# EVALUATION OF TUALANG HONEY AND PROPOLIS ON VAGINAL FLORA IN LOCALLY ADVANCED CERVICAL CANCER PATIENTS UNDERGOING CHEMORADIOTHERAPY


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## UNIVERSITI SAINS MALAYSIA

## 2018

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**by**


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**Thesis submitted in fulfilment of the requirements**

**for the Degree of**

**Master of Science**

**February 2018**

## ACKNOWLEDGEMENTS

In the name of Allah, The Most Gracious and The Most Merciful

All praise to Allah S.W.T., Lord of the universe and selawat to Prophet Muhammad P.B.U.H., messenger of Allah, for giving me this opportunity and strength to complete this study. Special thanks to my dear supervisor, Associate Prof. Dr. Kirnpal Kaur Banga Singh, for your guidance, knowledge and contribution on the project and content of this thesis. She is always full of mind and gave valuable suggestion and insight for this project to finish successfully.

To my co-supervisor Prof. Dr. Biswa Mohan Biswal, one of the main contributors for this research, before he left USM on 2014 to join KPJ Ipoh. Thank you for the lessons about nuclear medicine, radiotherapy and oncology, always giving me good tips in clinical field. Many thanks to all the staff from Nuclear Medicine, Radiotherapy & Oncology Clinic for helping during patient recruitment, especially to Staff Nurse Ramlah for her kindness and patience during this research period. My apologies for all the inconveniences caused by me or my study.

Also thanks to En. Nadzri, Mrs Rosliza Abd Rahman, and all the staff in Microbiology & Parasitology Department who was directly or indirectly involved and helped me in the lab, for lending me the facilities and knowledge, in order for me to complete my research comfortably. To Mr. Jamaruddin from Immunology Laboratory, thank you so much for guiding me with the immunology part. Many thanks to my postgraduate friends in Microbiology Laboratory for lots of advice and

help in completing my study. Good luck and all the best to all of you in your study. Last but not least, thanks to my family for the support and prayer, and also to my lovely wife, Siti Khatijah Abdul Razak for your contribution during my time in USM.

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| | |
|---|---|
| % | Percent |
| °C | Degree Celsius |
| µL | Microlitre |
| AIDS | Acquired Immune Deficiency Syndrom |
| AN | Anaerobic |
| AST | Antimicrobial Susceptibility Testing |
| BA | Blood Agar |
| BD | Bectone Dickinson |
| BHI | Brain Heart Infusion |
| BV | Bacterial vaginosis |
| CD4+ | Cluster of differentiation 4 |
| C | Control Arm |
| CAMHB | Cation Adjusted Mueller Hinton II Broth |
| CBA | Chocolate horse Blood Agar |
| CDDP | Cisplatin |
| CFU | Colony Forming Unit |
| CIN | Cervical Intraepithelial Neoplasia |
| $CO_2$ | Carbon Dioxide |
| CRP | C-Reactive Protein |
| ECOG | Eastern Cooperative Oncology Group |
| EDTA | Ethylenediamine Tetraacetic Acid |
| FAMA | Federal Agriculture Marketing Authority |
| FIGO | International Federation of Gynecology and Obstetrics |

| | |
|---|---|
| gm | Grams |
| GN | Gram Negative |
| GP | Gram Positive |
| Gy | Gray Unit |
| HBA | Horse Blood Agar |
| HVS | High Vagina Swab |
| HVW | High Vagina Wash |
| ICRT | Intracavitary Radiation Therapy |
| ICRU | International Commission on Radiation Unit and Measurement |
| L | Litre |
| MBC | Minimal Bactericidal Concentration |
| MHA | Mueller Hinton Agar |
| MHBA | Mueller Hinton Blood Agar |
| MIC | Minimal Inhibitory Concentration |
| mL | Mililitre |
| MR | Methyl Red |
| MV | Mega-electron Volts |
| nm | Nanometer |
| $O_2$ | Oxygen |
| OD | Optical Density |
| PBL | Peripheral Blood Lymphocyte |
| PPSP | Pusat Pengajian Sains Perubatan |
| rpm | Revolution per minute |
| S | Study Arm |
| SIM | Sulphate Indole Motility |

| | |
|---|---|
| TSB | Tryptone Soy Broth |
| TSI | Triple Sugar Ion |
| USA | United State of America |
| USM | Universiti Sains Malaysia |
| WCA | Wilkins-Chalgren Agar |
| WCB | Wilkins-Chalgren Broth |

# PENILAIAN RAWATAN MADU TUALANG DAN PROPOLIS TERHADAP MIKROFLORA FARAJ SEMASA RAWATAN KEMORADIOTERAPI PADA PESAKIT KANSER SERVIK PEMEREBAKAN SETEMPAT

## ABSTRAK

Kanser serviks adalah kanser yang paling lazim terjadi dalam kalangan wanita. Rawatan piawai bagi penyakit ini terdiri daripada radioterapi, kemoterapi dan brakiterapi. Kemoterapi selalunya diberikan kepada semua pesakit kanser serviks. Kemoradioterapi sering dikaitkan dengan kesan sampingan. Kemoradioterapi menyebabkan sistem imun menjadi lemah dan kesan sampingannya adalah seperti keguguran rambut, hilang selera makan yang mana boleh mengganggu kebanyakan pesakit kanser serviks. Terdapat kekurangan pengetahuan dalam bidang mikrobiologi terhadap jangkitan bakteria semasa kesan sitotoksik kemoradioterapi tersebut. Kajian klinikal ini dijalankan untuk mengetahui kesan madu Tualang dan propolis pada pencilan bakteria flora faraj semasa kemoradioterapi. Kajian *in vivo* telah dimulakan untuk menilai pengurangan pertumbuhan bakteria semasa rawatan piawai kemoradioterapi terhadap pesakit kanser servik. Seramai 30 pesakit telah berjaya direkrut (15 pesakit dalam kumpulan kajian, 15 pesakit dalam kumpulan kawalan). Untuk kumpulan kajian, rawatan kemoradioterapi telah disertakan dengan rawatan apiterapi, sementara untuk kumpulan kawalan hanya menerima rawatan kemoradioterapi sahaja. Swab pada kawasan faraj tinggi dan cecair basuhan faraj tinggi telah dikumpulkan sebelum dan selepas kemoradioterapi. Dua sampel ini telah diproses untuk melihat kadar pertumbuhan bakteria selama lima minggu berturut-turut terapi diberikan kepada pesakit. Perubahan dalam pertumbuhan bakteria telah

