ANTICANCER ACTIVITY OF *FICUS DELTOIDEA* AND TUALANG HONEY ON ORAL CANCER CELLS: AN *IN VIVO STUDY*

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ABSTRACT

The use of natural products have been gaining interest, notably in the area of cancer therapy and in this study, natural products rich in antioxidants such as Tualang Honey (Malaysian wild local honey) and *Ficus deltoidea* (Malaysian local herbal plant), were selected to assess their chemo-preventive and chemotherapeutic activities on oral cancer. The aim of this study is to evaluate the chemo-preventive and chemotherapeutic activities of *Ficus deltoidea* (FD) and Tualang Honey (TH) in an animal model induced for oral cancer using 4-Nitroquinoline-1-oxide (4NQO). A total of 70 male Sprague-Dawley (SD) rats were distributed into ten groups (n=7 per group); Group 1, (untreated group), Group 2, (control cancer group) received 4NQO only during 8 weeks in drinking water. Groups 3, 4, 5 and 6 (chemo-preventive) received 4NQO for 8 weeks and were simultaneously treated with FD extract at 250 and 500 mg/kg and TH at 1000 and 2000 mg/kg respectively by oral gavage. Group 7, 8, 9 and 10 (chemotherapeutic) received 4NQO for 8 weeks followed by the administration of FD extract at 250 and 500 mg/kg and TH at 1000 and 2000 mg/kg respectively, for another 10 weeks. All rats from all experiments were sacrificed after 22 weeks, and the histopathological changes and incidence of oral cancer were microscopically evaluated. An immunohistochemical evaluation was conducted to determine the effects of the FD extract and TH on the expression of tumour markers; cyclin D1, bcl2, p53, β- catenin and e-cadherin using a computerised image analyser system, while the RT^2 Profiler PCR Array method was employed in this study for gene expression analysis of *TP53*, *RAC1*, *COX-2, TWIST 1, CCND1* and *EGFR*. The results of the present study showed a beneficial regression effect of the FD extract and TH on tumour progression, especially in the chemo-preventive groups. The FD extract and TH significantly reduced the incidence of oral squamous cell carcinoma (OSCC)

from 100% to 14.3% in the high dose groups. The immunohistochemical analysis showed that the FD extract and TH had significantly decreased the expression of the key tumour marker cyclin D1 and had significantly increased the expression of the β-catenin and e-cadherin proteins that are associated with enhanced cellular adhesion. Based on the gene expression analysis, TH and FD extract had reduced the expression of the *TWIST1* and *RAC1* genes associated with epithelial-mesenchymal transition (EMT) and had significantly down-regulate the *COX-2* and *EGFR* genes associated with cancer angiogenesis, metastasis and chemo resistance. In conclusion, our data suggest that the FD extract and TH exert chemo-preventive and chemotherapeutic activities in an animal model induced for oral cancer using 4NQO and thus, have the potential to be developed as chemo-preventive and chemotherapeutic agents.

ABSTRAK

Salah satu kesan sampingan yang biasa semasa penggunaan ubat kemoterapi untuk rawatan kanser adalah ketoksikan ubat. Penggunaan produk semulajadi telah mendapat perhatian dan dalam kajian ini, produk semulajadi yang kaya dengan antioksidan seperti Tualang Honey (madu tempatan liar Malaysia) dan *Ficus deltoidea* (tanaman herba tempatan Malaysia) telah dipilih untuk menilai aktiviti anti kanser nya keatas kanser mulut. Objektif: Untuk menilai aktiviti kemopencegahan dan kemoteraputik *Ficus Deltoidea (FD)* dan Tualang Honey (TH) pada model haiwan yang telah diinduksi menggunakan 4-Nitroquinoline-1-oksida (4NQO). Kaedah: Sejumlah 70 ekor tikus jantan Sprague Dawley (SD) telah dibahagikan kepada sepuluh kumpulan (n = 7 setiap kumpulan). Kumpulan 1 (kumpulan tidak dirawat), Kumpulan 2 (kawalan) menerima air minuman mengandungi 4NQO selama 8 minggu, Kumpulan 3, 4, 5 dan 6 (kemopencegahan) menerima 4NQO selama 8 minggu dan secara serentak dirawat dengan ekstrak FD pada 250 dan 500 mg / kg dan TH pada 1000 dan 2000 mg / kg dengan pemberian gavage oral, Kumpulan 7, 8, 9 dan 10 (kemoterapeutik) menerima 4NQO selama 8 minggu diikuti oleh pemberian ekstrak FD pada 250 dan 500 mg / kg dan TH pada 1000 dan 2000 mg / kg, selama 10 minggu lagi. Semua tikus dari semua eksperimen dikorbankan selepas 22 minggu, dan kejadian neoplasma mulut dan perubahan histopatologi dinilai secara mikroskopik. Penilaian imunohistokimia telah dilakukan untuk menilai kesan ekstrak FD dan TH pada penanda tumor; cyclin D1, bcl2, p53, β- catenin dan e-cadherin dengan menggunakan sistem komputer penganalisis imej, manakala kaedah RT^2 Profiler PCR Array pula untuk mengkaji ekpresi gen terpilih iaitu *TP53*, *RAC1*, *COX-2*, *TWIST 1*, *CCND1* dan *EGFR*. Keputusan: Hasil kajian ini menunjukkan kesan regresi yang bermanfaat oleh ekstrak FD dan TH pada perkembangan tumor, terutamanya dalam kumpulan kemopencegahan.

TH dan FD secara signifikan mengurangkan kejadian karsinoma sel squamous oral (OSCC) dari 100% hingga 14.3% dalam kumpulan dos yang tinggi. Analisis imunohistokimia menunjukkan bahawa FD dan TH telah mengurangkan ekspresi penanda tumor utama cyclin D1 secara ketara, dan meningkatkan ekspresi β-catenin dan e-cadherin yang dikaitkan dengan penambahbaikan lekatan selular. Berdasarkan analisa RT^2 Profiler PCR, TH dan FD mengurangkan ekspresi gen *TWIST1* dan *RAC 1* yang berkaitan dengan peralihan epitelium-mesenchymal (EMT) dan dengan ketara menurunkan gen COX-2 dan EGFR yang berkaitan dengan angiogenesis, metastasis dan kemoresistan. Kesimpulan: Ekstrak FD dan TH mempunyai potensi antikanser pada karsinogenesis mulut. Data kami mencadangkan bahawa ekstrak *FD* dan TH adalah berkhasiat dalam kemopencegahan kanser mulut manusia dan mungkin mampu meningkatkan kualiti hidup pesakit-pesakit kanser. Oleh itu, mereka mempunyai potensi untuk dibangunkan sebagai agen semulajadi kemopencegahan dan kemoterapi untuk kanser mulut.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
^{o}C	:	Degree Celsius
µl	:	Microliter
4NQO	:	4-Nitroquinoline-1-Oxide
ANOVA	:	Analysis of variance
DAB	:	3,3'-diaminobenzidine
DNA	:	Deoxyribonucleic acid
FD	:	*Ficus deltoidea*
G1 phase	:	Gap 1 phase
H&E	:	Haematoxylin and eosin
HRP	:	Horseradish peroxidase
IHC	:	Immunohistochemistry
mg/kg	:	Milligram per kilogram
OSCC	:	Oral squamous cell carcinoma
$p < 0.05$	:	p value less than 0.05
RNA	:	Ribonucleic acid
rpm	:	Revolutions per minute
RT PCR	:	Reverse transcription polymerase chain reaction
SD	:	Sprague-Dawley
TH	:	Tualang Honey

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background

Approximately 94% of all oral malignancies consist of oral squamous cell carcinoma (OSCC). Squamous cells are epithelial cells that form the oral mucosa, thus oral squamous cell carcinoma is a malignancy of the surface epithelial cells. Oral cancer is the third most common cancer among the Malaysian Indian community in Malaysia (Zain et al., 1997) and it is highly associated with the practice of betel quid chewing, excessive alcohol consumption and tobacco smoking (Jemal et al., 2011).

The ultimate goal in cancer treatment is to eradicate the cancer, preserve or restore form and function, minimize the ramification of treatment and finally prevent the incidence of any subsequent new primary cancers. The currently available treatment modalities include radiotherapy, chemotherapy, surgery, combined modality treatments and chemoprevention, as well as prevention strategies involving lifestyle changes.

Despite recent advances in the treatment of oral cancer, the prognosis of cancer patients remains poor, and in about 65% of OSCC patients, the survival rate is only about 5 years. About 70% of patients with stage III and IV cancers cannot be cured, while for patients with stage I and II tumours, the percentage may be as low as 30% (Shah & Gil, 2009).

Over the years, the use of chemotherapeutic drugs such as cisplatin or allopathy medicine for treating cancer have been successful. However, this type of treatment modality is often associated with chemotherapeutic drugs toxicity, resulting in severe side effects.

As a result, researchers have tried to look for other types of treatment modalities and these include the use of natural products for treating cancer. Moreover, several characteristics of oral cavity make it an attractive site for clinical efforts in cancer prevention. Key risk factors for OSCC are well-known, and can be used to identify high risk individuals who are most likely to benefit from a chemo-preventive intervention (McCormick et al., 2015).

Ficus deltoidea (FD) is a common medicinal plant traditionally used in Southeast Asia countries including Malaysia. In Malaysia, the FD extract is used to heal wounds, sores, rheumatism and can also be used as an antidiabetic drug or as an after-birth tonic. Despite all these traditional claims, scientific studies of this plant are very limited with most studies focusing on evaluating its antioxidant, antihyperglycemic, antinociceptive, antihypertensive, wound and ulcer healing properties (Abdulla et al., 2010). It was reported in a study that 85 percent of the overall antioxidant activity of the FD extract was attributable to the flavan-3-ol monomers and the proanthocyanidins (Omar et al., 2011).

The anticancer activity of the FD extract against the human ovarian carcinoma cell line using a cell-based assay has been demonstrated (Akhir et al., 2011). Ware et al. (2015) reported that the FD extract possesses potent natural antioxidant and anticancer activity when tested on prostate cancer DU145 cell line.

