ANTIPROLIFERATIVE EFFECTS OF THE *ORTHOSIPHON STAMINEUS* AND TUALANG HONEY ON ORAL SQUAMOUS CELL CARCINOMA AND HUMAN OSTEOSARCOMA CELL LINES

BY

ABDULMLIK AL-GHASHM

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LIST OF ABBREVIATIONS

O. stamineus	*Orthosiphon stamineus*
MTT	3-(4,5-Di Methyl Thiazol-2-yl)-2,5-diphenyl Tetrazolium bromide
OSCC	Oral Squamous Cell Carcinoma
HOS	Human Osteosarcoma
LD_{50}	Lethal Dose 50
FITC	Fluorescein IsoThioCyanate
PS	PhosphatidylSerine
PI	Propidium Iodide
EDTA	EthyleneDiamineTetraAceticacid
PBS	Phosphate Buffered Saline
DMSO	Dimethyl Sulphoxide
DMEM/F12	Dulbecco's Modified Eagle Medium and Ham's F12
DMEM	Dulbecco`s Modified Eagle Medium
FBS	Foetal Bovine Serum
IC_{50}	Inhibitory Concentration 50%
O.D	Optical Density
IQR	Interquartile Range
ELISA	Enzyme-linked Immunosorbent Assay
v/v	volume/volume
VLB	Vinblastine
VCR	Vincristine
ROS	Reactive Oxygen Species
MMP	Matrix MetalloProtinases
GSH	Glutathione

KESAN ANTIPROLIFERATIVE OLEH ORTHOSIPHON STAMINEUS DAN MADU TUALANG KE ATAS SEL-SEL SKUAMOS KARSINOMA ORAL DAN OSTEOSARKOMA MANUSIA

ABSTRAK

Kanser adalah penyebab keempat kematian di Malaysia dan hampir 30,000 orang menghidap masalah ini setiap tahun. Salah satu bidang penyelidikan yang penting adalah kesan tumbuhan dan produk semulajadi sebagai agen kemoterapeutik yang efektif. Madu telah menunjukkan spektrum ciri terapeutik yang luas serta banyak kegunaan termasuk kesan antibaketria, antikulat, antikeradangan dan kesan antikanser yang sederhana. Ia mengandungi banyak bahan yang aktif secara biologi seperti asid caffeic, ester fenetil dan glikon flavanoid yang telah terbukti menghalang sel tumor membiak. *Orthosiphon stamineus* (*O. stamineus*) atau 'misai kucing' pula adalah herba tradisional yang popular diguna sebagai ubat herba sejak beberapa abad. Ia mempunyai ciri perubatan seperti aktiviti antioksidan, antikulat, diuretik, antidiabetik dan antikeradangan. Tidak ada kajian yg dpt telah dilakukan untuk menilai pengaruh madu Tualang dan *O. stamineus* ke atas sel-sel skuamos karsinoma oral (OSCC) and osteosarkoma manusia (HOS). Kajian ini bertujuan mengesahkan aktiviti antiproliferatif madu Tualang, *O. stamineus* dan beberapa kepekatan campuran madu Tualang dan *O. stamineus* ke atas OSCC dan HOS.

Beberapa dos madu Tualang (1-20%), *O. stamineus* (100µg/ml - 1mg/ml) dan beberapa kepekatan campuran kedua-duanya diuji ke atas kultur sel OSCC dan HOS untuk tempoh 3, 6, 12, 24, 48 and 72 jam. Ciri morfologi apoptosis dilihat dengan mikroskop cahaya dan stain Hoechst 33258 di bawah mikroskop fluoresen. Viabiliti sel diuji dengan pembenihan dan tambahan dos madu, *O. stamineus* dan beberapa kepekatan kedua-dua campuran. Selepas inkubasi, Metil Tiazol Tetrazolium (MTT) ditambah dan sel-sel diinkubasi untuk 4 jam lagi pada suhu 37°C sebelum

penambahan dimetil sulfoksid (DMSO). Analisis dibuat dengan alat ELISA pada absorban 570nm. Semua ujikaji dijalankan dalam triplikat. Apoptosis awal dikesan dengan aliran sitometri mengguna Kit Apoptosis I ApoAlert® Annexin V-FITC. Untuk kesan madu, aktiviti madu bukan peroksida dan cecair osmolar dibuat sebagai tambahan kepada ujian kesan selektif. Gambaran morfologi menunjukkan fragmentasi dan kondensasi kromatin nukleus seiring dengan pengurangan bilangan sel dan kesan ini bergantung kepada tempoh dan kepekatan bahan ujian yang ditambah. Bahan ujian menunjukkan kesan pertumbuhan inhibitori yang signifikan ke atas sel OSCC dan HOS. Ujian viabiliti sel menunjukkan kesan inhibitori yang bergantung kepada dos dan masa. Konsentrasi inhibitori 50% (IC_{50}) bagi OSCC dan HOS untuk madu Tualang adalah 4% and 3%, untuk *O. stamineus* adalah 700 dan 200 µg/ml dan campuran 0.35mg/ml *O. stamineus* + 3.5% madu Tualang untuk OSCC dan campuran 0.2mg/ml *O. stamineus* + 2% madu Tualang untuk HOS. Viabiliti sel menurun dengan peningkatan dos madu sekaligus mengesahkan kesan inhibitori madu ke atas kedua-dua sel OSCC dan HOS. Apoptosis awal jelas dilihat dengan aliran sitometri dan kesan ini adalah bergantung kepada masa dan kepekatan cecair campuran. Oleh itu madu Tualang, *O. stamineus* dan beberapa kepekatan tertentu campuran madu Tualang dan *O. stamineus* menunjukkan kesan antiproliferatif dan apoptosis ke atas sel-sel OSCC dan HOS.