dibandingkan diantara lima minggu tempoh rawatan untuk mengenalpasti keberkesanan *in vivo* madu Tualang dan propolis. Berbagai jenis bakteria telah dipencilkan dan dikenalpasti daripada sampel-sampel, mencadangkan bahawa telah berlaku perubahan dalam ekosistem faraj. Penemuan daripada kajian ini juga menunjukkan madu Tualang dan propolis mempunyai potentsi sebagai rawatan komplimentari semasa rawatan kemoradioterapi dilakukan. Pengurangan dalam pertumbuhan bakteria boleh dilihat pada pesakit yang dirawat dengan madu Tualang dan propolis (kumpulan kajian) berbanding dengan pesakit yang tidak dirawat dengan madu Tualang dan propolis (kumpulan kawalan). Hasil dari kajian ini, madu Tualang dan propolis mempunyai potensi sebagai agen anti-bakteria untuk digunakan sebagai rawatan komplementari. Kesan antibakteria seterusnya dibuktikan secara *in vitro* dengan menggunakan bakteria yang dipencilkan secara langsung daripada pesakit kanser servik dalam kajian ini. Dua penanda immunologi telah dikaji iaitu tahap protein CRP dan bilangan sel limfosit CD4+, yang dibandingkan dengan kadar pertumbuhan bakteria. Kajian ini menunjukkan bahawa kedua-dua protein CRP ($r=-0.3$, $p\leq0.05$) dan sel CD4+ ($r=-0.37$, $p\leq0.05$) tidak signifikan dengan kadar pertumbuhan bakteria. Tetapi untuk CRP protein, keputusan kajian menunjukkan hasil yang positif dan mungkin perlu dibuat kajian dengan lebih lanjut untuk kepastian kerana jumlah sampel yang sedikit bagi kajian ini. Bagi sel CD4+, ia tidak boleh digunakan sebagai penanda untuk mengesan jangkitan bakteria kerana ia terkesan dengan rawatan kemoradioterapi. Hasil daripada kajian ini, madu Tualang dan propolis boleh digunakan sebagai rawatan komplimentari untuk mencegah jangkitan bakteria semasa rawatan kemoradioterapi. Pengurangan dalam pertumbuhan bakteria dapat dilihat pada pesakit yang dirawat dengan madu Tualang dan propolis, selari dengan jumlah protein CRP didalam badan pesakit.

# EVALUATION OF TUALANG HONEY AND PROPOLIS ON VAGINAL FLORA IN LOCALLY ADVANCED CERVICAL CANCER PATIENTS UNDERGOING CHEMORADIOTHERAPY

## ABSTRACT

Cervical cancer is the most common cancer among women. The standard treatment consists of radiotherapy, chemotherapy and brachytherapy. Chemotherapy is invariably administered to all cervical cancer patients. Chemoradiotherapy agents often associated with side effects. Chemoradiotherapy induced immune system is one of the side effects despite of the hair loss, lost appetite which affects cervical cancer sufferers mostly. There is lack of knowledge in the field of microbiology that should focus on bacterial infection during the cytotoxic treatment with chemoradiotherapy. This prospective clinical trial was conducted to elucidate the effect of Tualang honey and propolis on the changes of the vaginal flora isolation in the high vaginal area during chemoradiotherapy treatment period. The *in vivo* study was embarked on to evaluate the reduction of bacterial growth during the standard chemoradiotherapy to cervical cancer patients. A total of 30 patients were successfully recruited (15 study arm, 15 control arm). In the study arm group, chemoradiotherapy was supplemented with additional apitherapy treatment, while the control arm group only received the chemoradiotherapy. High vaginal swab and high vaginal wash were collected before and after chemoradiotherapy from recruited patients to quantitate the bacterial growth, for five consecutive weeks of treatment. The changes in the bacterial growth were compared between the five weeks of treatment to determine the *in vivo*'s efficacy of Tualang honey and propolis. Various types of bacteria were isolated and

identified from samples, suggesting the alteration occurred in the vaginal ecosystem. Finding from this study also demonstrated that Tualang honey and propolis has potential as complementary treatment during chemoradiotherapy. Inhibition of bacterial growth were noted in patients treated with Tualang honey and propolis (study arm) compared to the untreated patients (control arm). The antibacterial effects of Tualang honey and propolis were subsequently proved via *in vitro* studies using bacteria that were isolated directly from cervical cancer patients. Two immunological markers namely CRP protein level and lymphocyte CD4+ cell count were tested whether correlated with the bioburden of bacteria. Finding showed that both CRP protein level ($r=-0.3$, $p\leq0.05$) and lymphocyte CD4+ cell count ($r=-0.37$, $p\leq0.05$) were not significant with the bioburden of bacteria. But for CRP protein, the results still showed a good result, and maybe can be tested further to confirm its role due to the low sample size in this research. For CD4+ cells, it cannot be used as a marker to monitor the bacterial infections because it was affected by chemoradiotherapy. In this study, Tualang honey and propolis can be used as a complementary treatment to prevent any bacterial infections during chemoradiotherapy treatment. Bacterial growth reduction can be seen in patients treated with Tualang honey and propolis, parallel with CRP protein level in patient's body.