Norrizah et al. (2012) observed that the cytotoxic activity aginst the HL-60 cell line of the FD Leaf extract was more potent than the fruit extract. Besides that, when tested against the male reproductive system of the rats, there was a significant effect of the FD extract on the testes and epididymis weight, sperm count and sperm viability (Norrizah et al., 2012).

Tualang Honey (TH), a natural product, has been reported to act as a natural vaccine against cancer because it improves the immune system, reduces cancer pathogenesis such as chronic inflammation and improves the healing of wounds and ulcers. TH demonstrates antiproliferative or antitumour activity against different kinds of cancer cells (Othman, 2012), exhibiting antiproliferative and early apoptotic effects against oral squamous cell carcinoma and human osteosarcoma cell lines (Ghashm et al., 2010), human breast cancer MCF-7 cells and cervical cancer cell lines (Fauzi et al., 2011). It would appear that the high content of phenolic and flavonoid antioxidants found in TH may have contributed to its anticancer activity.

Despite various *in vitro* studies demonstrating the anticancer activities of FD extracts and TH on various types of cancer cell lines, there have been no *in vivo* studies showing the effects of FD extracts and TH towards oral cancer. Hence, this study was conducted to assess the chemo-preventive and chemotherapeutic activities of the FD aqueous extract and TH in an animal model induced for oral cancer using 4NQO.

1.2 Justification and significance of the study

FD or Mas Cotek as locally known, from the family of Moraceae was chosen in this study since this fig tree has been widely used for treating cancer in the Malay community of the Malaysian population. It is also chosen due to its high content of antioxidant compounds such as the flavan-3-ol monomers and proanthocyanidins (Omar et al., 2011). Another natural product, TH was also included in this study as it has been reported to contain various compounds such as antioxidants (phenolics and flavonoid) and vitamins.

The development of cancer is very complex and the induction of oral cancer in animal models has been used extensively in many studies to represent the molecular and cellular changes associated with the initiation and progression of the human oral cancer. In this study, an animal model that had been induced for oral cancer using 4NQO was used to determine the the chemo-preventive and chemotherapeutic activities of the FD extract and TH.

1.3 Aim

The proposed study was designed to evaluate the chemo-preventive and chemotherapeutic activities of the FD extract and TH on oral cancer cells in an animal model induced for oral cancer.

1.4 Objectives of the research

1. To investigate the chemo-preventive effect of the FD extract and TH on oral cancer.
2. To investigate the chemotherapeutic effect of the FD extract and TH on oral cancer.
3. To investigate the antitumour activity of the FD extract and TH on the expression of selected genes and proteins associated with oral cancer.
4. To determine the effects of the FD extract and TH on oral cancer metastasis.

1.5 Hypothesis

The FD extract and TH possesses chemo-preventive and chemotherapeutic activities towards oral cancer and have the potential in being developed as chemo-preventive and chemotherapeutic agents for oral cancer.

1.6 Rationale

There has been no such study on this subject in Malaysia.

CHAPTER 2: LITERATURE REVIEW

2.1 Oral cancer

Oral cancer, which is a type of head and neck cancer, is related to any cancerous tissue growth located in the oral cavity. Oral cancer is considered as one of the top 10th most common cancers in the world and around 60% of these cancers are found in Asia (Jemal et al., 2011). Despite recent advancements in medical science, it has been reported that most of the patients die within 5 years of cancer detection (Funk et al., 2002 ; Stahl et al., 2004).

Up to this day, the overall mortality rate of oral cancer is still high, approximately 50%, depending on the advanced stage of the disease upon diagnosis. Oral cancer may arise as a primary lesion originating in any of the tissues in the oral cavity, or by metastasis from a distant site of origin, or by extension from other anatomical structures, such as the nasal cavity. The type of oral cancer that develops in the oral cavity and its histological features may vary depending on the source of the tissue involved; the development of lymphoma from the tonsillar tissues, melanoma from pigment-producing cells of the oral mucosa, adenocarcinoma derived from a major or minor salivary gland or from other lymphoid tissues (McMahon & Chen, 2003 ; Walker et al., 2003). There are many types of oral cancer, but the most common is SCC and the most common site for oral cancer is the tongue. Oral cancer may also occur on the cheeks, floor of the mouth, lips, palate or gingiva. The histological features for most oral cancers are similar when viewed under the microscope and are usually diagnosed as OSCC. It is estimated that more than 90% of all types of oral cancer consist of OSCC (Choi & Myers, 2008). In 2013, human death caused by oral cancer is around 135,000 compared to 84,000 deaths in 1990, while the five-year survival rates in the United States have been reported to be 63% (Naghavi et al., 2015).

2.2 Oral cancer: initiation, promotion and progression

Cancer is a multistage and polygenic disease. The multistage model of carcinogenesis consists of initiation, promotion and progression stages as first described by Armitage and Doll (1954). After repeated exposure to mutagens, normal cells undergo genetic alterations, and this constitute the initiation stage. During the tumour promotion stage, genetic and epigenetic changes accumulate in the cells, triggering an uncontrolled cell proliferation stage. Subsequently, the cellular and micro-environmental dynamics would further drive the tumour cells towards the advanced stages of cancer development leading to invasiveness and metastasis during tumour progression (Grizzi et al., 2006).

2.3 Molecular and genetic changes during carcinogenesis

During carcinogenesis (a multistage process), tumour cells develop genetic and epigenetic changes at the molecular level, and this would lead to a paradoxical state of dysfunctional self-sufficiency for promoting tumour growth and metastasis with sustained heterogeneity. Common genetic alterations resulting in tumourigenesis can be linked to, among others, three types of genes: oncogenes, tumour-suppressor genes and stability genes (Bishop, 1991; Kim et al., 2012) (Figure 2.1). Oncogenes consist of functionally heterogeneous group of genes, which are constitutively active in most of the cancer cells. These genes positively regulate complex cascades/mechanisms within the cancer cells which would additionally promote cell growth (Chial, 2008) .

In contrast, mutations in the tumour suppressor genes are desired as these can lead to a reduction in their activities. Such inactivation may result from either missense mutation at sites that are necessary for tumour-suppressor activity, or mutations that lead to the formation of truncated proteins or epigenetic silencing of these genes.

It is believed that the major anomalies in the genome leading to tumourigenesis result from mutations in the tumour suppressor genes (Todd et al., 1997). The third type of genes, termed as the stability genes, include those genes that are involved in the repair of damaged DNAs. These genes are not directly involved with cell proliferation, but instead maintain the genomic stability of the cells (Jaenisch & Bird, 2003).

Mutations in these genes may confer certain hallmark characteristics of cancer cells. These characteristics include sustained proliferation, immortality, evading tumour suppressor's activity, resistance to programmed cell death, angiogenesis and metastasis. In addition to these well-established hallmarks of cancer, the last few decades have witnessed remarkable progress in the field of cancer research including the discovery of certain distinctive features of cancer cells, such as the reprogramming of certain metabolism involved with energy production for sustainability, evading immune surveillance and remodelling of the tumour microenvironment (Hanahan & Weinberg, 2011).

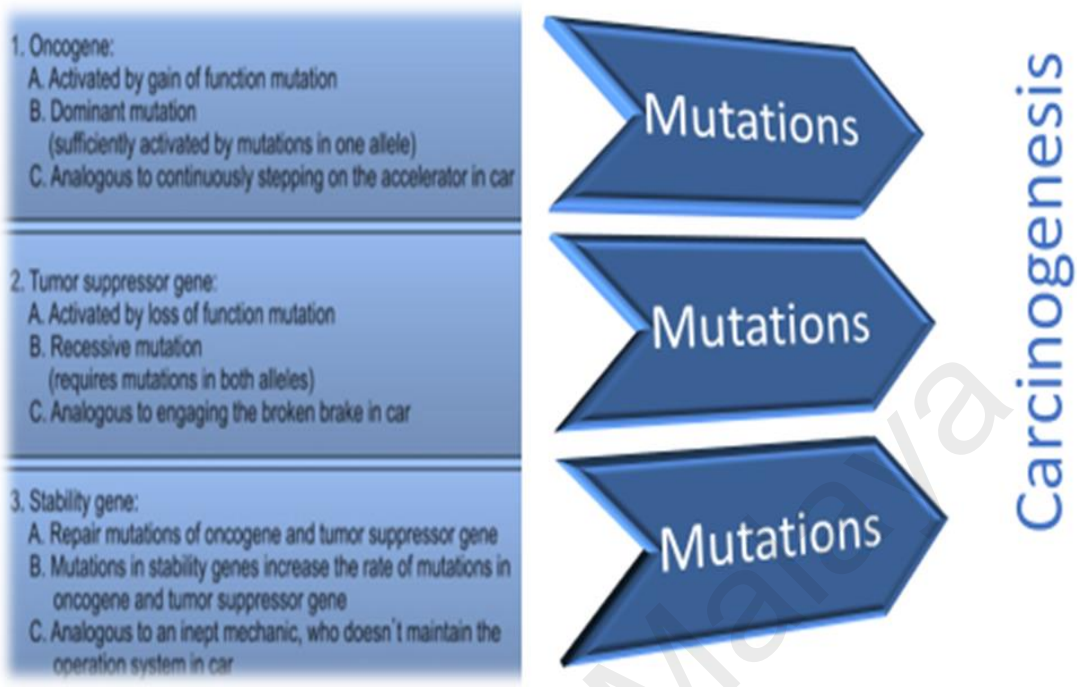


Figure 2.1: Three types of gene mutations responsible for tumour formation. Adapted from Kim et al., 2012.

2.4 Epidemiology of oral cancer

GLOBOCAN, an International Agency for Research in Cancer (IARC), estimates the incidence of cancer based on the availability of data obtained from each country. According to GLOBOCAN (2008), oral cancer is the 15th most frequent cancer around the world and is rated as the 10th and 13th most frequent cancer in men and women, respectively (Ferlay et al., 2010). A total of 263,000 new cases of oral cancer were reported in 2008 and of this, 64.8% were female. 65.4% of those cases have not included the developing nations such as China, Melanesia, Asia and Micronesia/Polynesia. Notably, a total of 128,000 deaths were projected in 2008 and nearly 80% of the deaths occurred in the less developed nations.

