin 0.3% sebagai kumpulan kawalan. Pemeriksaan klinikal secara bersiri menggunakan skor SLE telah dilakukan pada 24, 48, 72 jam, hari ke-5 dan hari ke-7. Semua

arnab telah dieutanasia pada hari ke-7 dan kornea mereka dituai. Kornea dituai telah diproses dan kiraan CFU telah dilakukan pada hari berikutnya. '*Repeated measure ANOVA'* dan '*Mann-Whitney'* telah digunakan untuk data analisis.

KEPUTUSAN

Tiada perbezaan yang ketara secara statistik untuk hasil klinikal (p = 0.434) dan kesan anti-bakteria (p = 0.198) antara madu Tualang dan madu Manuka sebagai rawatan tambahan dalam *Pseudomonas* keratitis.

KESIMPULAN

Madu Tualang adalah setanding dengan madu Manuka sebagai rawatan tambahan dalam *Pseudomonas* keratitis pada mata arnab.

KATA KUNCI

Pseudomonas keratitis, madu Tualang, madu Manuka, kesan klinikal, kesan antibakteria

ABSTRACT

INTRODUCTION

Honey is a known alternative therapy for varies diseases. Manuka honey antibacterial eye drop is established marketed honey for eye infection. We aim to explore the potential of Tualang honey to be commercialized in ophthalmic usage. Thus, we conducted an experimental study to compare the clinical and antibacterial effects of Tualang and Manuka honey as adjunctive treatment in *Pseudomonas* keratitis.

OBJECTIVE

Several in vitro studies documented that Tualang and Manuka honey had antibacterial effect towards *Pseudomonas aeruginosa*. This study is done to compare the mean Slit Lamp Examination (SLE) score and mean Colony Forming Unit (CFU) count of *Pseudomonas*-induced keratitis in rabbit eyes.

METHODS

This experimental animal study consisted of 20 rabbits induced with *Pseudomonas* keratitis that were randomly and equally divided into two groups. Group A was treated with a combination of Tualang honey 30% and gentamycin 0.3% eight times per day, while Group B was treated with a combination of Manuka honey and gentamycin 0.3% eight times per day. At the same time, a parallel study with similar methodology was conducted on ten rabbits which were treated with gentamycin 0.3% as a control group. Serial clinical examination using Slit Lamp Examination (SLE) score was done at 24, 48, 72 hours, Day 5 and Day 7. All rabbits were euthanized at Day 7 and their corneas harvested. The harvested

corneas were processed and Colony Forming Unit (CFU) counts were performed on the next day. Repeated measure ANOVA and Mann-Whitney test were used for the data analysis.

RESULTS

There was no statistically significant difference of clinical outcome (p=0.434) and antibacterial effect (p=0.198) between Tualang honey and Manuka honey as adjunctive treatment in *Pseudomonas* keratitis.

CONCLUSION

Tualang honey is comparable to Manuka honey as adjunctive treatment in *Pseudomonas* keratitis in rabbit eyes.

KEYWORDS

Pseudomonas keratitis, Tualang honey, Manuka Honey, Clinical effect, Antibacterial effect

CHAPTER 1:

INTRODUCTION

Pseudomonas keratitis is a common complication of contact lens and vegetative ocular injury (Hooi *et al*., 2005; Norina *et al*., 2008; Willcox., 2012). Rapid corneal perforation occurs due to a combination of *Pseudomonas aeruginosa's* protease enzymes and an intense host immune inflammatory reaction (Kessler *et al*., 1977). Therefore, urgent and prompt treatment is needed in order to minimize this devastating ocular complication.

A study by Faiz *et al*. (2007) has shown that only 33% of *Pseudomonas* keratitis is sensitive towards gentamycin. Similarly, a report by Bharati *et al*. (2010) documented that gentamycin only effective towards 55% of *Pseudomonas* keratitis patients. Therefore, this indicates an alarming phenomenon regarding resistance of antibiotic in the treatment of *Pseudomonas* keratitis.