# CHAPTER 1

# INTRODUCTION

## 1.1 Research background

Cervical cancer is very common in women. When the cancer grows, they involve adjacent pelvic structures cause pain, hydronephrosis, discharge and bleeding. The growth is very often involved with bacterial infection which goes unnoticed. Bacterial infestation usually leadings to foul smelling discharge, pain, tissue edema leading to less vascularity of tumor causing hypoxia (Biswal *et al.*, 2014). Hypoxia resulted in reduced effectiveness to radiation. Honey is a natural product which has wide-spectrum of activity including antibacterial properties (Estevinho *et al.*, 2008; Gheldof & Engeseth*.,* 2002; Irish *et al.*, 2008; Swellam *et al.*, 2003; Temaru *et al.*, 2007; Wang *et al.,* 2002). Propolis or bee glue is also very effective against variety of bacteria commonly associated with vaginal infection (Pratsinis *et al.,* 2010). In this study, Tualang honey and propolis were used as a complementary treatment during the routine cancer treatment using both chemotherapy and radiotherapy.

Universiti Sains Malaysia (USM) is currently engaged in a study on Tualang honey as an alternative treatment for bacterial infection. Malaysian Tualang honey is

believed and has been proven by the previous studies to possess good antibacterial properties (Tan *et al.*, 2009; Khoo *et al.*, 2010). Thus, this provides an opportunity to conduct this study and to prove the *in vivo* antibacterial properties of the Malaysian Tualang honey.

## 1.2 Literature Review

### 1.2.1 Cervical cancer

Cancer is the uncontrolled growth of cells coupled with malignant behaviour: invasion and metastasis (Hanahan *et al.*, 2000). It is caused by the interaction between genetic susceptibility and environmental factors (Hodgson, 2008; Perera, 1997). These factors lead to accumulations of genetic mutations in oncogenes (genes that promote cancer) and tumour suppressor genes (genes that help to prevent cancer), which gives cancer cells their malignant characteristics, such as uncontrolled growth (Kehe *et al.*, 2009).

Cervical cancer is the second most common malignancy among women worldwide including Malaysia (Ferlay *et al*., 2004; Lim *et al.*, 2008), accounting for 527,624 cases in 2012 and 560,505 new cases are estimated to occur in 2015 (Ferlay et al., 2010). However, knowledge on the illness still low which resulted in poor attitude towards its prevention. A woman's cervix (the opening of the uterus at the top of the vagina) is covered by a thin layer of tissue made up of cells. Healthy cells grow, divide, and are replaced as needed. Cancer of the cervix occurs when these cells change (ACOG, 2015). Cancer cells divide more rapidly and they may grow into

deeper cell layers or spread to other organs. The cancer cells eventually form a mass of tissue called a tumor. It often takes several years for cervical cancer to develop. During this time, the cells on or around the cervix becomes abnormal. The cell changes that occur before cancer is present are called dysplasia or cervical intraepithelial neoplasia (CIN). Screening for cervical cancer is based on the presence of cytomorphologically abnormal epithelial cells. Cervical cancer initially grows locally within the anatomical extent of the cervix, followed by permeation to the parametrium and other pelvic viscera in addition to lymph node metastases. One of the factors is the local growth harbor bacterial growth in the vaginal cavity, leading to pelvic inflammatory disease and hydrometra.

Globally, cervical cancer is very common cancer among women, and there has been a study done in developing countries where this type of cancer is the second most common virus related cancer (Parkin *et al.*, 2002). A report in 2004 indicated that there were approximately 12,000 incidences of invasive cervical cancer with 3850 deaths among American women (US Cancer Statistics Working Group, 2007). However, prevalence of cervical cancer reported by World Health Organization (WHO) in developing countries remains incomplete and fragmentary. Indeed, Garland *et al.*, (2008) estimated that 52% of all cervical cancer cases occur in the Asia-Pacific and Australasia region. Moreover, the same study estimated that if the current rate of cervical screening remains unchanged in this region, there will be a 62% increase in the burden of disease associated with cervical cancer by 2025. Malaysia is a fast-developing South-East Asian country with a medium level of GDP per capita and a significant burden of cervical cancer. In 2008, 76% of all cases were diagnosed in FIGO (International Federation of Gynecology and Obstetrics) stage 2

or higher (Othman *et al.*, 2009). This is reflective of the fact that the Asia-Pacific region bears a disproportionate amount of the global burden associated with cervical cancer especially in Malaysia.

The mortality rate in Malaysia due to cancer is approximately 8.4 per 100,000 which is similar to that of other countries in the region, such as Indonesia, Singapore and Thailand, but more than two-fold higher in comparison with Netherlands, United Kingdom and Finland (Othman *et al.*, 2009). In Malaysia, cancer of the cervix most commonly occurred in all the major ethnic groups which are Malay, Chinese and Indian (Lim *et al.*, 2002). Compared among the major races, Chinese women had the highest incidence for cervical cancer followed by Indian and Malay (National Cancer Registry, 2006) where the incidence rate increased with age after 30 years and has its peak at ages 60 to 69 years. In Malaysia also, cervical cancer is now becoming the second most common cancer and is the fourth common cause of death among women (Domingo *et al.*, 2008). In 2006, the incidence rate of cervical cancer in Peninsula Malaysia was 12.2 (9.1%) per 100,000 women (National Cancer Registry, 2006), but in 2008, the incidence rate leveled up to 17.9 per 100,000 women (Globocan, 2008).

Mikamo *et al.,* (1999) in their study indicate that prevalence of bacterial vaginosis (BV) among patients with cervical cancer varies from 50-80 %. BV resulted due to a change in vaginal ecosystem, where lactobacilli dominate, normal flora is absent or greatly reduced, and replaced with a mixed, predominantly anaerobic flora, consisting of *Gardnerella vaginalis, Mycoplasma hominis, Mobiluncus* sp*., Bacteroides* sp*., Prevotela* sp*., Peptostreptococcus* sp*., Fusobacterium* sp*., and Porphyromonas* sp. (Georgijevic *et al.*, 2000).