In general, a wide variability in the types of oral cancer has been discovered in the Oceania, Melanesia, and South-Central Asia with age-standardized rate (ASR) of 7.1, 17.8, and 7.4 per 100,000 inhabitants respectively. Europe, and the Northern America have comparatively higher ASR which range between 4.9-4.6 per 100,000 inhabitants (Ferlay et al., 2010).

According to the data obtained from GLOBOCAN, oral cancer is rated as the 13th most frequent cancer in Malaysia with the ASR of 3.5 per 100,000 inhabitants (Figure 2.2). According to gender, oral cancer ranked 10th among men and 12th among women. Since the Malaysian population comprises of three main racial groups: Malay (51.0%), Chinese (24.2%) and Indian (7.1%), it would be important to determine the prevalence of oral cancer for each of the racial groups. Notably, the Indian female community in Malaysia is the most affected (Zainal, 2011). The Malaysian National Cancer Registry records cancer cases in government hospitals in Malaysia with the exclusion of Sabah and Sarawak. It was reported that the ASR of esophageal cancer, excluding tongue cancer for Indian females has been exceptionally high with an ASR of 14.4 per 100,000 population, in comparison to the Malay and Chinese with just 0.8 and 0.6 per 100,000 population respectively. In reality, this value is much greater than the value estimated by GLOBOCAN 2008 for oral cancer prevalence in Melanesia which has the greatest ASR score among females, thereby indicating the massive burden of oral cancer among the female Indian ethnic group in Malaysia (Zainal, 2011).

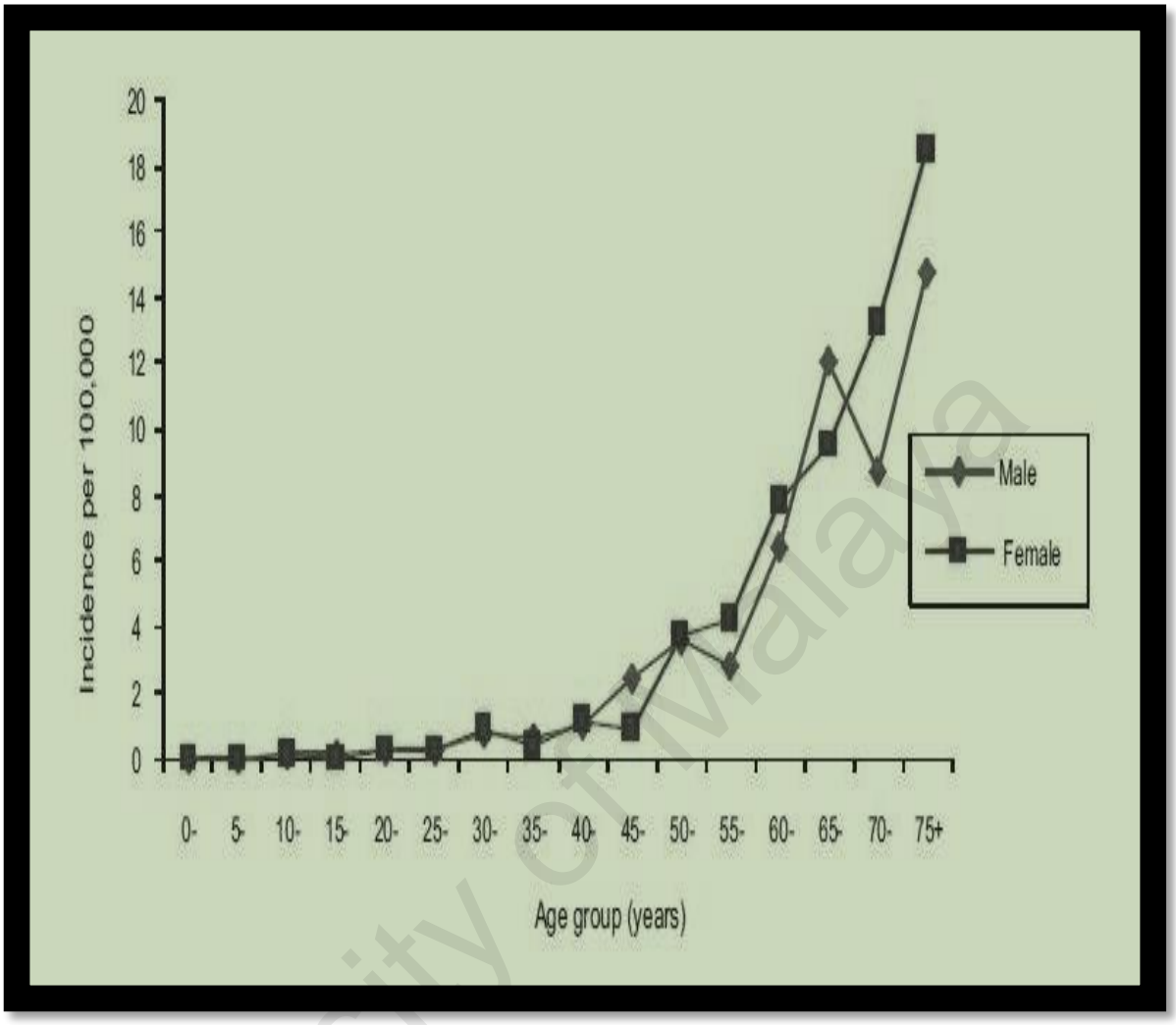


Figure 2.2: Incidence of Age-specific oral cancer by sex per 100,000 population in Malaysia 2007. Adapted from the Malaysian National Cancer Registry Report (MNCR) 2007-2011 (Zainal, 2011).

2.5 Risk factors for oral cancer

Alcohol ingestion, smoking, betel quid usage, HPV disease and microbial infection are the significant risk factors for oral cancer (Andre et al., 1995; Blot et al., 1988; Cogliano et al., 2004; Nair & Pillai, 2005; Bakri et al., 2014). However, the contribution of each risk factor to the burden of oral cancer changes across geographical regions (Jemal et al., 2011). It has been reported that other features such as occupational hazard, nutrition and diet, resistant

disturbances, poor oral health care and hereditary effects may also contribute towards oral cancer growth (Ram et al., 2011; Warnakulasuriya, 2009; Xia et al., 1997).

The association between smoking and oral cancer is well documented. Past studies have revealed that the possibility of developing oral cancer is five to eight times higher in smokers compared to non-smokers, and this risk is increased to approximately 17 times higher among heavy smokers who consume 80 or more cigarettes daily (Blot et al., 1988; Andre et al., 1995). It has been noted that tobacco chewing is also related to a greater risk of oral cancer. Tobacco smoke contains more than 300 pro-carcinogens and carcinogens that can contaminate the saliva and elevate the level of DNA adducts in the human tissues, resulting in greater risk of cancer (Löfroth, 1989). It is also noted that certain pro-carcinogens that are specifically associated with tobacco such as nitrosamine (e.g. 4- methyl nitrosamine), polycyclic aromatic hydrocarbons (e.g. benzo (a) pyrene), and aromatic amines (e.g. 4-aminobiphenyl) require metabolic activation via xenobiotic enzymes, specifically cytochrome p450, a key enzyme in cancer formation and treatment (Hecht, 1999) .

Alcohol consumption has been proven to have a role in oral cancer independent of tobacco usage. The incidence of developing oral cancer in heavy drinkers who consume more than 100 mg of alcohol daily, is reported to be three to 30 times higher than the average drinkers (Blot et al., 1988; Andre et al., 1995). Alcohol itself is not carcinogenic, but can be metabolised into acetaldehyde, a known carcinogen, through the enzymatic activity of the human oral mucosa or microorganisms present in the oral cavity (Bakri et al., 2015).

Almost all types of carcinogens and pro-carcinogens require for activation by the xenobiotic enzymes, and this has led to many extensive studies connecting genetic polymorphism of the xenobiotic enzymes and also its capacity to modify an individual's reaction to these carcinogens (Shimada, 2006).

An example of an enzyme involved in alcohol metabolism is alcohol dehydrogenase, which has been extensively studied, especially in the Japanese population which is associated with alcohol dehydrogenase (ADH) polymorphism (Stamatoyannopoulos et al., 1975). Alcohol consumption when combined with tobacco smoking can increase the threat of oral cancer up to 100 times in heavy smokers and heavy drinkers (Blot et al., 1988; Andre et al., 1995). Alcohol consumption affects mucosal permeability, and this could lead to improved penetration of carcinogens into the oral mucosa (Walker et al., 2003). Additionally, during alcohol metabolism, acetaldehyde is generated and this can lead to the creation of free radicals and hydroxylation of all DNA bases, which additionally causes cellular DNA damage (Scully & Porter, 2000).

Another risk factor associated with oral cancer is betel quid chewing. A workshop held in Malaysia in 1996 recommended that the term "quid" should be used to describe a substance or mixture of compounds that is placed in the mouth that comprise of at least one or both fundamental components; tobacco or areca nut (Zain et al., 1999). The components of the betel quid may change in various cultures; however, it generally contains betel nut, piper betel plant (inflorescence, leaf or root) and slaked lime (either as a powder or paste). In most nations, the component may also comprise of snuff and spices since betel chewing is associated with adding snuff. Similar chewing habits, such as khat chewing, may also contribute to oral cancer in certain areas (Fasanmade et al., 2007). Arecoline, chief alkaloid of the betel nut, has been reported to block tumour suppressor genes through hypermethylation, and therefore inhibits DNA repair (Tsai et al., 2008) . In Malaysia, the areca nut (taken either dried or fresh), betel leaf and slaked lime are occasionally folded in betel leaves, like small parcels and chewed. On the other hand, the quid from various cultural groups may vary and the quid mixtures may contain added ingredients such as spices and tobacco (Zain et al., 1999).

The chewing substance can be obtained as mass-produced packets and consist of proprietary mixtures called pan masala or gutka (IARC, 2004). The development of oral cancer has also been linked with the ingestion of betel nut (Areca), a habit that is associated with certain population found in India and other parts of Southeast Asia (Cogliano et al., 2004; Zhang & Reichart, 2007).