Apitheraphy is an alternative medicine that uses bee products especially honey. The unique properties are determined by the bee's floral and geographical area of origin (Taormina *et al*., 2001). Honey has been proven to have antibacterial, healing properties, anti-inflammatory anticarcinogenic, and antifungal (Dustman., 1979; Sadagatullah *et al*., 2011; Bashkaran *et al*., 2011; Kadir *et al*., 2013; Sayadi *et al*., 2015).

Tualang honey is a local Malaysian honey while Manuka honey is a well commercialized honey from Australia and New Zealand. Tualang honey is a multifloral honey with higher phenolics, flavonoids and 5-hydroxymethyl-furfural than Manuka honey (Sarfarz *et al*.,

2013). Meanwhile, Manuka honey is a monofloral honey with its antibacterial properties derived from high non hydrogen peroxide and methylglyoxal activity (Khan *et al*., 2007).

In vitro study by Tan *et al*. (2009) demonstrated that Tualang and Manuka honey have similar level of Minimum Inhibitory Concentration against *Pseudomonas aeruginosa*. In fact, Sadagatullah *et al*. (2011) proved that the wound healing properties of Tualang honey are comparable to Manuka honey in post debridement diabetic foot wounds.

Manuka honey has been well established medical honey in many fields including ophthalmology. Malhotra *et al*. (2016) showed that Manuka honey has healing effect towards bilateral upper blepharoplasty scar. On the other hand, Albietz *et al*. (2015) demonstrated that Manuka honey was a safe and effective adjunctive therapy for persistent post-operative corneal oedema.

To date, there is limited data available on the use of Tualang honey in ophthalmology practice. Recently, Bashkaran *et al*. (2011) reported that Tualang honey has corneal healing effect in experimental alkaline corneal injury in rabbits. However, we have not encountered literature comparing the clinical and antibacterial effect of Manuka and Tualang honey as adjunctive treatment in *Pseudomonas* keratitis.

CHAPTER 2:

STUDY PROTOCOL

DISSERTATION PROTOCOL

A Comparison of the Clinical and Antibacterial Effect between Tualang Honey and Manuka Honey as Adjunctive Treatment in *Pseudomonas* Keratitis in Rabbit Eyes

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2.1 INTRODUCTION

2.1.1 *Pseudomonas* Keratitis

Corneal ulcer and keratitits cause by *Pseudomonas aeruginosa* is a common type of contact lens related ulcer (Choy *et* al., 2008). Most of the patients are young and healthy. It is known to cause rapid cornea perforation due to collagenase activity of the *Pseudomonas*. Urgent and intensive treatment is needed to prevent this vision threatening event. In practice, topical gentamycin and flouroquinolone are proven as standard treatment (Willcox., 2012). However, the issue on antibiotic resistance in treatment of keratitis is on the rise. The antibiotic resistance in ocular infection is contributed by prolonged and overuse therapy (Sharma., 2011).

2.1.2 Honey

Recently, modern medical therapy is developing their new treatment from natural source of product such as honey as it is cost effective and unlikely to develop resistance and side effects. Honey is a natural product of bees. The antibacterial activity of honey was first recognized in 1892 (Dustmann., 1979). Honey can inhibit the growth of bacteria, fungal and protozoa (Molan., 1992). The difference of its properties is determined by different floral and geographical area of origin of the bees (Taormina *et al*., 2001). Many studies were conducted to prove its anti-inflammatory, healing properties, antibacterial, antioxidant, anticarcinogenic and antifungal (Molan., 1992; Medhi *et al*., 2008; Tan *et al*., 2009; Bashkaran *et al*., 2011; Kadir *et* al., 2013; Sayadi *et al*., 2015). The successful of honey in treatment of surgical

(Bansal *et al*., 2005) and diabetic wound healing (Makhdoom *et al*., 2009) encourage the initiatives to prove its effectiveness in other field including ophthalmology.