The Centers for Disease Control and Prevention (CDC), which is a part of the principal agency in the United States government for protecting the health and safety of all Americans and for providing essential human services, have recently included BV on their list of emerging infectious diseases (CDC, 2002). Even though bacterial growth will be reduced by 47 % during radiotherapy, 24% of the patients developed fever possibly due to bacterial pathogenesis after brachytherapy procedure (Choo *et al.*, 2008). BV in cervical cancer induces inflammation, increase tissue pressure, and oxygenation thereby reduces radiation tolerance.

### 1.2.2 Chemotherapy

Chemotherapy is the treatment of cancer with one or more cytotoxic anti-neoplastic drugs (chemotherapeutic agents) as part of a standardized regimen. In the broad sense, most chemotherapeutic drugs work by impairing mitosis (cell division), effectively targeting fast-dividing cells. As these drugs cause damage to cells, they are also called cytotoxic. Chemotherapy treatment is about using chemical and biological agents to treat cancer. Since the first dose of cytotoxic chemotherapy was given in 1942, hundreds of thousands of chemical and biological agents have been tested for their activity in destroying cancer cells (David *et al.*, 2003). They prevent mitosis by various mechanisms for examples like damaging DNA and inhibition of the cellular machinery involved in cell division (Malhotra *et al.*, 2003; Kehe *et al.*, 2009).

One of the theories why these drugs can kill cancer cells is that they induce a programmed form of cell death known as apoptosis (Makin *et al.*, 2000). As

chemotherapy affects cell division, tumours with high growth rates (such as acute myelogenous leukaemia and the aggressive lymphomas, including Hodgkin's disease) are more sensitive to chemotherapy, as a larger proportion of the targeted cells are undergoing cell division at any time. Malignancies with slower growth rates, such as indolent lymphomas, tend to respond to chemotherapy much more modestly (Corrie and Pippa, 2008).

### 1.2.3 Radiotherapy

Radiotherapy is the treatment of disease using radiation. Radiotherapy uses radiation such as x-rays, gamma rays, electron beams or protons, which to kill or damage cancer cells and stop them from continue growing and multiplying. It can be given in two ways which are by external radiotherapy (a machine from outside the body aims radiation beams towards the cancer and surrounding tissues where the cancer may have spread) and by internal radiotherapy or also called brachytherapy (a radiation source is put inside the body on or near the cancer area). The aim of radiotherapy is to destroy cancer cells with as little damage as possible to normal cells. With the right amount of treatment, cancer cells do not recover from radiotherapy. Meanwhile, damage to normal cells causes side-effects. Usually the normal tissue recovers quickly and the side-effects do not last long. Sometimes the damage takes longer to repair and the side-effects may prolong on patients. Radiotherapy is very specific and only affects the area that is being treated. It is unlike chemotherapy which can affect the cells in your entire body (Antoinette, 2009).

### 1.2.4 Apitherapy

Apitherapy is a branch of alternative medicine that uses honey and bee product such as propolis. These apitherapy products are well-known potential natural agents that have been shown to have good antibacterial properties. It is recognized for its benefits in medical uses, due to evidence of its antibacterial properties, which inhibit a broad spectrum of bacterial species (Molan and Russell, 2001).

Honey is still being used not only as a natural sweetener or for its nutritional values, but also as a curative agent especially for bacterial infection. This was proved in the past decade where it has been an increase in the use of traditional and complementary or natural systems of medicine (The Landmark Report, 1998; Wilkinson *et al.*, 2001; Thomas *et al.*, 2001). Physical characteristics of honey such as its high osmolarity and acidity of honey also contributed to its antibacterial properties (Basualdo *et al.*, 2007). The content inside honey is a bundle collection of nectar from many sources of plants by honey bees and this natural product is well known for its high nutritional and prophylactic medicinal value (Abou El-Soud Neveen, 2012).

The first discovery of antibacterial activity of honey was in 1892 (Dustmann, 1989), but due to the lack of scientific support, the medicinal purpose was very limited (Ali *et al.,* 1991). Recently, honey has been scientifically tested and confirmed to possess functional and biological properties such as anti-oxidant, anti-inflammatory, anti-bacterial, anti-viral, anti-ulcerative activities, anti-lipid and anti-cancer properties (Estevinho *et al*., 2008; Gheldof *et al*., 2002; Irish *et al*., 2008; Swellam *et al*., 2003;

Temaru *et al*., 2007; Wang *et al*., 2002). All of these properties are mainly attributed to the phenolic constituents such as flavonoids that have antioxidant properties and radical-scavenging activities. These flavonoids are observed in all types of honeys in different proportions depending on the geography, food source for the honeybee, and climate (Beretta *et al*., 2007; Hegazi *et al*., 2009; Viuda-Martos *et al*., 2008). Malaysian tualang honey is collected from the combs of Asian rock bees (Apis dorsata), which build their hives high up in the tualang tree (Koompassia excelsa). Tualang honey is used commonly as a medicinal product and as food in Malaysia. However, little scientific information about its microbiological properties has been published to date (Tan *et al.,* 2009).

Propolis is a hive product collected by honeybees from various floral plant sources. It is a hard, resinous material derived by bees from plant juices and used to seal openings in the hives. It was a long history of being used in folk medicine dating back to at least 300 BC (Ghisalberti, 1979) and also has been reported to possess various biological activities, namely anticancer, antioxidant, anti-inflammatory, antibiotic, antifungal and anti-hepatotoxic (Ghisalberti, 1979; Burdock, 1998). Propolis also contains pollen, resins and waxes and large amounts of flavonoids which are benzo-y-pyrone derivatives found in all photosynthesizing cells. Flavonoids have many biological effects in animal systems but have received relatively little attention from pharmacologists (Havsteen, 1983). There are lots of propolis in the market, and this propolis that used in this study were bought trusted researcher in France. In many clinical studies, honey and propolis were found to control infections, including surgical wound and respiratory tract infections (Grange, 1990; Molan, 2002; Paul, 2007; Ramanauskiene, 2009). Both products have wide-

spectrum of antibacterial activities. They also posseses cytostatic and anti-angiogenic properties and reduce inflammation (Banskota, 2002; Isla, 2009; Pratsinis, 2010).