The association between the Human Papilloma Virus (HPV) and oral or pharyngeal cancer was initially suggested by Syrjänen et al. (1983), and was supported by numerous researches (Ha & Califano, 2004; Nair & Pillai, 2005). The HPV strains associated with oral cancer are HPV-16 and HPV-18. It has been reported that HPV-16 is associated with approximately 50 percent of the carcinomas of the oropharynx (Weinberger et al., 2006).

The greater prevalence of OSCC and its association with HPV, and its occurrence in young men and women is noted especially when the most frequent risk factors are not present. HPV 16 oncoproteins encoded by the genes E6 and E7, cause genomic instability and inactivate the tumour suppressor genes (Hübbers et al., 2015; Weinberger et al., 2006).

2.6 Oral cancer management

The treatment modality for oral cancer depends on many factors, among others, the clinical staging and histological evaluation of the tumour, tumour volume and the presence of metastatic tumours. Depending on the severity of the disease, patients with oral cancer may have varied response towards the various types of treatment modalities available for treating cancer. Among the current available treatment modalities include surgery, radiotherapy, chemotherapy, a combination of treatment modalities and prevention strategies involving chemoprevention or lifestyle changes.

Despite the recent advances in cancer treatment, the success rate of treating patients with oral cancer remains a challenge as patients are often presented with the advanced stage of the tumour (Scully & Porter, 2000). Pre-malignant lesions such as dysplasia, can undergo malignant transformation and therefore, the presence of pre-malignant lesions may serve as a prognostic tool for early cancer detection. Although cancer development may progress from pre-malignant lesions, not all pre-malignant lesions undergo malignant transformation. Hence, the search for molecular markers for early cancer detection has generated a lot of interests and this still continues to this day (Bankfalvi & Piffko, 2000; Van't Veer et al., 2008).

2.7 Natural products with chemo-preventive activity

Many plants and organic products have been identified for their chemo-preventive activities and this had led to the development of drugs for cancer treatment (Mans et al., 2000). Active compounds such as podophyllotoxin and lignans, isolated from the mayapple plant (*Podophyllum peltatum*) has led to the development of drugs for the treatment of small cell lung cancer and testicular cancer (Cragg & Newman, 2005). Chemo protective activity from the plant such *Abrus precatorius* has been reported to be effective against fibrosarcoma, ascites tumour cells and Yoshida sarcoma have been studied (Reddy & Sirsi, 1969) while *Alstonia scholaries*, have also been shown to be effective against stomach carcinoma in mice (Jagetia et al., 2003). Other plants with anticarcinogenic properties that have been shown to be effective against specific types of tumour are; *Peaderia foetida* in human epidermoid carcinoma, *Picrorrhiza kurroa* in hepatic cancers, *Boswellia serrata* in human epidermal carcinoma, *Erthyrina suberosa* in sarcoma, *Anacardium occidentale* in hepatoma, *Euphorbia hirta* in Freund virus leukemia, *Gynandropis pentaphylla* in hepatoma, *Nigella sativa* in Lewis lung carcinoma, *Asparagus racemosa* in human epidermoid carcinoma and *Withania somnifera* in various types of tumours (Desai et al., 2008).

The natural products that have been assessed in this study are rich in antioxidants (also known as "free radical scavengers"). Free radicals are highly reactive chemicals that have the potential to harm cells. They are created when a molecule either gains or loses an electron. Free radicals are formed naturally in the body and play an important role in many normal cellular processes. However, at high concentrations, free radicals can be hazardous to the body and damage all components of cells, including DNA, cell membranes, and proteins. The damage to cells caused by free radicals, especially the damage to DNA, may play a role in the development of cancer. Abnormally high concentrations of free radicals in the body can be caused by exposure to some environmental toxins, such as cigarette smoke, some metals, and high-oxygen atmospheres (Diplock et al., 1998).

Antioxidants are chemicals that interact with and neutralize free radicals, thus preventing them from causing damage. The body makes some of the antioxidants that it uses to neutralize free radicals. These antioxidants are called endogenous antioxidants. However, the body may also rely on other sources such as external (exogenous) sources, which primarily comprise of the diet that is being consumed. In laboratory and animal studies, the presence of increased levels of exogenous antioxidants has been shown to prevent certain types of damage caused by free radical damage and associated with cancer development (Valko et al., 2007).

2.7.1 *Ficus deltoidea*

FD, a herbaceous type of plant, can grow up to about 2 meters tall with thick leaves that are deltoid in shape, rounded at the apex and tapered at the base (Figure 2. 3). The upper surface of the plant's leaves is dark shining green, while the lower surface is golden yellow with black spots. The male and female plants are physically distinctive, with the leaves of female plants being big and round, while the leaves of the male plants are small, round and long.

The plant grows wild in the eastern part of Peninsular Malaysia (Kelantan, Terengganu) and also in Borneo (Sabah, Sarawak and Kalimantan) and is used by traditional medical practitioners in these regions. FD comes from the family of Moraceae genus Ficus. In Malaysia, it is known as Mas Cotek other than FD, the vernacular names are Delta Fig, Fig Shrub, and Mistletoe Fig.

It is well distributed throughout Southeast Asia and can also be found in Africa. There are about 13 varieties of FD, based on the morphology of the leaves and figs (Corner, 1969). FD has been proven to have many pharmacological effects such as antimicrobial, antinociceptive, antioxidant as well as anti-inflammatory. It has been reported that the FD extract has anticancer effect against ovarian carcinoma and leukemic HL-60 cell line (Norrizah et al., 2012).

Figure 2.3: *Ficus deltoidea* plant

When the body contains high amounts of free radicals, it can cause cellular harm specifically to the genomic part of the cell, and this can lead to the development of cancer. There have been many studies related to the antioxidant and phenolic content of plants and it was reported that the phenolic compounds can inhibit the initiation, progression and spread of cancer. A phenolic compound of FD, the eicosonoid, has anti-inflammatory effect and therefore can help to inhibit the development of blood vessels. The development of blood vessels or angiogenesis is associated with tumour growth, and it has been reported that eicosonoid may aid in inhibiting the growth of tumours (Wahle et al., 2010). It has also been stated that the antioxidant activities of the phenolic compounds from plants are also known for their antioxidant activity and thus, it is possible that FD, which is rich in antioxidants, may be beneficial in reducing the risk of cancer (Ware et al., 2015).

FD is one of the most well-known and available Ficus species in Malaysia, which is traditionally used by the Malay community for its health maintenance purposes. The botanical name for *Ficus deltoidea* is *Ficus deltoidea* Jack, while its local names are given according to the different parts of the world. In Malaysia, *Ficus deltoidea* is locally known as "Mas Cotek", "Serapat Angin", "Sempit-sempit" and "Telinga Beruk". In Indonesian, this species is called "Tabat Barito", "Ara Jelatih", "Ara Tunggal", "Apiapi Gajah" or "Api-api Telinga Kera". In Africa and Philippines, it is known as "Kangkalibang" and "Angulora", respectively. Among some of the subspecies of *Ficus deltoidea* are as follows: *Ficus deltoidea* var Deltoidea, *Ficus deltoidea* var augustifolia, *Ficus deltoidea* var angustissima, *Ficus deltoidea* var arenaria, *Ficus deltoidea* var bilobota, *Ficus deltoidea* var borneensis, and *Ficus deltoidea* var oligoneura (Bunawan et al., 2014).

2.7.1.1 Biochemical content of *Ficus deltoidea*

A comprehensive study on the volatile compounds produced by the fruits of FD began in 2002 (Grison-Pigé et al., 2002). In the study, volatile compounds associated with floral fragrances such as terpenoids and aliphatic groups, have also been identified in FD. In 2011, an anti-bacterial compound known as lupeol was isolated from the leaves of FD (Suryati et al., 2011). Two other bioactive compounds found in the leaves of FD, vitexin and isovitexin, were isolated and were found to be useful for the treatment of diabetes (Choo et al., 2012).

Phytochemical studies have revealed that the antioxidant activity of the FD extract is attributed to the presence of flavonoids, phenolics, vitamin C, terpenoids, alkaloids, saponin and tannins (Omar et al., 2011). The leaves of FD have also been shown to contain enzymatic antioxidants such as ascorbate oxidase, peroxidase, catalase, and ascorbate peroxidase (Hakiman & Maziah, 2009).

In a cell culture study, it was observed that some of the flavonoid compounds such as rutin, quercetin and naringen found in FD can be influenced by the different carbon sources found in the different types of medium used for growing the cells (Pick et al., 2011) .

Three major groups of the phytochemicals present in FD are the phenolic compounds, tannins and phenylisopropanoid (Abdullah et al., 2009). The phenolic compounds accumulate in different parts of the plant tissues and provide many biological activities. Localisation of the phenolic compounds in the cell walls, vacuoles and cell nuclei can contribute to the anti-inflammatory and anti-septic properties (Yi et al., 2005). The chemical constituents of the phenolic compounds that have been identified are catechin, flavones, naringin, vitexin, isovitexin, anthocyanins and proanthocyanins. The beneficial role of naringin is reported in reducing the cholesterol level in the body by lowering plasma lipid concentrations (Choi et al., 2001).

Strong antioxidant properties of vitexin and isovitexin have also been demonstrated (Abdullah & Ismail, 2009). Velayutham observed that the supplementation of tannins from FD can reduce the levels of plasma glucose and lipid profile, as well as attenuating the oxidative stress level of hypercholesterolemia associated diabetes in an animal model study (Velayutham et al., 2012).

2.7.1.2 Ethnomedicinal and pharmacological activities of *Ficus deltoidea*

According to tradition, different parts of this plant have been used to treat various kinds of ailments. The powdered form of the root and leaves are applied externally to wounds, on sore and rheumatism joints, while the fruits are chewed to relieve toothaches, headaches and colds.

Decoctions of the boiled leaves are taken during the confinement period to constrict the womb and strengthen the vaginal muscles, as well as to treat menstrual cycle problems. The entire plant, on the other hand, can be used as an aphrodisiac tonic to enhance sexual desire (Bunawan et al., 2014).