ANTIPROLIFERATIVE EFFECTS OF THE *ORTHOSIPHON STAMINEUS* AND TUALANG HONEY ON ORAL SQUAMOUS CELL CARCINOMA AND HUMAN OSTEOSARCOMA CELL LINES

ABSTRACT

Cancer is the fourth leading cause of death in Malaysia with almost 30,000 people contracting the disease annually. One of the important research fields is the beneficial effects of plants and natural products as effective chemotherapeutic agents. Honey exhibits a broad spectrum of therapeutic properties including antibacterial, antifungal, anti-inflammatory and moderate anticancer effects. It contains many biologically active compounds such as caffeic acid, caffeic acid phenethyl ester and flavonoid glycones which have been proven to exhibit inhibitory effect on tumor cell proliferation. *Orthosiphon stamineus* (*O. stamineus*) or 'misai kucing' is a popular traditional folk medicine used as medicinal herb for many centuries. It has many medicinal properties such as antioxidant activity, antifungal activity, diuretic activity, antidiabetic activity, anti-inflammatory activity. No previous study has been done to evaluate the effect of Tualang honey and *O. stamineus* on oral squamous cell carcinoma (OSCC) and human osteosarcoma (HOS). This study was aimed to verify the antiproliferative activity for several concentrations of Tualang honey, *O. stamineus* and mixed concentrations of honey and *O. stamineus* on OSCC and HOS cell lines. Several doses of Tualang honey (1% - 20%), several concentrations of *O. stamineus* (100µg/ml to 1mg/ml) and several mixed solutions of both were applied on OSCC and HOS cell culture for 3, 6, 12, 24, 48 and 72 hours. Morphological characteristics were assessed under light microscopy and with Hoechst stain 33258 under fluorescence microscope. Cell viability assay was performed whereby OSCC

and HOS cell lines were seeded in 96-well plates and doses of treatment were added. After incubation period, Methyl Thiazole Tetrazolium (MTT) was added and incubated for a further 4 hours at 37°C prior to addition of dimethyl sulfoxide (DMSO). Analysis was done by using ELISA reader at absorbance 570nm. All experiments were performed in triplicates. Detection of early apoptosis was done using ApoAlert® Annexin V-FITC Apoptosis Kit I for flow cytometry. For honey the non-peroxidase honey activity and osmolar solution control was examined in addition to selectivity effect.

Morphological appearance showed fragmented and nuclear chromatin condensation with reduction in cell number depending on time and concentration of treatment applied. Treatments showed significant growth inhibitory effect on OSCC and HOS. Cell viability assay showed a dose and time-dependent inhibitory effect on both cell lines. The 50% inhibitory concentration (IC_{50}) was 4% and 3% for Tualang honey, 700 and 200µg/ml for *O. stamineus* and (0.35mg/ml *O. stamineus* + 3.5% Tualang honey) and (0.2mg/ml *O. stamineus* + 2% Tualang honey) for mixed solutions of *O. stamineus* and honey on OSCC and HOS respectively. Cell viability decreased as the dosage of honey was increased showing growth inhibition effect of honey on both cell lines. Early apoptosis was evident by flow cytometry. This effect was time and concentration-dependent. In conclusion *O. stamineus* alone have weak antiproliferative effect while Tualang honey and several specific concentrations of mixed solutions have good antiproliferative and apoptotic effect on OSCC and HOS cell lines.

CHAPTER ONE
INTRODUCTION

In Malaysia, cancer is the fourth leading cause of death. Almost 30,000 people contract the disease each year, with lung cancer being the most common. Most patients are diagnosed with cancer during the late stages of the disease. Programs are focused to combat cancer, including early diagnosis, treatment, and palliative care, and to improve the quality of life of cancer patients (Lim, 2002). In standardized incidence rate for all cancers in the year 2003 was 134.3 per 100,000 males and 154.2 per 100,000 females (Chye and Yahaya, 2004).

Over the centuries, no fewer than 3,000 plant species have been used to treat cancer. Worldwide use of these plants products generated untold wealth, relieved much suffering and saved millions of lives. Furthermore, the use of herbal drugs from these plants led to the successful habitation of vast areas of tropical to warm temperate regions, making possible new opportunities for progress and riches (Lewis and Elvin-Lewis, 2003).