Most of the bacterial pathogens could be controlled with apitherapy including resistant organisms and there are many types of honey that can be found all over the world. Each honey has different antimicrobial potency depending on its sources either polyfloral or monofloral, types of bees and environmental conditions, as well as its harvesting processes and storage conditions (Sherlock *et al.*, 2010).

### 1.2.5 Microbiological ecology of vagina

The vaginal ecosystem is a complex biosphere, which made up of variety of constituents existing in a delicate equilibrium of many types of bacteria that are constantly secreting and releasing metabolic products and cellular debris from the disruption of dying bacterial cells. This vaginal secretion contains a variety of compounds such as proteins, carbohydrates, urea and fatty acids (Sumawong *et al.*, 1952; Paavonen, 1983). While the host vagina is also constantly secreting metabolic products and cellular debris into the ecosystem (Sebastian, 2004).

The vaginal microflora consists of among other organisms, Gram-positive and Gram-negative aerobic, facultative and obligate anaerobic bacteria, which are non-pathogenic and potentially pathogenic bacteria. In a healthy vaginal ecosystem, the microflora is dominated by *Lactobacillus* sp. (Sebastian, 2004). But when the ecosystem becomes disrupted or unbalanced, the pathogenic bacteria gain dominance and pose a potential threat to the individual's general health and this condition will

lead to BV (Sebastian 2004). BV may cause damage to the vaginal epithelium by degrading the cervical mucus with proteases and changing the physicochemical and immunological environment of the vaginal niche, allowing the entry of pathogens (Rodriguez-Cerdeira *et al*., 2012).

### 1.2.6 Cluster of differentiation 4 (CD4+)

In molecular biology, CD4+ is a glycoprotein which can be found on the surface of immune cells such as T helper cells, monocytes, macrophages, and dendritic cells. It was discovered in the late 1970s and was originally known as leu-3 and T4 (after the OKT4 monoclonal antibody that reacted with it) before being named CD4 in 1984 (Bernard *et al.*, 1984). In humans, the CD4 protein is encoded by the *CD4* gene (Isobe *et al.,* 1986; Ansari *et al.*, 1996).

CD4+ T helper cells are white blood cells that are an essential part of the human immune system. They are often referred to as CD4+ cells, T-helper cells or T4 cells. They are called helper cells because one of their main roles is to send signals to other types of immune cells, including CD8 killer cells, which then destroy the infectious particle. If CD4+ cells become depleted, for example in untreated HIV infection, or following immune suppression prior to a transplant, the body is left vulnerable to a wide range of infections that it would otherwise have been able to fight (Christophe *et al.,* 2004). The role of CD4+ levels measured in this study is to see whether it can act as a suitable marker to monitor BV.

### 1.2.7 C-reactive protein (CRP)

CRP is an acute phase protein present in low concentrations in healthy individuals (Price *et al.*, 1999). Any pathological condition associated with invasive bacterial infection, inflammation or tissue destruction is accompanied by elevation of the CRP level in the patient's serum. The rise in CRP levels is rapid, and increased levels can be detected within 6 to 12 hours of the onset of the inflammatory process (van Leeuwen *et al.*, 1994).

These acute phase protein synthesized in the liver. Production of CRP is rapidly induced in response to infection, inflammation and tissue injury. Measurement of CRP may be helpful in the clinical management of a patient with symptoms of an infection. When evaluated in the light of the patient's clinical condition, measurement of CRP can assist healthcare professionals in differentiating between bacterial and viral infections and in rationalising antibiotic therapy. Monitoring CRP levels also provides an objective means for assessing treatment response, as CRP levels fall rapidly as a result of an effective therapy.

Quantitative measurement of the CRP concentration has been reported to be a sensitive indicator of the efficacy of antimicrobial therapy and the course of bacterial infections, as well as an effective tool in controlling and monitoring postoperative infections (van Leeuwen *et al.*, 1994; Olaison *et al.,* 1997; Peltola *et al.,* 1997; Philip *et al.,* 2000; Pepys, 2003). CRP levels were measured in this study to determine whether it has potential as a marker for BV detection.

## 1.3 Purpose of the study

This prospective study is conducted to observe the local application of Tualang honey as vaginal packing and propolis vaginal pessary would completely inhibit bacterial growth from the high vaginal area in the study arm of patients compared to control arm patients, on the prevention of BV in invasive cervical cancer patients undergoing concurrent chemoradiotherapy.

## 1.4 Hypothesis

Tualang honey and propolis can inhibit the growth of the vaginal flora during chemoradiotherapy treatment in locally advanced cervical cancer.

## 1.5 Specific objectives

1) To determine the bioburden and to compare the bacterial growth between control arm and study arm of cervical cancer patients.
2) To determine the antibacterial activities of Tualang honey and propolis against bacteria isolated from cervical cancer patients via *in vitro* by using antimicrobial susceptibility testing (AST).
3) To quantitate and to correlate the C-reactive protein (CRP) and CD4+ cell (lymphocyte) in cervical cancer patients with the bioburden of bacteria.