A study demonstrating the FD's potential as an antidiabetic agent has been reported (Adam et al., 2012). The antihyperglycemic activity of FD is mediated through the stimulation of insulin secretion from pancreatic β cells, thereby increasing the glucose uptake by the adipocytes cells and the augmentation of the adiponectin secretion from the adipocytes cells.

The rational in using FD for treating microbial infections was supported by an *in vitro* study, where the antimicrobial activity of the chloroform, methanol and aqueous extracts of this medicinal plant on fungus, Gram-positive and Gram-negative bacteria strains was found to be efficient (Abdsamah et al., 2012).

FD has been reported to have anti-melanogenic activity by suppressing the tyrosinasegene expression of B16F1 in melanoma cells (Oh et al., 2011). Thus, it was suggested that the FD extract has the potential to be used as a novel depigmenting agent for cosmetic use. With regards to the reproductive system, FD has the potential to be developed as a natural uterotonic agent to facilitate the delivery process and also for the treatment of post-partum haemorrhage (Amiera et al., 2014). During contraction, the uterotonic effect of FD is mediated by multiple uterotonin receptors (muscarinic, oxytocin and prostaglandin) (Salleh & Ahmad, 2013).

Extensive pharmacological studies have shown that the FD extract contain the following properties; anti-inflammatory and antinociceptive (Abdullah et al., 2009), antiphotoaging (Oh et al., 2011) and antiulcerogenic agent (Fatimah et al., 2009). In addition, the FD extract has also been shown to have antibacterial (Abdsamah et al., 2012), wound healing activity (Abdulla et al., 2010), anticancer and cytotoxic activity (Akhir et al., 2011; Ware et al., 2015)

2.7.2 Honey

For many centuries, honey has been famous for its medicinal and health promoting properties. It contains several types of phytochemicals with a high phenolic and flavonoid content that contribute to its high antioxidant action. It is a powerful agent due to its high content of antioxidant, and thus has the capability to avoid cancer development due to the presence of free radicals and oxidative stress that are substantially involved in inducing the formation of cancer. The phytochemical content available in honey can be narrowed down into phenolic acid and polyphenols. The variants of polyphenols in honey have been reported to possess antiproliferative property against various types of cancer (Othman, 2012).

Polyphenols found in honey, specifically, pinobanksin (PB), chrysin (CR), caffeic acid (CA), apigenin (AP), caffeic acid phenyl esters (CAPE), kaempferol (KP), acacetin (AC), pinocembrin (PC), galangin (GA) and quercetin (QU) have been associated with antiproliferative properties (Jaganathan & Mandal, 2009). Various types of honey from all over the world have been tested for their antioxidant properties; such as the Trigona carbonaria honey from Australia (Oddo et al., 2008), or the different types of honey from Spain as reported by Pérez et al. (2007). Dark honey has been reported as possessing greater phenolic compounds and antioxidant activity than clear honey, and that the amino acid composition of honey is also a sign of its capacity in scavenging for free radicals (Pérez et al., 2007).

2.7.2.1 Honey and cancer treatment

Honey may offer the foundation for the development of novel therapeutics for individuals with cancer and cancer-associated tumours. The jungle honey from Nigeria has been shown to possess chemotactic induction for neutrophils and reactive oxygen species (ROS), demonstrating its antitumour action (Takeuchi et al., 2011).

In a related study, the Malaysian jungle honey has revealed significant anticancer activity when tested against the human breast, cervical, oral and osteosarcoma cancer cell lines (Fauzi et al., 2011). Honey has also been shown in an *in vivo* and *in vitro* studies as possessing antineoplastic activity in the bladder of an animal model (Swellam et al., 2003). Another constituent of honey, flavonoids, has produced a great deal of interest among researchers due to its anticancer properties.

The mechanism of action for flavonoids may vary and include different signaling pathways; the stimulation of TNF-alpha (tumour necrosis factor-alpha), inhibition of cell proliferation, induction of apoptosis, cell cycle arrest and inhibition of lipoprotein oxidation (Tonks et al., 2001). Honey is thought to possess these valuable effects due to its content such as chrysin and other flavonoids.

The effects of various types of honey may also vary due to the difference in composition and this can be attributed to the different types of floral resources involved during the formation of each type of honey.

2.7.2.2 Tualang Honey (TH)

Malaysian TH is collected from the honeycombs of the Asian rock bees (Apis dorsata), which build their hives high up in the Tualang tree (Koompassia excelsa). TH is used commonly as a medicinal product in Malaysia and is also widely consumed as a food supplement (Figure 2. 4). TH is reported to possess antiproliferative effect against oral cancer and osteosarcoma cell lines (Ghashm et al., 2010). TH has been shown to possess anti-inflammatory, antimicrobial, antimutagenic, antitumour, antioxidant and antidiabetic properties, along with wound-healing attributes (Ahmed & Othman, 2013).

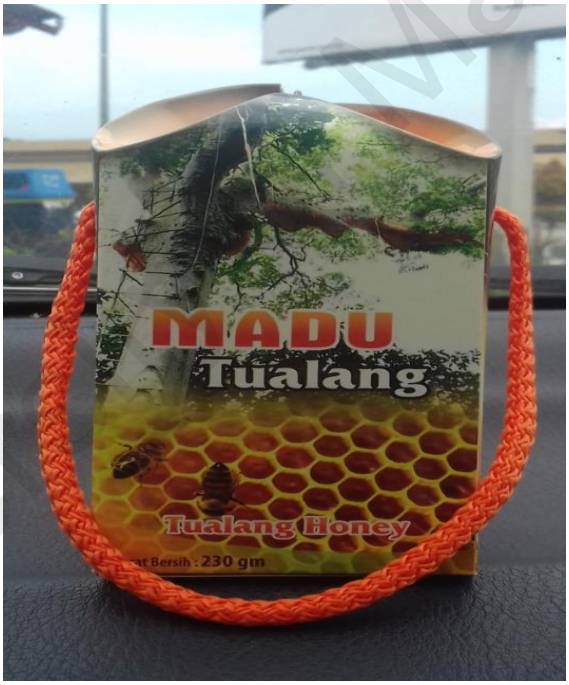


Figure 2. 4: Tualang honey (Agromas, Malaysia)

When compared to Manuka honey, TH has higher content of phenolics, flavonoids, and 5-(hydroxymethyl) furfural (HMF). TH was also found to be more effective against several gram-negative bacterial strains in burn wounds (Ahmed & Othman, 2013). Kishore et al. (2011) indicated that the raised free-radical scavenging and antioxidant action found in TH are the result of the growing amount of phenolic substances. Besides the antibacterial, anticarcinogenic and

anti-inflammatory properties, the results obtained from the study emphasised on the significant positive antioxidant properties of TH towards human nourishment and wellness (Kishore et al., 2011). In a related study, Fauzi et al. (2011) reported that TH has significant anticancer activity against the human breast and cervical cancer cell lines (Fauzi et al., 2011). The phenolic content, antioxidant capacity, chemical compositions, volatile compositions and hydroxymethylfurfural have been extensively studied in TH, and the correlation between the total phenolics and flavonoids contents and antioxidant activity have been reported (Chua et al., 2013; Khalil et al., 2011; Kishore et al., 2011).

Interestingly, it was shown that TH has better antioxidant effect against a variety of reactive oxygen species (ROS) compared to other local Malaysian honey such as Gelam honey, Commercial honey and Pineapple honey (Kishore et al., 2011). A cancer research group claimed that TH has the potential to induce anticancer activity in the breast cancer cell lines via the upregulation of double strand DNA repair enzymes, thereby preserving the cellular DNA integrity (Yaacob & Ismail, 2014), or by promoting apoptotic cell death (Yaacob et al., 2013).

In an ultraviolet B (UVB) radiation study, it has been found that the treatment using TH towards PAM212 mouse keratinocyte cell line resulted in the induction of a number of cyclobutane pyrimidine dimers and 8-oxo-dG-positive cells (biomarkers of oxidative damage) due to the improvement of DNA repair (Ahmad et al., 2012).

TH has been shown to have antimicrobial properties on full-thickness burnt wound in rats (Khoo et al., 2010), and also has the potential to be used as a therapeutic agent for treating diabetic wounds on the foot (Alam et al., 2014). The combination in taking TH as a supplement with exercise have also been reported to be beneficial on the tibial bone mineral density (Kiew Ooi et al., 2011).

Honey has been proven to be an effective agent against yeast infection and can reduce apoptosis in leukemia cells (Nik Man et al., 2015). As a prophylactic measure, TH was also reported to be significantly effective in decreasing the symptoms of acute respiratory symptoms among Malaysian Hajj pilgrims (Deris et al., 2010).

2.8 Carcinogenesis induction in animal models

Since the 1950s, many researchers in the field of cancer research have carried out experiments using chemicals for the induction of cancer. Initially, the use of chemicals for cancer induction in animal models contributed to the identification of carcinogens/chemical components of tobacco and alcohol, the main etiologies of human SCC of the oral cavity (Mognetti et al., 2006). Various types of animal models have been developed for *in vivo* studies and this would involve the use of chemicals, transplantation of cancer cells and also various methods involving genetic manipulation (Mognetti et al., 2006; Morris et al., 1960) (Figure 2. 5).