Flavonoids are a group of more than 5000 polyphenolic compounds that occur naturally in foods of plant origin. These compounds possess a common phenylbenzopyrone structure (C6–C3–C6), and are categorized according to the saturation level and opening of the central pyran ring, mainly into flavones, flavanols, isoflavones, flavonols, flavanones and flavanonols (Harborne and Williams, 2000; Ren *et al.*, 2003). These polyphenolic compounds have several biological activities that include antimutagenic,

antiproliferative, and antioxidant effects as well as involvement in cell signaling, cell cycle regulation and angiogenesis (Nijveldt *et al.*, 2001).

Most of the researches for new drugs to be used in oncology have refocused on natural products, which led to the finding of some new compounds such as taxanes and camptothecins (anticancer drugs derived from plants). In particular, interest has intensified on the class of flavonoid compounds present in normal human diet and in many folk medicines still in use. Flavonoids and flavonoid-rich extracts have been implicated as beneficial agents in a multitude of disease states (Havsteen, 2002).

Dietary flavonoids have preventive and therapeutic potential against malignant tumors (Horinaka *et al.*, 2006) and some pharmacological studies have demonstrated that flavonoids possess antiproliferation effects of tumor cells and induces cell apoptosis (Hsu *et al.*, 2004). Dietary flavonoids have been demonstrated to exert several beneficial biological activities in various cell types such as anti-inflammation, antioxidation, antiproliferation and pro-apoptosis (Ross and Kasum, 2002).

Examples of commercialized plant products, Vinblastine (VLB) and Vincristine (VCR) are powerful anticancer drugs in the treatment of acute leukemia, lymphomas, rhabdomyosarcomas, and Wilma’s tumors in children, Hodgkin's disease, non-Hodgkin lymphomas, testis carcinomas, breast cancer and choriocarcinomas. During the last 40 years, these drugs have been used for the treatment and cure of thousands of patients, both because of their unique mode of action and their effectiveness. VLB and VCR are natural products belonging to the group of terpenoid-indole alkaloids. They are isolated

from the pan tropical plant *Cathranths. roseus* (Madagascar periwinkle, formerly known as Vinca rosea), in which they are present only as minor constituents of the complex mixture of about 130 alkaloids produced by this plant (van Der Heijden *et al.*, 2004).

Apoptosis is a mechanism by which cells undergo death to control cell proliferation or in response to DNA damage. The basis of this study was to find out the antiproliferative and apoptotic effect of *O. stamineus* and Tualang honey, which may be beneficial in anticancer therapy.

Apoptosis is one of the two mechanisms by which cell death occurs (the other being the pathological process of necrosis). It is the mechanism responsible for the physiological deletion of cells and appears to be intrinsically programmed. It is characterized by distinctive morphologic changes in the nucleus and cytoplasm, chromatin cleavage at regularly spaced sites and the endonucleolytic cleavage of genomic DNA or DNA fragmentation at internucleosomal sites. This mode of cell death serves as a balance to mitosis in regulating the size of animal tissues and in mediating pathologic processes associated with tumor growth (NIH, 2004).

Cell death has been shown to occur by two major mechanisms namely, necrosis and apoptosis. Classical necrotic cell death occurs due to noxious injury or trauma, while apoptosis takes place during normal cell development, regulating cellular differentiation and number. While necrotic cell death results in cell lysis, cellular apoptosis is characterized morphologically by cell shrinkage, nuclear pyknosis, chromatin

condensation and blebbing of the plasma membrane. It seems that all known anticancer drugs kill cancer cells predominantly through apoptosis (Bremer *et al.*, 2006).

1.2 Statement of the problem

The use of herbal extracts and natural products as a complementary to modern medicine is gaining increased popularity, while knowledge about the beneficial effects of certain materials is mainly transmitted by personal communication. Therefore, it remains unknown to the general population. Several studies on anticancer effects of herbal extracts and natural products using cancer cell lines suggested that more studies are needed to promote the usage and application of herbs for anticancer purposes. To the knowledge of the authors, there are no previous experiments to evaluate the effects of *O. stamineus* and Tualang honey on OSCC and HOS cell lines.

1.3 Justification of study

The main challenges in cancer treatment strategy are producing curative medicine with less side effects and the use of available raw materials. Therefore, there is a great need for more experimental research to find natural substitutes in chemotherapy. The literatures, some past and ongoing researches have focused on the medicinal effects of these plants and natural products.

There is need for clarifying scientifically the anticancer effect of these local herbs and honey. This may open other extensions in researches for their good use. Thus, the

exploration of cell death induced by these materials will be valuable to design more effective chemotherapeutic agents.

There is thus a great need for detailed scientific clarification of the anticancer effects of *O. stamineus* and Tualang honey. These effects may enlighten new research areas for the exploration of the beneficial effects of plants and natural products as affective chemotherapeutic agents, there are no previous experiments to evaluate the effects of *O. stamineus* and Tualang honey on OSCC and HOS cell lines.

1.4 Objectives

1.4.1 General objective

To assess the antiproliferative effect and apoptotic activity of *Orthosiphon stamineus*, Tualang honey and a mixture of these products on Oral Tongue Squamous Cell Carcinoma and Human Osteosarcoma cell lines.