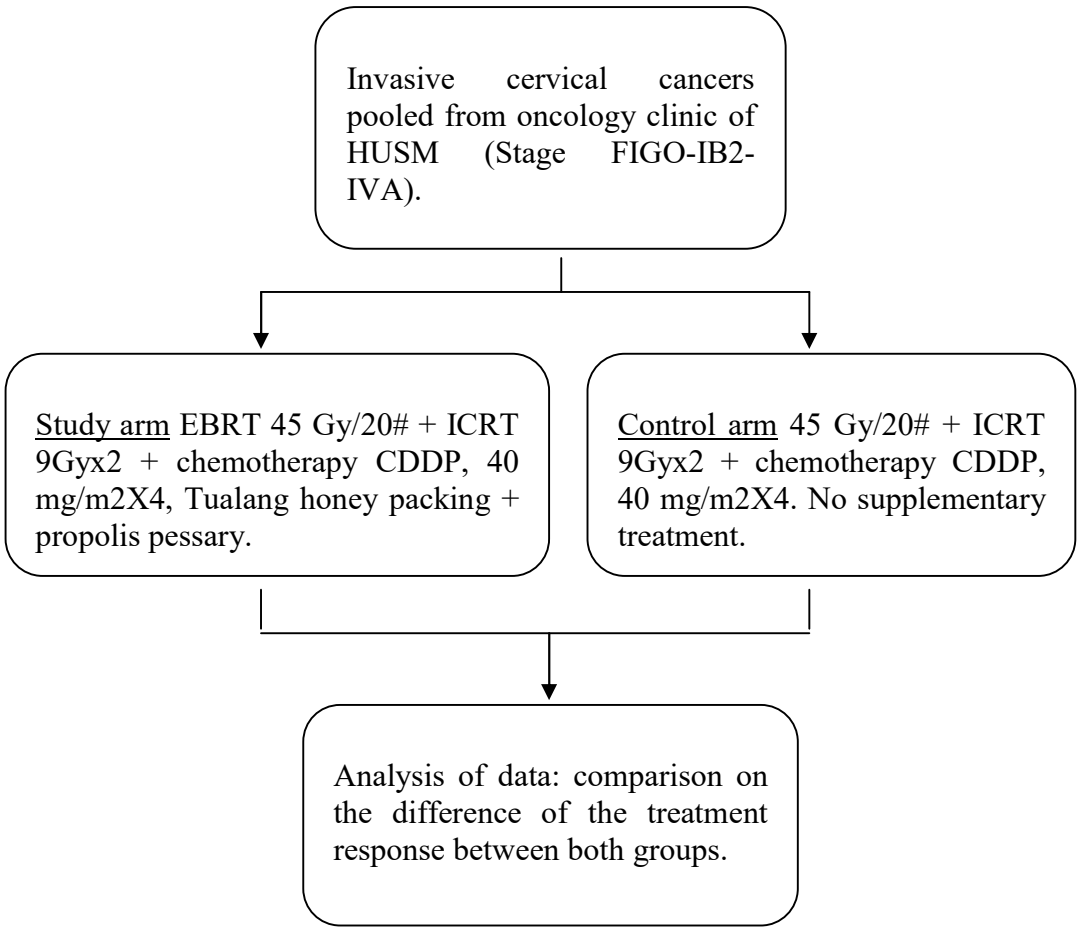


**Figure 1.1:** An overview of the study design

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Study design

An *in vivo* and *in vitro* study was carried out to assess the antimicrobial activities of Tualang honey and propolis on a range of selected clinical bacterial strains isolated from cervical cancer patients. A total of 30 patients were successfully recruited (15 study arm, 15 control arm). Patients were evaluated once a week day for consecutive five weeks period and samples were collected by appointed staff nurse. All collected samples were immediately sent to microbiology lab for processing to evaluate the bioburden, and blood samples for immunological indicators such as CD4+ and CRP levels were sent to immunology lab for processing. For CD4+ and CRP, samples were taken on the first, third and fifth week of the study period. Throughout the evaluation period, patients were advice not to take any medicine prescription in order to see the effectiveness of apitherapy given. The overview of the experimental design is as shown in Figure 2.1.