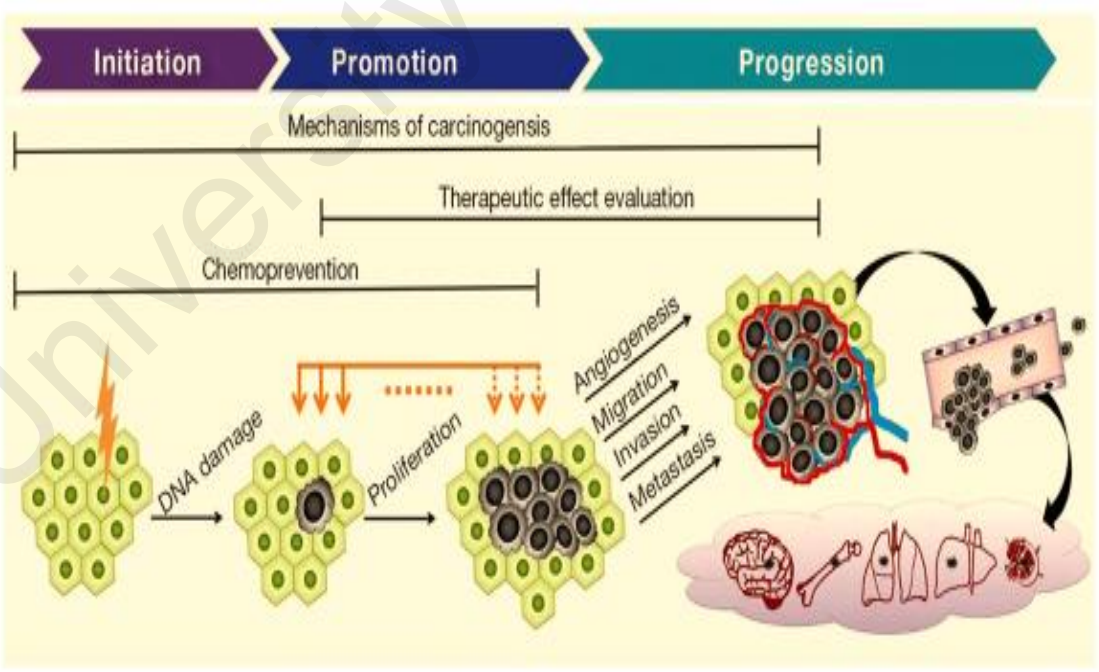


Figure 2.5: Three-phase process of carcinogenesis upon carcinogen administration and the various types of research applications based on animal models of primary cancers.

Adapted from Liu et al., 2015

2.8.1 4-Nitroquinoline-1-Oxide (4NQO)

The 4NQO carcinogen is defined as a synthetic derivative of quinoline which is soluble in water and sensitive to high temperature and light (Wilkey et al., 2009). It is a well- known and potent carcinogen present in cigarette smoke (LaVoie et al., 1983). The administration of this carcinogenic agent to rodents in drinking water will lead to damaged DNA and simulate tongue carcinogenesis in rodents (Minicucci et al., 2009).

The mechanism of action of 4NQO takes place by generating reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as superoxide radicals, hydrogen peroxide and nitric oxide inducing an intracellular oxidative stress (Nunoshiba & Demple, 1993). ROS/RNS can cause adducts by directly and indirectly damaging macromolecules including DNA and the joining of predominantly guanine residues (Kanojia & Vaidya, 2006). This may lead to mutations in the nitrogenous bases of DNA, genetic deletions and chromosomal aberrations, as well as breaks and chromatin changes of the nucleus.

There have been many methods for applying 4NQO in experimental studies on carcinogenesis. From the reviewed literature, the application of 4NQO in the oral cavity was initially traced to 1965 when Fujino et al. used the carcinogen diluted to 0.25% in benzene for topical application (Fujino et al., 1965). It was reported that the best results were achieved when 4NQO was dissolved in the drinking water of the animals (Tang et al., 2004).

Other researchers have also reported that oral route for the administration of 4NQO through liquid solution was preferable (Vered et al., 2005; Ribeiro et al., 2004). Lekholm et al. (1975) diluted 4NQO in 0.5% propylene glycol and applied 4NQO three times a week using a brush.

Henriques et al. (2011) on the other hand, diluted 4NQO in 0.5% propylene glycol and brushed the left side edge of the tongue (Henriques et al., 2011). Ribeiro et al. (2004) reported that DNA damaged sites was evident in just 4 weeks of 4NQO consumption including incomplete repair (Ribeiro et al., 2004).

Apart from 4NQO, such as polycyclic aromatic hydrocarbons, dimethylbenzanthracene, methylcholanthrene and benzpyrene have also been used for the induction of oral carcinogenesis in animal models (Tanaka et al., 2011).

2.9 Metastasis

Generally, OSCC is characterised by a high rate of recurrence and metastasis to the regional lymph nodes. Lymph node metastasis is considered to be a significant prognostic indicator (Noguti et al., 2012). Metastatic cancer cells must undergo certain processes, such as interaction with the local microenvironment, migration, invasion, resistance to apoptosis and induction of angiogenesis (Geiger & Peeper, 2009).

The epithelial tissues associated with tumours are capable of forming relatively rigid sheets of cells which are separated from the stroma by a basement membrane and are arranged as lateral belts of cell-cell adhesion complexes.

During the invasion period, the epithelial tumour cells are released from their adjacent cells and there is a break in the basement membrane. It has been suggested that epithelial-mesenchymal transition (EMT) plays a key role during this process and the EMT genes, associated with cancer metastasis, were suggested to be activated during the late stages of malignancy (Christofori, 2006; Tsai & Yang, 2013).

During metastasis, the epithelial cells can be triggered to undergo transformation and in the process, acquire the phenotype of a mesenchymal cell that is associated with increased motility and invasiveness (Singh & Settleman, 2010). Lymph node metastatic tumours occur in 40% of OSCC patients (Noguti et al., 2012), and up to 25% of OSCC cases have evidence of distant metastasis (Woolgar, 2006).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Animals

A total of 70 healthy male Sprague-Dawley (SD) rats (6-8 weeks old with body weight between 200-250 g) were obtained from the Animal Experimental Unit, Faculty of Medicine, University of Malaya, Kuala Lumpur.

Experimental procedures were conducted according to the approval of the Institutional Animal Care and Use Committee (IACUC), University Malaya (Ethic reference no. 2016-190607/DENT/R/AMHS and 2016-171103/DENT/R/AMH).

All experimental procedures were performed according to the FOM IACUC guidelines. All animals received human care based on the criteria outlined in the "Guide for the care and use of laboratory animals" (USA) (National Research Council, 2010).

The animals were maintained under environmental condition of the animal house, had free access to standard ad libitum rat chow diet and fresh reverse osmosis (RO) water. They were housed in animal cages in an air-conditioned area at 22 ± 30 ºC and with a 12 h light/dark cycle in an experimental room.

The animals were acclimatized to laboratory conditions for 7 days prior to the experiments.

3.1.2 Tualang honey (TH)

TH was purchased from the Federal Agricultural Marketing Authority (FAMA), under the Ministry of Agriculture and Agro-Based Industry, Malaysia (Figure 3.1). TH is a wild type multifloral honey collected from Apis dorsata's beehive that is built on giant Koompassia excels tree (locally known as Tualang tree) in the rainforest of Kedah, Malaysia. The wild local Tualang honey was chosen for this study as the purity and quality of this product are guaranteed and fully monitored by the Malaysian Agricultural Research and Development Institute (MARDI).

At the honey-processing center in Kuala Nerang, Kedah, TH was processed through several stages of quality inspection, dehydration, packaging and labelling. TH was filtered to remove solid particles and was concentrated in an oven at 40°C. TH was subjected to commercialised sterilisation procedure using γ irradiation (25 kGy) at Sterilgamma (M) Sdn. Bhd. (Selangor, Malaysia). The water concentration of TH was standardized by FAMA at 18%.

Figure 3. 1: Tualang honey processing-center in Kuala Nerang, Kedah (penpasksgb.blogspot.com)

3.1.3 Aqueous extract of *Ficus deltoidea*

The aqueous extract of FD was purchased from HCA Products Sdn Bhd. Universiti Putra Malaysia, Selangor, Malaysia (Figure 3.2). The quality of the aqueous extract of *Ficus deltoidea* has been guaranteed by the Institute of Bioproduct Development (IBD), Universiti Kebangsaan Malaysia (UKM), Malaysia. The production of this product was carried out in accordance with the ISO Certified Laboratory protocol, which follows the international standard guidelines.

The aqueous extraction was done using FD leaves obtained from Herbagus Sdn Bhd according to the standardised 100% pure extraction method. The leaves of the plant were dried and powdered mechanically. The dried powdered leaves (100 g) was infused in distilled water at 60 °C for 4 hours, filtered, and the liquid extract content was spray dried for 6 hours at 80 °C.

Figure 3.2: Processing of *Ficus deltoidea* at the HCA Products Sdn. Bhd. Universiti Putra Malaysia, Selangor, Malaysia

3.1.4 4-Nitroquinoline-1-oxide

4-Nitroquinoline-1-oxide (4NQO) is a carcinogenic chemical. 4NQO may naturally occur in the environment but is typically manufactured for research purposes. 4NQO (Cas No. N-8141, Sigma Aldrich) was purchased from Labchem Sdn. Bhd., Malaysia.

3.2 Methods

3.2.1 Study design

3.2.1.1 Grouping of animals

After acclimatisation for one week, a total of 70 male Sprague-Dawley (SD) rats were distributed into ten groups (n=7 per group). Group 1 (untreated group) and group 2 (cancer control) received 4NQO in their drinking water for 8 weeks. Group 3, 4, 5 and 6 (chemo-preventive) received 4NQO for 8 weeks and were simultaneously treated with FD extract at 250 and 500 mg/kg and TH at 1000 and 2000 mg/kg respectively by oral gavage for 10 weeks. Group 7, 8, 9 and 10 (chemotherapeutic) received 4NQO for 8 weeks, followed by the administration of the FD extract at 250 and 500 mg/kg and TH at 1000 and 2000 mg/kg respectively for another 10 weeks.

The oral route was chosen to mimic the most likely route for human food consumption. The administration of FD extract and TH was performed once daily in the morning (between 09:00 and 10:00 am). Throughout the administration period, the body weight of the individual rats was recorded once a week. All rats from all experiments were sacrificed after 22 weeks. The study design is shown in a flow chart in Figure 3.3.