2.1.3 Tualang and Manuka honey

Tualang honey is a Malaysian honey derived from *Koompassia excelsa* (Tualang tree). It is a natural honey that is comparable to other types of honey. Properties that contribute to its beneficial effects are from the non-enzymetic and enzymetic activities. It is rich in protein especially proline, vitamins, minerals, flavonoid and alkaloid. Enzymetic properties are hydrogen peroxide, oxidase, invertase, catalase, and amylase (Molan., 1992). Brudzynski (2006) reported that dilution of honey enhances its antibacterial activity due to hydrogen peroxide enzyme.

Tumin *et al*. (2005) have conducted a study regarding antibacterial properties of different types of honey in Malaysia. Tualang honey is the most acidic local honey with pH 3.55. Acidity contributes for the antibacterial activity. They reported that Tualang honey has the lowest Minimum Inhibitory Concentration (MIC) of 48.75µg/ml for *Pseudomonas aeruginosa* compared to other local type of honey 57.50 µg/ml, 70 µg/ml, 80 µg/ml and 135 µg/ml respectively for Hutan, Gelang, Pucuk daun and Ee Feng Gu.

Manuka honey is an Australian and New Zealand honey that has been proven to be effective in treating many types of disease by many studies. Manuka honey demonstrates both peroxide and non peroxide activity (Molan., 1992). Thus, this suggests its antibacterial activity will still be effective in the absence of hydrogen peroxide enzyme.

Research has been done for Tualang and Manuka honey to compare their ability in treating infected wound and healing process (Sadagatullah *et al*., 2011; Sarfarz *et al*., 2013). The comparison between these honeys in ophthalmology practise is still lacking.

2.2 RESEARCH OBJECTIVE

2.2.1 General Objective

To evaluate the clinical response and antibacterial effects of Tualang honey and Manuka honey as adjunctive treatment in *Pseudomonas*-induced keratitis in rabbit eyes.

2.2.2 Specific Objectives

i. To compare the mean Slit Lamp Examination (SLE) score between Tualang honey and Manuka honey as adjunctive treatment in *Pseudomonas*-induced keratitis at 24, 48, 72 hours, 5th and 7th day.

ii. To compare the mean Colony Forming Unit (CFU) count of *Pseudomonas*-induced keratitis following treatment of Tualang honey and Manuka honey at Day 7.

2.3 RESEARCH HYPOTHESIS

1. There is a significance difference in the SLE score of *Pseudomonas*-induced keratitis in rabbit eyes between Tualang honey and Manuka honey as adjunctive treatment.
2. There is a significance difference in CFU count of *Pseudomonas aeruginosa* between Tualang honey and Manuka honey as adjunctive treatment.

2.4 DEFINITION OF TERMS

2.4.1 Tualang honey

Tualang honey is obtained from Federal Agriculture Marketing Authority (FAMA), Kedah Malaysia which was collected from *Koompassia excelsa* (Tualang tree).

2.4.2 Manuka honey

Manuka honey is an Australian or New Zealand's honey that commercially available in viscous eye drop manufactured by Melcare Biomedical Pty Ltd. Each sterilized tube contains 98% *Leptospermum scoparium.*

2.4.3 Clinical response

Clinical response is examination of rabbit's eye using a portable slit lamp based on SLE score system (Dong *et al*., 2012).

2.4.4 Colony forming unit

Calculation of viable *Pseudomonas aeruginosa* in rabbits harvested corneas post treatment.

2.5 METHODOLOGY

2.5.1 Study Design

Randomized control trial

2.5.2 Study Location

Animal Research and Study Centre (ARASC), Microbiology and Parasitology laboratory, Universiti Sains Malaysia, Health Campus, Kubang Kerian

2.5.3 Study Duration

September 2014 - August 2016

2.5.4 Study population

New Zealand white adult rabbits will be obtained from ARASC, Universiti Sains Malaysia, Health Campus, Kubang Kerian.

2.5.5 Sampling Method

Randomization of 20 New Zealand white adult rabbits (aged 8 - 10 months, weighing between 2.0 and 2.5kg) into 2 groups (A and B) consisting of 10 rabbits in each group.