1.4.2 Specific objectives

1. To evaluate the morphological changes on Oral Squamous Cell Carcinoma and Human Osteosarcoma cell lines induced by *Orthosiphon stamineus,* Tualang honey and mixtures of these products under light and flourocense microscope.
2. To evaluate the antiproliferative activity of *Orthosiphon stamineus*, Tualang honey and mixtures of these products on Oral Squamous Cell Carcinoma and Human Osteosarcoma cell lines by Methyl Thiazole Tetrazolium (MTT) assay.
3. To evaluate the apoptotic effect of *Orthosiphon stamineus*, Tualang honey and mixtures of these products on Oral Squamous Cell Carcinoma and Human Osteosarcoma cell lines by flow cytometry.

CHAPTER TWO
LITERATURE REVIEW

2.1 *Orthosiphon stamineus Benth*

Orthosiphon stamineus Benth *(O. stamineus)*, which belongs to the *Lamiaceae* family, is a native plant in tropical Asia. It is a popular medicinal herb in Southeast Asia, known locally as ‘‘Misai kucing’’ or ‘‘Kumis kucing’’ in Malaysia and Singapore or **“**cat whiskers”. It grows well on wet soil and can be found in both temperate and tropical gardens (Leng and Keng, 2004).

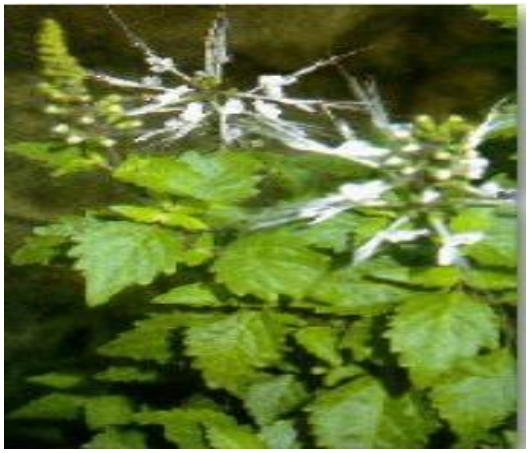

Fig. 2.1 *O. stamineus* plant (http://misaikucing.tripod.com)

2.1.1 Traditional medicinal uses

O. stamineus is a popular traditional folk medicine that has been used as medicinal herbs for many centuries in Southeast Asian countries like Indonesia and Malaysia. It is used as a remedy for catarrh of the bladder and as a medicine for various disorders such as nephritis, nephrolithiasis, hydronephrosis, vesicle calculi, arteriosclerosis, gout, jaundice and rheumatism.

In Malaysia, the tea prepared from the leaves is taken as a beverage to improve health and to treat kidney and liver disease, bladder inflammation, diabetes, hypertension, tonsillitis and menstrual disorder (Eisai, 1995; Awale *et al.*, 2003a; Yam *et al.*, 2007). In the aerial part of Vietnam this plant is known by the name "Rau meo" and used for the treatment of urinary lithiasis, edema, eruptive fever, influenza, hepatitis, jaundice (Tran, 1990).

In Myanmar, this plant is known as "Secho" or "Myit-shwe". The leaves are reputed to be an antidiabetic drug and decoctions of the air-dried leaves are used to cure eruptive fever, epilepsy, gallstone, hepatitis, rheumatism, hypertension, syphilis, gonorrhea and renal calculus (Bwin and Gwan, 1967).

It is reported that *O. stamineus* leaves have been introduced in to Europe and Japan as a health tea. According to traditional phytotherapy, *O. stamineus* tea is beneficial in removing uric acid stones from kidney and has been reported to be active against kidney and bladder inflammation because of its diuretic activity. It is also used for the treatment of diabetes and hypertension (Leng and Keng, 2004).

2.1.2 Antioxidant activity

Methanolic extract of *O. stamineus* samples collected from different places in Malaysia have been studied. Twenty phenolic compounds were isolated from *O. stamineus* extract and showed antioxidant properties for phenolic compounds of all samples (Akowuah *et al.*, 2004). It was concluded that *O. stamineus* leaf extract possessed antioxidant and free

radical scavenging activities and has a protective effect on hepatic damage (Yam *et al.*, 2007).

2.1.3 Antifungal activity

Sixty nine compounds were identified from crude methanolic extract of leaves and stems of *O. stamineus*. The essential oils and crude extract have an antifungal effect against phytopathogens that cause severe diseases in the plants. It was also found that the crude methanol extract of *O. stamineus* showed higher antifungal effect than essential oils (Hossain *et al.*, 2008).

2.1.4 Diuretic activity

Several classes of compounds have been identified from *O. stamineus* including flavonoids, terpenoids, saponins, hexoses, organic acids, caffeic acid derivatives, chromene and myo-inositol (Tezuka *et al.*, 2000; Olah *et al.*, 2003). Among these compounds, the flavonoids and caffeic acid derivatives were found to possess potential therapeutic properties, as they were shown to exert diuretic and uricosuric actions in rats (Olah *et al.*, 2003). The main flavonoids in *O. stamineus* extract 0.15%, 0.21% and 0.05%; (w/w) of sinensitin, eupatorin and 3(-hydroxy-5,6,7,4)-tetramethoxyflavone, respectively and was analysed by the plasma oral bioavailability in rats (Loon *et al.*, 2005).