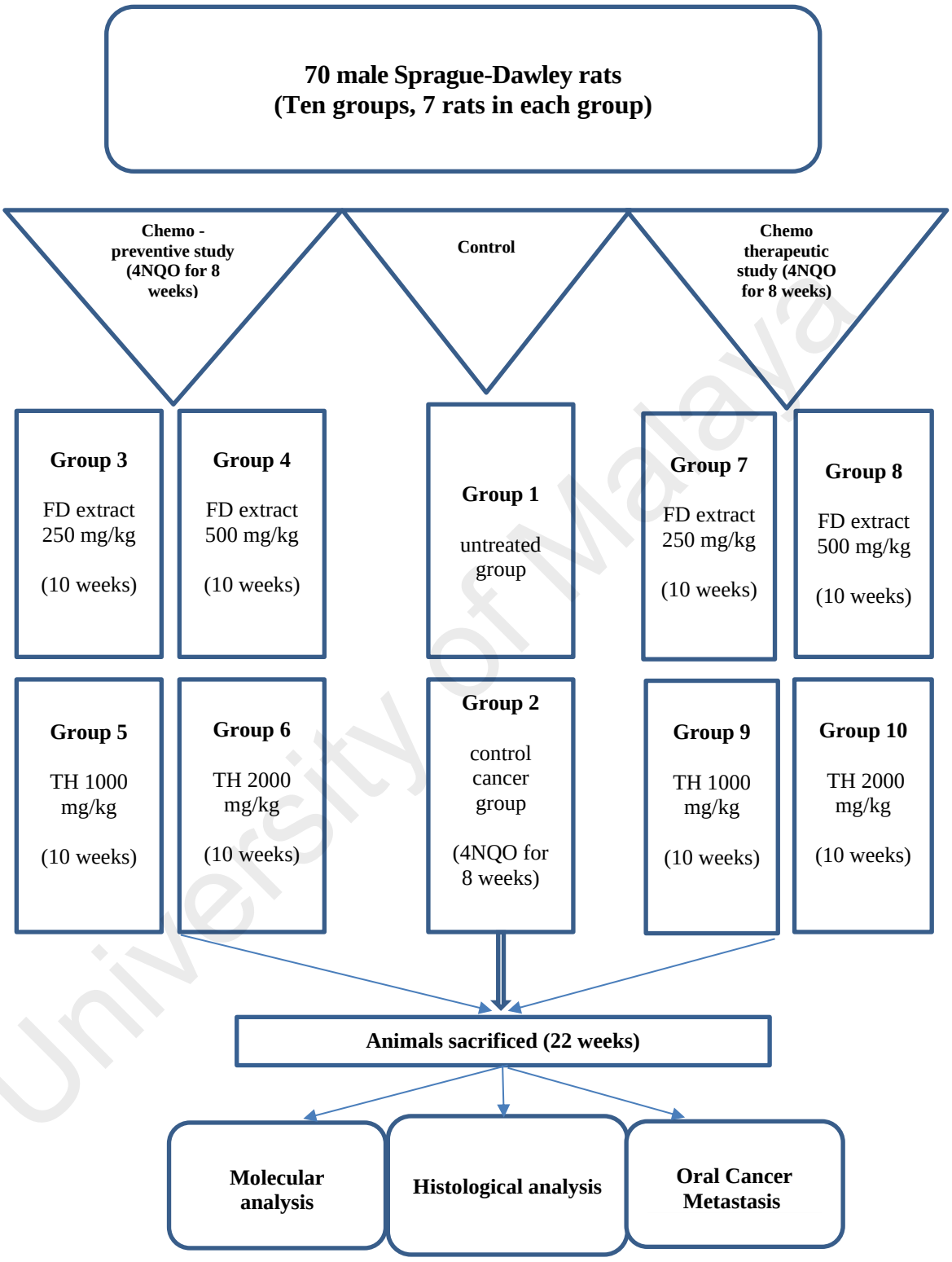


Figure 3.3: Flow chart showing the study design employed in this study

3.2.1.2 Induction of oral carcinogenesis using 4NQO

4-Nitroquinoline-1-oxide (4NQO), a water-soluble quinoline derivative, can produce a spectrum of preneoplastic and neoplastic lesions in the oral cavity, especially on the tongue. Drinking water containing 4NQO was freshly prepared twice a week in RO water and was administered to the rats in light-shielded water bottles (to protect the prepared solution from any unwanted effect of light exposure) at the concentration of 20 ppm for 8 weeks (Kanojia & Vaidya, 2006). The water consumption by each rat was measured and reported before cancer induction (30 ml /day/animal) and during cancer induction each rat was separated in a cage and has given only the same amount of water mixed with 4NQO at 20 ppm without any other source of water to ensure each rat received all and similar amount of 4NQO.

3.2.1.3 Chemo-preventive study

In order to evaluate the effect of the FD extract and TH during the initiation phase of 4NQO-induced oral carcinogenesis, a chemo preventive study was designed in male SD rats. A total of 42 male SD rats were randomised into 6 groups of 7 rats per group. Group 1 was given normal RO water (untreated control) while the remaining 5 groups of rats (Group 2, 3, 4, 5 and 6) were given 4NQO (Sigma Aldrich) solution as drinking water for 8 weeks as previously described (Tanaka et al., 1998; Zhang et al., 2013). The 5 groups also received vehicle (water), FD extract at 250 and 500 mg/kg and TH at 1000 and 2000 mg/kg respectively, daily in a volume of 10 ml/kg body weight, starting one week before the consumption of 4NQO and this was continued for another week when the consumption of 4NQO has completed.

Following this, the animals in all groups were switched back to RO water and this continued until the end of this study at 22 weeks. The oral administrations of the FD extract and TH to the rats were done using gastric intubation by force-feeding using needle size 18 G (Harvard Apparatus, INC).

3.2.1.4 Chemotherapeutic study

This study was designed to evaluate the effect of the FD extract and TH in the post-initiation phase of 4NQO-induced oral carcinogenesis in SD rats. A total of 28 male SD rats from groups 7, 8, 9 and 10 were given 4NQO (Sigma Aldrich) solution as drinking water for 8 weeks. Following this at the 9th week, groups 7, 8, 9 and 10 were administrated with the FD extract at 250 and 500 mg/kg and TH at 1000 and 2000 mg/kg respectively, for another 10 weeks. Oral administration to the rats was carried out using gastric intubation by force-feeding using needle size 18 G (Harvard Apparatus, INC) starting 1 week after cessation of the 4NQO treatment (Tanka et al., 1998; Zhang et al., 2013).

Both of the control groups (Group 1- untreated and Group 2- cancer induced) that were involved in the chemo-preventive study were also used in the chemotherapeutic study since both experiments were carried out at the same time (Figure 3.4). For both chemo-preventive and chemotherapeutic studies, the observation of the overall health and behaviour of the rats was done once a day to identify possible toxicities that may have resulted from the administration of the 4NQO carcinogen. The body weight of the rats were recorded every week during the study. The experiment was terminated on the 22nd week.

General anesthesia was induced through the administration of an intramuscular injection of 50 mg/kg of ketamine 100 mg/ml and 5 mg/kg of xylazine 100 mg/ml. As previously described by Stokes et al. (2009), the rats were sacrificed through cervical dislocation, and was followed by the excision of the whole tongue and vital organs (liver, kidney, lung and lymph nodes).

The tongue was sectioned longitudinally in to two half, one half was fixed in formalin for the histological analysis and the other half was used for the molecular analysis.

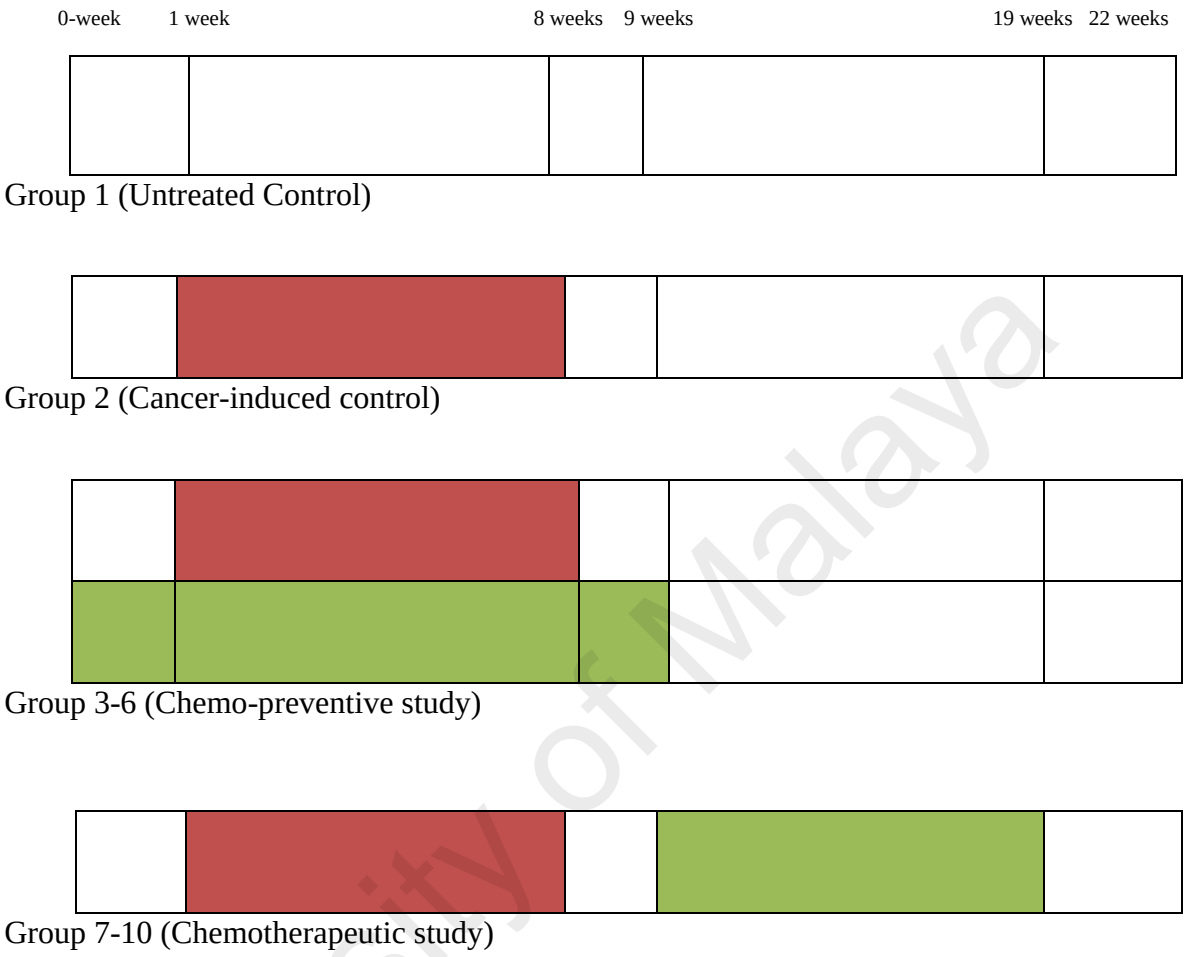


Figure 3.4: Diagrammatic representation of experimental protocol showing the administration of 4NQO, FD extract and TH to SD rats.