2.5.6 Inclusion & Exclusion Criteria

Inclusion Criteria

1. All healthy adult rabbits with clear corneas that are successfully induced with *Pseudomonas* keratitis.

Exclusion Criteria

1. Rabbits with cornea perforation.
2. Rabbits with systemic infection of *Pseudomonas aeruginosa.*

2.5.7 Determination of Sample Size

Sample Size Calculation

Objective 1: To compare the mean SLE score between Tualang honey and Manuka honey as adjunctive treatment in *Pseudomonas*-induced keratitis at 24, 48, 72 hours, 5th and 7th day.

Effect size = 0.4, Power = 80%, Alpha level = 5% (0.05)

Number of group = 2, Number of evaluation = 5

Calculated sample size is 10 rabbits per group

Objective 2: To compare the mean CFU count of *Pseudomonas*-induced keratitis following treatment of Tualang honey and Manuka honey at Day 7.

Effect size = 0.4, Power = 80 %, Alpha level = 5% (0.05)

Number of group = 2

Calculated sample size is 52 rabbits per group

The sample size is chosen based on first objective as the numbers of the rabbits are smaller

Calculated sample size is 10 rabbits per group

30% dropout rate = 7

Total number of rabbits needed = (10 x 2) + 7 = 27

2.5.8 Randomization Method

Twenty New Zealand white adult rabbits (aged 8 - 10 months) weighing between 2.0 and 2.5kg will be used in this study. The rabbits will be randomly divided into 2 groups (A and B) consisting of 10 rabbits in each group.

a) Group A will be treated with a combination of one drop of topical Tualang honey 30% and one drop of topical gentamycin 0.3%.

b) Group B will be treated with a combination of one drop of topical Manuka honey and one drop of topical gentamycin 0.3%.

2.5.9 Research Tools

A. Animals

1. New Zealand white adult rabbits
2. Rabbit cage
3. Pellet and bedding

B. Drugs and Solutions

1. Ketamine hydrochloride (Troy Laboratories, New South Wales, Australia)
2. Xylazine hydrochloride (Troy Laboratories, New South Wales, Australia)
3. Pentobarbital sodium (Troy Laboratories, New South Wales, Australia)
4. Buphrenorphine (Troy Laboratories, New South Wales, Australia)
5. Proparacaine hydrochloride 0.5% eye drops (Alcon-Couvreur Puurs, Belgium)
6. Gentamycin 0.3% eye drops (FDC Ltd)
7. Topical Tualang honey 30% (FAMA)

8. Topical Manuka honey (Melcare Biomedical Pty Ltd, Australia)
9. Trypticase Soy broth and agar plates (Thermo Scientific Microbiology Sdn. Bhd)
10. 1% fluorescein strip (Chauvin Pharmaceuticals Ltd, England)
11. Povidone iodine

C. Ophthalmic instrument for routine clinical examination

1. Portable slit lamp (Kowa SL-15)

D. Surgical instruments

1. Needles 27G and 30G
2. Syringes 1ml and 5ml
3. Dressing set
4. Caliper
5. Barraquer wire speculum (paediatric)
6. Conjunctival forcep
7. Surgical glove
8. Surgical drape
9. Shaver blade

10. Corneal scissors

E. Laboratory instruments

1. Centrifuge tube
2. Repeat pipette tip
3. Weighing scale machine
4. Disposable biohazard bag

2.5.10 Examiner

1. Primary Investigator
2. Trainee Pathologist

2.5.11 Study Procedures

The study will be conducted after obtaining approval from the Animal Ethics Committee Universiti Sains Malaysia.

A. Preparation of $10^{\wedge 3}$ CFU of *Pseudomonas aeruginosa*

a. *Pseudomonas aeruginosa* (ATCC27853) will be grown overnight at 37ºC in a trypticase soy agar (TSA).

b. The inoculum will be diluted with trypticase soy broth (TSB) to yield $10^{\wedge 3}$ CFU/ml.