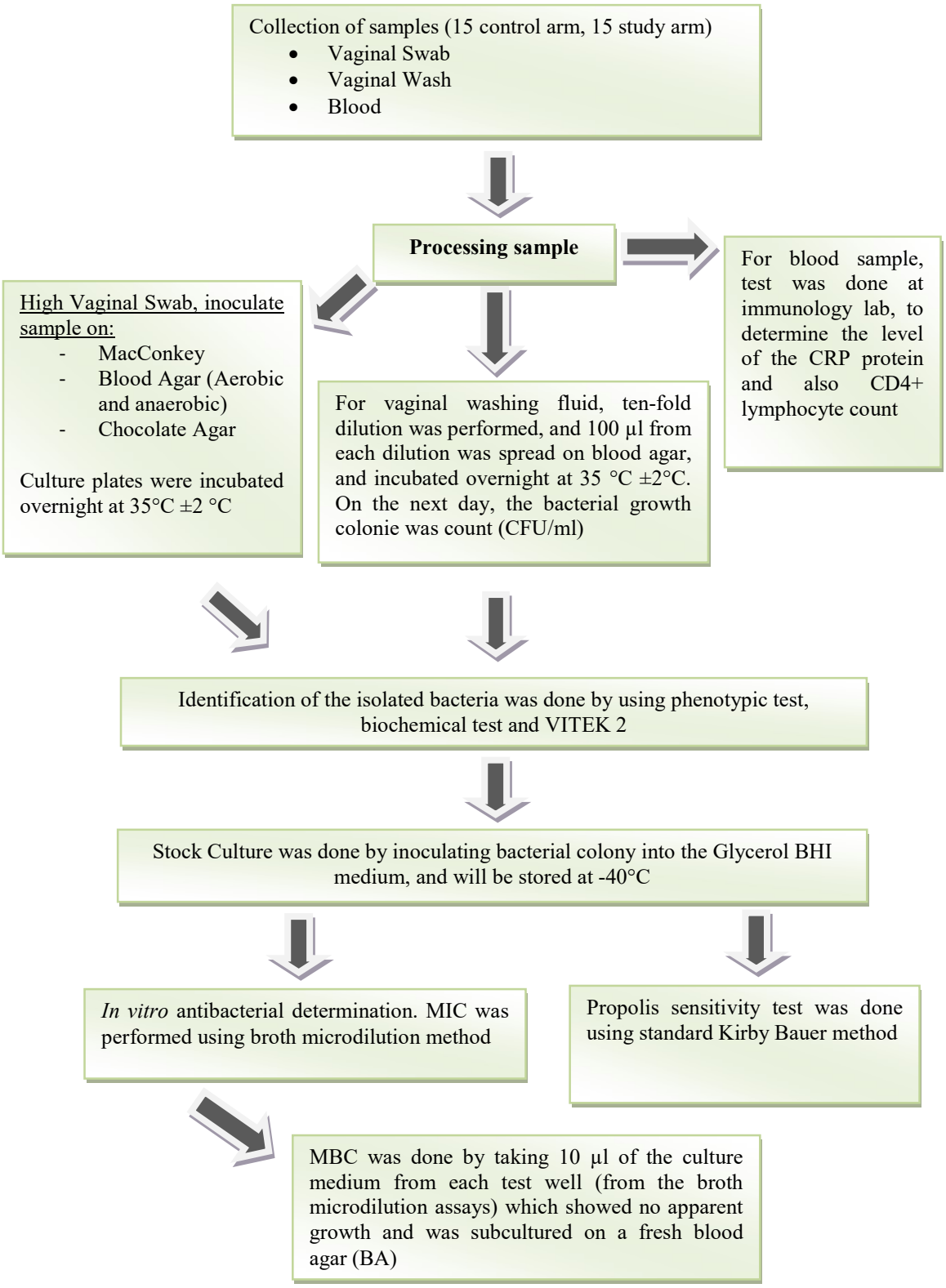


**Figure 2.1:** Research flowchart.

## 2.2 Materials

### 2.2.1 *In vivo* study design and materials

#### 2.2.1.(a) Experimental human model

This was a clinical trial study which targeted patients with cervical cancer stage Ib2 to IVA. The state of the cancer was based on International Federation of Gynecology and Obstetrics (FIGO) staging of cervical carcinomas as decribed in Table 2.1. A total of 30 clinically diagnosed patients with cervical cancer, age between 25 to 75 years were successfully recruited in this study. All patients were selected based on inclusion and exclusion criteria as described in the Table 2.2. The study was performed in the Hospital Universiti Sains Malaysia, Kubang Kerian, Kelantan where patients were recruited in Nuclear Medicine, Radiotherapy and Oncology Clinic.

It was conducted for the duration of 3 years from June 2011 until December 2014. Patients were distributed into two arms, first was control arm where all patients were given standard procedure for chemoradiotherapy treatment. While for study arm, patients were also given the same standard chemoradiotherapy treatment as in control arm, but they were supplemented with apitherapy consisted of Tualang honey and propolis ovules or provules, the mixture of propolis, cocoa butter, and beeswax.

**Table 2.1:** FIGO staging of cervical carcinomas.

| Cancer Stage | Description |
|---|---|
| **Stage I**<br>Cervical carcinoma confined to the cervix (disregard extension to the corpus) | • **Stage IA**: Invasive carcinoma diagnosed only by microscopy; stromal invasion with a maximum depth of 5.0 mm measured from the base of the epithelium and a horizontal spread of 7.0 mm or less; vascular space involvement, venous or lymphatic, does not affect classification<br>• **Stage IA1**: Measured stromal invasion ≤ 3.0 mm in depth and ≤ 7.0 mm in horizontal spread<br>• **Stage IA2**: Measured stromal invasion > 3.0 mm and ≤ 5.0 mm with a horizontal spread ≤ 7.0 mm<br>• **Stage IB**: Clinically visible lesion confined to the cervix or microscopic lesion greater than T1a/IA2<br>• **Stage IB1**: Clinically visible lesion ≤ 4.0 cm in greatest dimension<br>• **Stage IB2**: Clinically visible lesion > 4.0 cm in greatest dimension |
| **Stage II**<br>Cervical carcinoma invades beyond uterus but not to pelvic wall or to lower third of vagina | • **Stage IIA**: Tumor without parametrial invasion<br>• **Stage IIA1**: Clinically visible lesion ≤ 4.0 cm in greatest dimension<br>• **Stage IIA2**: Clinically visible lesion > 4.0 cm in greatest dimension<br>• **Stage IIB**: Tumor with parametrial invasion |
| **Stage III**<br>Tumor extends to pelvic wall and/or involves lower third of vagina and/or causes hydronephrosis or nonfunctional kidney | • **Stage IIIA**: Tumor involves lower third of vagina, no extension to pelvic wall<br>• **Stage IIIB**: Tumor extends to pelvic wall and/or causes hydronephrosis or nonfunctional kidney |
| **Stage IV**<br>Tumor invades mucosa of bladder or rectum and/or extends beyond true pelvis (bullous edema is not sufficient to classify a tumor as T4) | • **Stage IVA**: Tumor invades mucosa of bladder or rectum (bullous edema is not sufficient to classify a tumor as T4)<br>• **Stage IVB**: Tumor extends beyond true pelvis |

Adopted from Cecelia *et al.,* 2015.

**Table 2.2:** Inclusion and exclusion criteria.

| Inclusion Criteria | Exclusion Criteria |
|---|---|
| - Diagnosis of cervical cancer<br>- Histopathological proof of squamous cell or adenocarcinoma<br>- Age between 25 to 75 years<br>- ECOG PS 0-1<br>- Stage of cancer based on FIGO stageIb2-IVA<br>- Adequate renal function | - Small cell cervical cancer<br>- Prior radiotherapy<br>- Prior chemotherapy<br>- Age < 25 years and >75 years<br>- Uncontrolled medical condition<br>- Pregnancy<br>- Allergy or sensitivity to bee products<br>- AIDS |

### 2.2.1(b) Apitherapy materials (Tualang honey and propolis ovules)

Local Malaysian Tualang honey (Agromas® pure Tualang honey) was supplied by the Federal Agriculture Marketing Authority (FAMA) Negeri Kedah Darul Aman, Malaysia. Tualang honey was subjected to sterilization by gamma-irradiation (25kGy) at Malaysia Nuclear Agency. These honey samples were obtained in the proper bottle container and kept in the laboratory at 20°C away from direct sunlight (Figure 2.2). Tualang honey was used as a supplement in study arm treatment directly after chemoradiotherapy treatment.


**Figure 2.2:** Tualang honey and propolis pessary.

Propolis ovules, also called provules, were made of mixture of propolis, cocoa butter and beeswax. The propolis was bought from the Bee Healthy Farm, USA, under consultation of Jean-François Lariviere (Figure 2.2). These provules were kept in room temperature before were used as a supplement in the study arm treatment directly after chemoradiotherapy treatment.