The main polyphenols (flavonoids): sinesetine, eupatorine, rosmarinic-, cichoric- and caffeic-acids were identified and quantitatively determined in *O. stamineus* and tested on

rats for pharmacological action as diuretic and uricosuric (increase volume of urine – increase uric acid excretion) were proven by Olah *et al* (2003). Arafat *et al* (2008) showed the high tendency of *O. stamineus* towards diuretic property and provided evidence for hypouricemic (reduced uric acid formation) activity of *O. stamineus* extract using animal models.

Sodium citrate is a known inhibitor of calcium oxalate crystal formation that forms kidney stones. *In-vitro* study conducted to compare the effect of sodium citrate and *O. stamineus* extract found that the *O. stamineus* extract was a more powerful inhibitor for calcium oxalate crystal formation than sodium citrate (Dharmaraj *et al.*, 2006).

2.1.5 Antidiabetic activity

One study in Thailand determined that the total phenolic compound in 1 gram of *O. stamineus* extract contained 13.24±0.33mg gallic acid and 1.73±0.147µg flavonoids. The authors evaluated the effect of the extract on normal and diabetic rats, where they found *O. stamineus* extract starting from 100µg/ml potentiated glucose-induced insulin secretion and in general markedly reduced hyperglycaemia in diabetic rats, decreased plasma triglyceride and increased plasma HDL-cholesterol concentrations. These findings suggested that *O. stamineus* extract is useful in the control of diabetes mellitus (Sriplang *et al.*, 2007).

2.1.6 Anti-inflammatory activity

Diterpenes were proven to be important compounds extracted from *O. stamineus* crude extract. Awale *et al* (2003a) and (2003b) isolated 16 diterpenes from the *O. stamineus* extract and proved their inhibitory effect on nitric oxide production in lipopolysaccharide-activated macrophage-like J774.1 cell line (antioxidant effect) which verifies the anti-inflammatory activity of *O. stamineus.*

The same results were proven for 47 diterpenes isolated from *O. stamineus* extracts from Indonesia, Okinawa, Myanmar and Vietnam, all possesing the same inhibitory effect on nitric oxide production. MTT assay of cell viability showed that many diterpenes displayed cytotoxic property at 200mM concentration, while quite a few diterpenes were found to be cytotoxic even up to 50mM in concentration (Awale *et al.*, 2003b). Another diterpene (neoorthosiphonone A) has been discovered as the strongest inhibitor for nitric oxide production (Awale *et al.*, 2004).

2.1.7 Safety and toxicity

O. stamineus in doses from 0.5, 1.0, 3.0 and 5g/kg is safe and have been used in young female Sprague Dawley rats. Fourteen days oral administration of methanol extract of *O. stamineus* in these rats did not produce any death or cause any adverse effects on body weight, food consumption, water intake and relative organ weight (Chin *et al.*, 2008).

Standardized extract of *O. stamineus* had no observable acute effect on the experimental animals and was therefore deemed non-toxic. This finding has provided scientific

information on the acute effect of the *O. stamineus* given in a single dose orally at 5000mg/kg body weight on rats since it did not produce any mortality or alter the behavior patterns of the rats. Thus, it was concluded that lethal dose 50 (LD_{50}) of the *O. stamineus* extract is more than 5000mg/kg body weight (Abdullah and Ismail, 2009). Accidently, a third confirmatory observation reported that the LD_{50} of the *O. stamineus* extract is much higher than the highest dose employed in a study by Abdullah and Ismail (2009). This was proven while they were investigating the antipyretic effect of *O. stamineus* on rats. They showed that the leaves extract of *O. stamineus* showed a significant effect in yeast-induced elevation of body temperature (Yam *et al.*, 2009).

2.2 Honey

Honey is a natural foodstuff and it is considered as the most popular balanced diet for all ages. Characteristic features include: it does not get spoiled, does not need refrigeration and can be stored at room temperature. Honey is made by honey-bees from the nectar of flowers or secretions from other parts of the plants. Honey is stored in the honeycomb of beehives. One tablespoon of honey contains 64 calories. It is an average mixture of 82% carbohydrates, 17% water, proteins, amino acids, vitamins and minerals. Honey is also used as medicinal treatment for clinical conditions such as gastrointestinal tract problems, ophthalmic lesions, wound healing and burns (Khan *et al.*, 2007).

Tualang honey come from Malay language with reference to the number of giant honeybee colonies in Tualang trees. "*Lebah camok"* refers to less than seven colonies on a tree, while "*Lebah* Tualang*"* is a much larger aggregation found only in extensive forest areas on the huge *Koompassia* tree, usually a few dozen to nearly 200 colonies as shown in Fig. 2.2 (Roubik *et al.*, 2005).

Fig. 2.2 Aggregation of honeybee nests (*Apis dorsata)* in the high branches of a forest tree, *Koompassia excelsa.* Photograph by T. Inoue (Roubik *et al*, 2005).