B. Induction of *Pseudomonas* keratitis

a. The rabbits will be anaesthetized by subcutaneous injection of a mixture of xylazine hydrochloride (100mg/ml) and ketamine hydrochloride (100mg/ml).
b. Proparacaine hydrochloride will be applied to the rabbit's eye before intrastromal injection.
c. Right cornea epithelium of each rabbit will be debride using 27G needle followed by intrastromal injection of 0.1ml ($10^{\wedge 3}$ CFU/ml) *Pseudomonas aeruginosa*.
d. Intramuscular buphrenorphine of 0.02-0.1mg/kg (every 6-12 hours) will be administered for pain relief.

C. Preparation of 30% topical Tualang honey

a. 100% Tualang honey will be given Gamma irradiation at a dose of 25kGy followed by sterility test.
b. Tualang honey will be diluted with water for injection until become 30% (1.5ml water for injection + 3.5ml Tualang honey). 30% concentration is used due to its lowest concentration for bactericidal effect towards *Pseudomonas aeruginosa* is 25% (Tan *et al*., 2009).

D. Preparation of Manuka honey

a. Manuka honey is manufactured by Melcare Biomedical Pty Ltd, Australia and available in the form of viscous eye drop with concentration of 98% *Leptospermum scoparium*.

E. Instillation of topical eye drops

a. Block randomization of 20 rabbits into two groups: Group A (n=10) and B (n=10).
b. Topical eye drops will be instilled eight times per day after 24 hours.
c. Group A will be started with topical Tualang honey 30% + topical gentamycin 0.3%
d. Group B will be started with topical Manuka honey + topical gentamycin 0.3%

F. Slit lamp examination score

a. The rabbits will be examined at 24, 48, 72 hours, 5th and 7th days.
b. The SLE score as below (Dong *et al.,* 2012)

Table 1: Slit lamp examination score

Grade	Focus of infection		
0	No focus of infection		
1	Corneal infiltrate	1.25	Limited in the inoculated area
		1.50	≤ ½ corneal thickness
		1.75	> ½ corneal thickness
2	Corneal ulcer	2.25	Diameter ≤ 3mm
		2.50	> 3mm - < 5mm
		2.75	Diameter ≥ 5mm
3	Hypopyon	3.25	≤ ⅓
		3.50	⅓ - ½
		3.75	> ½
4	Hypopyon and corneal perforation		

G. Cornea harvesting

a. All rabbits will be euthanized by an intravenous injection of a pentobarbital sodium solution (100mg/ml) at Day 7.
b. The entire cornea of right eye will be excised at the clear limbal margin, excluding any sclera or conjunctiva.
c. The excised cornea will be rinsed with 5 ml of balanced salt solution, minced and placed in a test tube with 2 ml of phosphate buffered saline and homogenized.
d. Aliquots of the cornea will be serially diluted and plate in TSA.

H. Colony Forming Unit count

a. The TSA plates containing diluted aliquots of harvested cornea will be incubated for 18 hours at 37ºC.

b. Colonies will be counted and mean *Pseudomonas* CFU will be determined.

2.5.12 Statistical Analysis

First Objective : To compare the mean SLE score between Tualang honey and Manuka honey as adjunctive treatment in *Pseudomonas*-induced keratitis at 24, 48, 72 hours, 5th day and 7th day.

Table 1: The comparison of the mean SLE score between Tualang honey and Manuka honey as adjunctive treatment in *Pseudomonas*-induced keratitis.

Time	Groups	SLE score Mean (SD)	Mean difference (95% CI)	F-stat (df)	P value
24 hours	A B				
48 hours	A B				
72 hours	A B				
Day 5	A B				
Day 7	A B				

Repeated measure ANOVA or Friedman test will be used for the statistical analysis

Second Objective : To compare the mean CFU count of *Pseudomonas*-induced keratitis followng treatment of Tualang and Manuka honey at Day 7.

Table 2: The comparison of the mean CFU count of *Pseudomonas*-induced keratitis following treatment of Tualang honey and Manuka honey at Day 7.