### 2.2.2 Instruments and apparatus

Various instruments and apparatus used in this study were listed in the Table 2.3. These instruments were situated in the Medical Microbiology and Parasitology Laboratory, Central Research Laboratory and Immunology Laboratory, School of Medical Sciences, Universiti Sains Malaysia (USM).

**Table 2.3:** List of instruments and apparatus used for this study.

| Name | Manufacturer |
|---|---|
| Biological Microscope CX31 | Olympus Corporation, Japan |
| Bunsen burner (FIREBOY) | INTEGRA Biosciences, Switzerland |
| Class II Biological Safety Cabinet | NuAire, USA |
| $CO_2$ Incubator | Forma Scientific, USA |
| DensiCHEK plus | bioMérieux, USA |
| Dispensette bottle-top dispensers | BRAND, Germany |
| O2 Incubator | Memmert, Germany |
| PhoenixSpecNephelometer | Becton Dickinson, USA |
| Test tube with screw-neck, borosilicated glass | KIMBLE Chase, USA |
| VITEK 2 Automated Microbial Identification System | bioMérieux, USA |
| Vortex REAX top | Heidolph Instruments, Germany |
| Thermoscientific Varioskan Flash | Thermo Fisher Scientific, USA |
| Quickread CRP Instrument | Orion, Finland |
| Flow Cytometer FACSCanto II | Becton Dickinson BD, USA |

### 2.2.3 Consumables

All media and consumables used in this study are research grade (Table 2.4).

**Table 2.4:** Details of media and consumables used in this study.

| Name | Manufacturer |
|---|---|
| Alcohol based hand rub (70%) | Pharmacy, Hospital USM |
| Vaginal speculum | Mediscience Sdn. Bhd. Malaysia |
| Amies Transport Medium with Charcoal (ready-made) | LabChem Sdn. Bhd., Malaysia |
| Anaerogen compact | Oxoid Ltd., United Kingdom |
| Chocolate blood agar with horse blood plate (ready-made) | Oxoid Ltd., United Kingdom |
| Columbia horse blood agar plate (ready-made) | Oxoid Ltd., United Kingdom |
| Disposable sterile pipette | Oxoid Ltd., United Kingdom |
| Microscopic glass slide (frosted end) | Ideal Healthcare Sdn. Bhd., Malaysia |
| Mueller Hinton agar plate (ready-made) | Oxoid Ltd., United Kingdom |
| Nugard prepowdered examination gloves | Sun Healthcare Sdn, Bhd., Malaysia |
| Peptone water (3 ml) | Oxoid Ltd., United Kingdom |
| Wilkins-Chalgren agar (WCA) | (Neogen Corporation, USA |
| Wilkins-Chalgren broth (WCB) | (Neogen Corporation, USA) |
| Plastic pouch | Oxoid Ltd., United Kingdom |
| Surgical Scrub 4% | Steriline Sdn. Bhd. Malaysia |
| Vitek 2 GN ID test card | bioMérieux, USA |
| Vitek 2 GP ID test card | bioMérieux, USA |
| Vitek 2 AN ID test card | bioMérieux, USA |
| BDMultitest™ IMK kit | Becton Dickinson BD, USA |
| QuickRead CRP kit | Orion, Finland |
| Nunc$^{TM}$ MicroWell$^{TM}$ 96 well microplates | Thermo Scientific, USA |

### 2.2.4 Media and reagents for microbiological analysis

#### 2.2.4(a) Horse blood agar (HBA)

Ready to use enrichment media plates of horse blood agar (Cat. No. PB0114, Oxoid, Ltd, England) was used for the cultivation of fastidious bacteria and other microorganisms, an enrichment media.

#### 2.2.4(b) Chocolate horse blood agar (CBA)

Ready to use media plates of chocolate horse blood agar (Cat. No. PB0124, Oxoid, Ltd, England) was used for the cultivation of fastidious bacteria and other microorganisms.

#### 2.2.4(c) MacConkey agar No. 3 (MAC)

This agar was prepared by suspending 51.5 gm of MacConkey powder (Cat. No. CM0115, Oxoid, Ltd., England) in 1 L of distilled water and was boiled to dissolve completely. It was then sterilized by autoclaving at 121°C for 15 minutes. The mixture was left to cool to 60°C before pouring approximately 15 ml of the agar into sterile petri dishes. The agar was left to cool and harden completely at room temperature and incubated at 35°C ± 2°C for 24 hours (for quality control to check the sterility of the plates).

### 2.2.4(d) Wilkins-Chalgren broth (WCB)

Preparation was made by suspending 33 gm of WCB powder (Code No. 7233A) from Acumedia Manufacturers (Neogen Corporation, USA) in 1 L of distilled water. The mixture was boiled until completely dissolved and sterilized at 121°C for 15 minutes by autoclaving. The broth was left to cool and incubated at 35°C ± 2°C for 24 hours (quality control) before stored in cold room at 5°C and ready to use.

### 2.2.4(e) Wilkins-Chalgren agar (WCA)

Preparation was made by suspending 33 gm of WCB powder (Code No. 7233A) from Acumedia Manufacturers (Neogen Corporation, USA) and 15 gm agar powder (Oxoid Ltd, England) in 1 L of distilled water. The mixture was boiled until completely dissolved. Sterilization was done by autoclaving at 121°C for 15 minutes. The mixture was left to cool to 60°C before pouring approximately 15 ml of agar into sterile empty petri dishes. The agar was left to cool and harden completely at room temperature and incubated at 35°C ± 2°C for 24 hours (quality control) before stored in cold room at 5°C and ready to use.

### 2.2.4(f) Tryptone soy broth (TSB)

Preparation was made by suspending 30 gm of TSB powder (Oxoid Ltd, England) in 1 L of distilled water. The mixture was boiled until completely dissolved. Sterilization was done by autoclaving at 121°C for 15 minutes. The broth was left to