2.2.1 Medicinal uses

Honey is important not only for its nutritional properties but also for its functional and biological properties like antioxidant, anti-inflammatory, antibacterial, antiviral and antiulcerous activities as well as have the capacity for the inhibition of enzymatic browning which is a reaction during fruit and vegetable processing for obtaining juices and preserves. These activities are mainly attributed to the phenolic compounds such as flavonoids which are present in all types of honey with different percentage depending on the geographical areas, source of honeybee food and climate (Viuda-Martos *et al.*, 2008).

2.2.2 Antimicrobial activity

Many literatures on the antibacterial effect of honey emphasize on the type of honey and geographical region. Most literature found that the antimicrobial effect of honey depends on the hydrogen peroxide content of honey in some types or due to phytochemical or entomological (bees) factors. These researches are still ongoing (Allen *et al.*, 1991; Miorin *et al.*, 2003; Temaru *et al.*, 2007; Irish *et al.*, 2008).

2.2.3 Antioxidant activity

Antioxidant properties of seven types of honey from various floral sources were determined in comparison with sugars analogue by Gheldof and Engeseth (2002). All types of honey were more effective as antioxidant than the sugars analogue.

Different types of honey come in dark and light colours. The phenolic compounds from honey extract were proven as antioxidant and antimicrobial agents. The dark coloured honey has stronger antibacterial effect than the light coloured honey. Moreover, these compounds are reported to exhibit anticarcinogenic, anti-inflammatory, antiatherogenic, antithrombotic, immune modulating and analgesic as well as antioxidant activities (Estevinho *et al.*, 2008).

2.2.4 Anticarcinogenic activity

Antimutagenic properties of honey were detected using Ames assay by Wang *et al*, (2002). Regarding the anticancer effects of honey, very few resources are available to substantiate the antineoplastic activity on cancer cell lines *in-vitro* and *in-vivo*. When honey was applied on bladder cancer cell lines that were tested for antiproliferative effect through MTT assay and flow cytometry, results showed significant inhibition of the proliferation of bladder cancer cell lines more than the mixed sugar solution. It showed an important conclusion that hyperosmolarity of honey alone cannot explain the drastic inhibitory effect on tumours. Therefore, flow cytometry might be useful to confirm the apoptotic effect of honey on bladder cancer cell lines (Swellam et al, 2003).

Swellam *et al,* (2003) implanted bladder cancer cell lines suspension in 100 abdomens of mice. After the tumour reached 100 -150 mm^3, the mice were divided into groups depending on honey concentration fed orally in comparison with control (with no honey treatment). Their results showed significant difference between the final tumour volume

and weight in the honey treated groups and the control group. This confirmed the inhibitory effect of honey on tumour growth *in-vivo*.

In an *in-vivo* study, mice were injected with single cancer cell suspension subcutaneously and intravenously to generate metastasis. Honey products were applied orally and systemically before and after the cancer inoculation. The results showed reduced tumour growth and inhibited metastasis formation with honey products (Orsolic *et al.*, 2005).

The use of honey in cancer surgery was assessed where local honey treatment was applied pre-and post-cancer inoculation on experimental mice. Tumour growth could not be detected even microscopically when treated with honey while in all control group with no honey treatment, the tumour was palpable and confirmed histopathologically. These findings indicated that honey might prevent tumour implantation when applied locally (Hamzaoglu *et al.*, 2000).

2.2.5 Wound and burn healing and surgery

Many trials for the effect of honey on burn healing were reviewed by Wallace (2009). She concluded that honey application on burns make better healing than conventional dressing. Honey enzyme content like amylase play an important role in synergistic antibacterial effect when honey was mixed with starch, which can be applied safely on wounds or inserted into cavities and sinuses to clear out infection (Boukraa and Amara, 2008).

2.2.6 Synergistic activity

The antimicrobial effect of honey on some oral bacterial species was investigated using different types of honey with control solution from carbohydrate concentrations. Some bacterial species were found sensitive to honey at concentrations lower than that observed for the carbohydrate control. This indicated that there may be unidentified components present in honey that is active against these organisms (Basson and Grobler, 2008).

The effect of honey on coagulase-negative staphylococci was tested *in-vitro* in comparison with osmolar solution equivalent to the osmolarity of honey. It was shown that the effect of honey was eight times potent in killing of the antibiotic resistant bacteria than osmolar control solution, indicating that honey applied to skin at insertion points of medical devices may have a role in the treatment or prevention of infections by coagulase-negative staphylococci (French *et al.*, 2005).

Commonly, honey is used in the mixed form with other food or medicinal herbs for the synergistic effect of honey (Boukraa and Amara, 2008). Many researches are targeting to find a novel synergistic medicine by mixing flavonoids to get the maximum effect with no harm on human health (Harborne and Williams, 2000; Campbell *et al.*, 2006). However, these researches are still ongoing.

2.3 Oral Squamous Cell Carcinoma

Oral Squamous Cell Carcinoma (OSCC) is the most common cancer of the head and neck. Although oral cancer that is detected early is highly curable, more than 50% of oral cancer patients present with advanced disease, and fewer than 30% of these patients can be cured (Marcus *et al.*, 2004). Studies in the past decade showed that concurrent chemo-radiotherapy has the potential to improve the overall survival of patients with head and neck OSCC (Pignon *et al.*, 2000).