Treatment Group	Colony Forming Unit (CFU) count	Mean Difference (95% CI)	F-stat (df)	P value
	Mean (SD)			
Group A				
Group B				

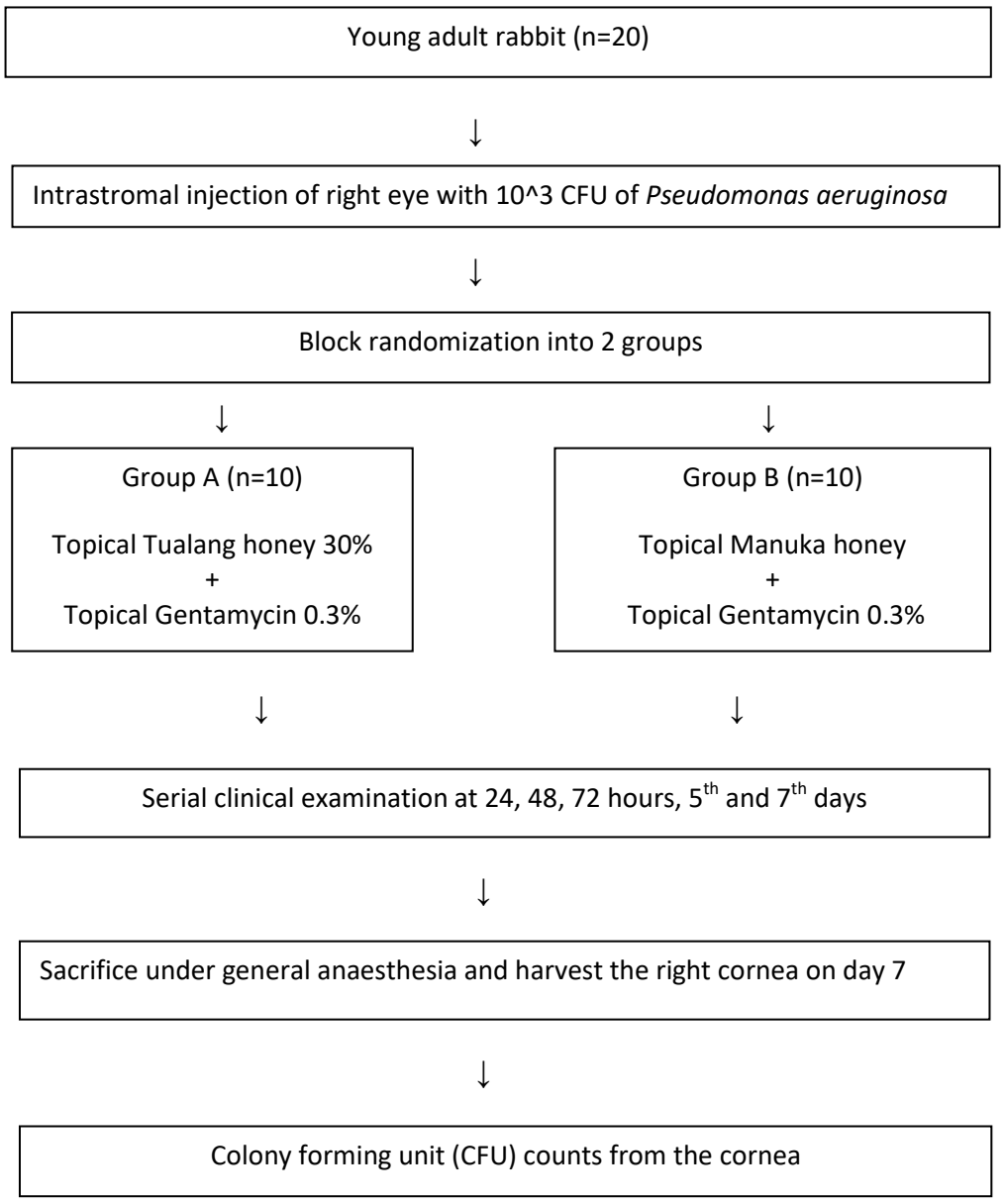
Independent t-test or Mann-Whitney test will be used for the statistical analysis

2.5.13 Methods to minimize study errors

A. Serial clinical examination will be done by a primary investigator who is blinded.

B. Colony forming units count will be done by a trainee pathologist who is blinded.

2.5.14 Flow Chart



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