OSCC presents a significant health problem that affects over half a million people in the world each year (Parkin *et al*., 1993). General treatment modalities of OSCC are surgery, radiotherapy and chemotherapy. A number of chemotherapeutic agents have been used to treat OSCC (Forastiere *et al*., 1992).

The survival rate of OSCC patients has not improved with current chemotherapeutic agents. For the improvement of clinical outcome, it is necessary to verify the molecular mechanisms of chemotherapeutic agents and to apply the combination of chemotherapeutic agents that induce synergistic antitumor activity (Kim *et al*., 2007). Many recent researches use OSCC to detect the anticancer activities of such drugs or herb extracts (Tong *et al.*, 2000; Zhang *et al.*, 2003; Xu *et al.*, 2005; Kim *et al.*, 2007; Otsubo *et al.*, 2007; Pan *et al.*, 2007; Wu *et al.*, 2007; Yang *et al.*, 2007; Lee *et al.*, 2008; Yang *et al.*, 2008).

OSCC represents a malignant transformation of keratinocytes in stratified squamous epithelium. It is the most common cancer of the oral cavity, accounting for 90% of all diagnosed oral malignancies, and it accounts for approximately 3% of all human malignancies. Some OSCCs invade adjacent bone, some metastasize to cervical lymph nodes or distant organs, while other OSCCs follow a more indolent course (Erdem *et al.*, 2007).

Recently, cancer chemoprevention using food and medicinal herbs has been regarded as one of the most visible fields for cancer control (Jo *et al.*, 2004). Considering their safety and fewer side effects, some herbal drugs have been reported to possess chemotherapeutic effects which exhibit effective anticancer activity on head and neck squamous cell carcinoma *in-vitro* and *in-vivo* (Zhang *et al.*, 2003).

Furthermore, a growing number of *in-vitro* studies have been conducted on the potential anticancer activity of flavonoids in various cell systems including human oral cancer (Ren *et al.*, 2003; Hsu *et al.*, 2004). For example, Elattar and Virji (2000) found inhibitory effects of tea polyphenols, curcumin, genistein, quercetin and cisplatin on the growth of oral cancer cell lines SCC-25. In addition, (Haghiac and Walle, 2005) also gave evidence that quercetin (flavonoid) induces necrosis and apoptosis in SCC-9 (Yang *et al.*, 2008).

2.4 Human Osteosarcoma

Osteosarcoma is a highly malignant and rare disease with an incidence of around 3 per million (Dorfman and Czerniak, 1995). Osteosarcomas that arise from the jaw account for 2.1% of all malignant oral and maxillofacial tumours (Chen *et al.*, 2008b). In a recent systemic review of prognostic factors in osteosarcoma, it was concluded that chemotherapy may assume independent prognostic factor where the development of novel therapeutic agents targeting the malignant behaviour of osteosarcoma cells is important to improve the prognosis of patients (Bramer *et al.*, 2009).

The risk of early metastases particularly in the lung is high with 20% of all patients with osteosarcoma having solid metastases when first diagnosed. The rate of micrometastases is estimated to be 80%. The 5-year survival of the typical osteosarcoma is now 60% – 70%. The prognosis for recurrences and tumours with primary metastases is still poor (Carrle and Bielack, 2006).

Isoflavone derivatives that belong to flavonoids were proven as anticancer agents by inducing apoptosis on osteosarcoma cell lines. This indicates that isoflavone is a promising chemotherapeutic agent worthy of further development for treatment of human osteosarcoma cells (Chen *et al.*, 2008a; Hou *et al.*, 2008).

2.5 Apoptosis

Apoptosis or programmed cell death is an essential physiological process that plays a critical role in development and tissue homeostasis (Zimmermann *et al.*, 2001). The goal of cancer chemoprevention is to inhibit the induction and suppress the progression of pre-neoplastic lesions to invasive cancer by using specific natural or synthetic chemicals (Sun, 2001). Further understanding of the effects of potential chemopreventive agents on specific components of the pathways that lead to apoptosis may provide a rational approach to use such agents either alone or in combination with other agents to enhance apoptosis as a strategy for effective chemoprevention of cancer (Jo *et al.*, 2004).

There are two main pathways leading to apoptosis. The first of these depends on the participation of mitochondria and the second pathway involves the interaction of death receptors with its ligands (Li *et al.*, 2007a).

2.5.1 Apoptosis features

Apoptosis, a physiological cell suicide plays an important role in embryogenesis, metamorphosis, cellular homeostasis and as a defensive mechanism to remove infected, mutated or damaged cells. It is characterized by loss of cellular contact with the matrix, cytoplasmic contraction, chromatin condensation, plasma membrane blebbing and DNA fragmentation into large and small oligosomes. There are two major pathways of apoptosis: the death receptor pathway and the mitochondrial pathway. There is recent

evidence to suggest that these two pathways may be linked in certain cell types (Gupta, 2000a; Gupta, 2000b).

Membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation followed by rapid engulfment of cell debris by neighboring cells was also explained by Borner (2003).

In the early stage of apoptosis, which occurs at the cell surface, one of the plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell. Annexin V-FITC is a phospholipid-binding protein with a high affinity for PS. Previous molecular process can be identified by flow cytometry analysis to quantify the percentage of apoptotic cells, the simplest way to look for apoptosis bodies which remain as a gold standard with light microscopy or by the Hoechst stain under fluorescence microscope (Vermes *et al.*, 2000).

2.5.2 Importance of apoptosis

Defects in the regulation of apoptosis contribute to many diseases including pathologies associated with cell loss (e.g. stroke, heart failure, neurodegeneration, AIDS) and disorders characterized by a failure to eliminate harmful cells (e.g. cancer, autoimmunity). Progress in identifying apoptosis-relevant targets for drug discovery opens wide opportunities for new and more effective treatments for many medical illnesses currently lacking adequate treatments (Reed, 2001).

Apoptotic cell death is a physiological mechanism that eliminates unwanted cells by triggering the cell's intrinsic suicide program. Impairment of the apoptotic mechanism ultimately generates a pathological condition that includes developmental defects like autoimmune diseases, neurodegeneration or cancerous neoplasia (Reed, 2001).

2.5.3 Apoptosis inducers

Anticancer researches are directed towards innovations to find safe anticancer agents or drugs for the human body and harmful only for cancer cells by making selective mechanism which produce apoptotic effect on cancer cells. Most of these efforts focus on the natural plants that have medicinal uses.

Apoptosis has been characterized as a fundamental cellular activity to maintain the physiological balance of the organism. It is also involved in the immune system and plays a necessary role as a protective mechanism against carcinogenesis by eliminating damaged cells or abnormal excess cells which have proliferated owing to various inductions of chemical agents. Emerging evidence has demonstrated that the anticancer activities of certain chemotherapeutic agents are involved in the induction of apoptosis, which is regarded as the preferred way to manage cancer (Schuchmann and Galle, 2004).

In-vitro and *in-vivo* studies suggest that dietary flavonols could inhibit cancer in humans (Hollman and Katan, 1999). A prospective study involving about 10,000 men and women aged 15±99 years was carried out in Finland. After 24 years of follow-up, a

reduction in risk of lung cancer of about 50% was found in the highest quartile of those with flavonol intake (Knekt *et al.*, 1997).

There are already numerous natural products known, which control apoptotic pathways at different points. Progress in this field will depend on the cooperation of natural product chemistry, synthesis and medicinal chemistry. Natural products are potential lead structures for future drugs to cure apoptotic misregulation (Daniel *et al.*, 2006).

Many recent *in-vitro* and *in-vivo* studies have concentrated on the beneficial effect of dietary and herbal flavonoids. The effect was related to cancer prevention and treatment (Kanno *et al.*, 2005) on hepatoma, breast, cervix, stomach and pancreas cancer cell lines. Those flavonoids induced a positive apoptotic effect on all previously mentioned cancer cell lines. Horinaka *et al* (2006) studied the natural flavonoid (apigenin) that induced apoptotic effect on leukemia, prostate and colon cancer cell lines. Flavonoids have been shown to induce apoptosis in some cancer cell lines while sparing normal cells. The molecular mechanisms by which flavonoids induce apoptosis have not yet been clarified. Flavonoids are generally nontoxic and manifest a diverse range of beneficial biological activities.

The role of dietary flavonoids in cancer prevention has been widely discussed. There is much evidence that flavonoids have important effects on inhibiting carcinogenesis. Epidemiological studies have provided data that high dietary intake of flavonoids with fruits and vegetables could be associated with low cancer prevalence in humans. This is supported by a multitude of *in-vitro* and *in-vivo* studies, which showed that flavonoids

may inhibit various stages in the carcinogenesis process, namely tumour initiation, promotion and progression (Ren *et al.*, 2003).

Based on the *in-vitro* and *in-vivo* studies, it is understood that many mechanisms of action may be involved. These include carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis and differentiation, inhibition of angiogenesis, antioxidation and reversal of multidrug resistance or a combination of these mechanisms.

Tumor cell apoptosis is characterized by cellular rounding-up, cytoplasmic contraction, plasma membrane blebbing, chromatin condensation, DNA fragmentation and many biochemical characteristics, including the activation of death receptor pathway, mitochondrial pathway and/or caspases cascades (Gupta, 2001; Daniel *et al.*, 2006).

Ueda *et al* (2002) investigated Vietnamese plants extracts and their apoptotic effect on fibrosarcoma. Herbal drugs that were used traditionally tested on ovarian cancer cell lines showed apoptotic effect (Zhu *et al.*, 2005). Moreover, the herbal extract fractions were tested for apoptotic effect on hepatoma and colorectal cancer cell lines (Wang *et al.*, 2005). Studies have shown that *litchi* fruits (*Litchi chinensis*) gave apoptotic effect on oral cancer cell lines (Xu *et al.*, 2005). The same effect was noticed with *antrodia cinnamomea* (*antrodia camphorate*) on hepatoma cancer cell lines (Kuo *et al.*, 2006).

In the progress for plant medical industry, their pure compounds of fractions are formulated for medical uses. Before this stage, it should pass through many experiments