3.2.2 Administration dose for 4NQO

The dose of 4NQO used in the present study was 20 ppm and this dose was chosen based on many previous studies as it has been shown that oral cancer was successfully induced in Sprague Dawley rat model when 20 ppm of 4NQO agent dissolved in drinking water was administrated for 8 weeks (Tanaka & Ishigamori, 2011; Kanojia & Vaidya, 2006).

3.2.3 Administration dose for Tualang honey and *Ficus deltoidea*

In this study, the dose of 250 and 500 mg/kg were chosen for the FD extract based on the previous studies. The dose of 2500 mg/kg when used in a sub chronic toxicity study, was discovered to be nontoxic (Farsi et al., 2013; Ilyanie et al., 2011). Thus, we have chosen to use 1/10th of maximum tolerable dose for therapeutic purpose, which were 250 and 500 mg/kg representing the low and higher doses (Chevret, 2014).

The doses of TH administrated to the rats in this study were at 2000 mg/kg and 1000 mg/kg of body weight. The doses were chosen based on a previous study that showed the inhibitory effects of TH on breast cancer in an animal model study (Kadir et al., 2013).

In order to prevent any loss of the antioxidant content, the working dose of the FD extract and TH were freshly prepared every morning through dissolution in deionised water.

3.2.4 Tumour volume

The animal's tongue was examined during and after the experimental period and the tumour volume was recorded in each group. In order to determine if the tumour volume was affected by the TH and FD extract administration, the tumour volume was measured using the formula ($\pi/6$ × width × length × height) as mentioned previously by Wali et al. (2012) in 4NQO cancer-induced rats (Wali et al., 2012).

3.2.5 Histopathological examination

3.2.5.1 Fixation and processing of the samples

The rat organs were placed in a container containing 50 ml of 10% natural buffer formalin (NBF) and were fixed for 24 hours. The ratio of tissue to the fixative volume was 1:50. The samples were processed using a Leica TP 1020 tissue processor. This is an automatic machine where the specimens (organs) undergo a long cycle schedule. The samples were treated through twelve stations containing graded concentrations of ethanol (70 %, 95 %, 100 %, 100 % and 100 %) and xylene, and followed by Paraplast wax (56°C melting point). Finally, the samples were embedded in molten (56°C) paraffin wax. The blocks were stored in an airtight container for future use.

3.2.5.2 Sectioning of samples

A microtome (Leica, Germany), with disposable microtome blade (Leica 818 high profile disposable microtome blade) was used in sectioning the tissues. A ribbon of sections, four μm thin, were floated on a water bath (45°C). The forceps was then used for placing a section onto a glass slide and was followed by a drop of 20 % alcohol.

3.2.5.3 Staining of samples

The fixed sections were then placed in fresh xylene bath and were incubated at room temperature for 5 minutes. The previous step was repeated with fresh xylene for another 4 minutes. The excess liquid was drained, and the slides were placed in fresh absolute ethyl alcohol bath followed by 95 %, 70 % alcohol bath for 3 minutes and then in running water bath for 3 minutes at room temperature. The sections were stained with Harris' hematoxylin working solution for 5 minutes.

Following this, the sections were washed in slow running tap water for 3 minutes to remove the excess blue stain. The sections were then dipped in 0.5% acid alcohol and were washed well in running tap water for 3 minutes. The sections were then dipped in 2% sodium acetate (4 dips) and were washed well in running tap water (3 minutes).

The slides were then placed in 80 % alcohol bath for 1 minute, and following this, the sections were stained with eosin working solution for 2-3 minutes. The slides were then placed in 95 % alcohol bath for four dips, which was then followed by 100% alcohol bath twice for 2 minutes. The sections were then placed in xylene I, II and III baths for 3 minutes and finally, the stained tissue sections were mounted and cover slipped with DPX mounting medium and checked under the microscope. The hematoxylin and eosin stained slides were then stored for microscopic examination.

The tissue sections were examined by a pathologist who was not aware of the treatment received by the rats. The tongue sections were analyzed and graded as normal, hyperplasia, dysplasia or squamous cell carcinoma per animal as described by Ribeiro et al. (2007) and El-Rouby (2011).

3.2.6 Histological evaluation of oral cancer metastasis

In this study, the cervical lymph nodes, kidneys, liver and lungs of all animals of each group were excised at the time of necropsy when the animals were sacrificed and submitted for histological evaluation. All specimens were fixed in 10% formalin, embedded in paraffin and the serial sections were cut and stained with hematoxylin and eosin as explained in section 3.2.5 and were examined in detail by a qualified pathologist to identify foci of the metastasis.

3.2.7 Immunohistochemical evaluation

Immunohistochemical evaluation was conducted in the present study to determine the effect of the FD extract and TH on the expression of tumour markers; cyclin D1, bcl2, p53, β- catenin and e-cadherin in the tongue tissue sections of the Sprague Dawely rats. Formalin-fixed, paraffin-embedded tissue sections (4 μm) were cut from the paraffin blocks and deparaffinized and rehydrated. In order to recover antigen reactivity from the formalin fixed paraffin embedded tissue, the heat induced epitope antigen retrieval (HIER) method was applied according to the manufacturer's guideline. The cell conditioner solution (CC1) (Roche, Ventana Medical), was then applied at 95°C for 44 minutes. For blocking endogenous peroxides and protein, ChromoMap inhibitor (CM) (Roche, Ventana Medical) was used at 37°C for 4 minutes. The antibodies were then applied according to the manufacturer's protocol at the pathology laboratory, Department of Pathology, Faculty of Medicine, University of Malaya. Immunohistochemistry was carried out using Ventana Benchmark XT autostainer (Ventana Medical Systems Inc., Tucson, Arizona) with the following mice monoclonal antibodies; p53 (clone DO-7, Dako Japan), e-cadherin (clone NCH-38, Dako Japan), β-catenin (clone b-Catenin-1, Dako Japan), bcl2 (clone 124, Dako Japan) and cyclin D1 (clone SP4, Thermo Fisher Scientific).

Quality controls such as negative control (rat tongue specimen that is not exposed to the primary antibody) have been performed to identify any non-specific staining or false positive results. A positive control (human breast cancer tissue) that is known to express the protein of interest was also included in this study for validation of the procedure and to verify the negative results.

The IHC staining was carried out based on the automated process for routine staining at the Department of Pathology, Faculty of Medicine, University of Malaya.

3.2.7.1 Digital image analysis of immunohistochemical expression

Verification of the immunohistochemical reaction of cyclin D1, p53, β-catenin, e-cadherin and bcl2, was performed using a light microscope and scanned using a digital slide scanner (3DHISTECH Ltd., Budapest, Hungary).

Histological slides obtained from five rats from each animal group were analysed using IHC. For each slide, 5 pictures were taken from different regions of the slide as representing the whole slide area. The 5 picture consists of one picture from the middle and four pictures from all the four corners of the slide.

The results were then assessed using computer-assisted image analysis (ImageJ; National Institutes of Health, Bethesda, MD, USA). The image threshold was adjusted using the automated color detection and semi-automatic color selection of the computer-assisted image analysis system, until all the stained areas were selected and assessed and the results obtained were displayed with the assistance of a histogram display. The threshold setting that was used to detect and quantify the staining was then duplicated for every image for comparison purpose as described previously (Jayash et al., 2017; Jensen, 2013). Finally, the mean and standard deviation were calculated for each sample.

3.2.8 Real Time PCR

3.2.8.1 RNA Extraction

The RNA extraction from the tongue samples was carried out using the RNeasy Plus Universal Mini Kit (Qiagen, USA). Thirty mg of tongue tissue that was preserved in half ml of RNAlater solution (Qiagen, USA) for 16 hours at 4ºC then transferred to -80ºC. The tissue when thawed was homogenised in QIAzol Lysis Reagent using tissue ruptor. The lysate was then centrifuged at 12000 rpm. The supernatant was transferred into a new 1.5 ml tube and one volume of 70% ethanol was added. This sample was then transferred into an RNeasy spin column, centrifuged and the flow-through discarded. Subsequently, buffer RPE was added to the RNeasy spin column, centrifuged for one minute at 10000 rpm and the flow-through was again discarded. This step was repeated but the centrifugation step was carried out for two minutes.

Finally, the spin column was placed in a new 1.5 ml tube, added with 30 µl of RNase-free water and centrifuged at 10000 rpm for one minute to elute the RNA. The concentration and purity of the RNA samples were done twice for each sample using Nano Drop (BioTek, USA) at A260:A280 ratio of absorbance. An average of the two readings were then averaged for each sample.

The quality and purity of the RNA samples were found to be within the range of 1.8 to 2.0 of absorbance ratio. The total RNA yields for all samples were calculated manually based on the concentration and volume of the RNA obtained. The RNA integrity was accessed using the Agilent Bioanalyzer-2100 (Agilent Technologies, CA, USA) to determine the quality of the extracted RNA. The Agilent Bioanalyzer-2100 software (Agilent Technologies, CA, USA) can detect the ratio of the 18S and 28S ribosomal RNA (rRNA) and generate the RNA integrity

number (RIN). This RIN is classified from 1 to 10, with 1 indicating the most degraded form of RNA and 10 the most intact. RNA integrity number ≥ 7 is generally considered as sufficient. All RNA samples were kept at -80ºC until further analysis.

3.2.8.2 Reverse transcription of RNA to cDNA

The RT^2 first strand kit was used for cDNA synthesis according to the manufacturer's guidelines (Qiagen). Briefly, a reverse transcription mixture was prepared by adding 4 µl of Buffer BC3 x 5, 1 µl of Control P2, 3 µl of RNase free water, and 2 µl of reverse transcriptase RE3 mix. This reverse transcription mixture (10 µl) was then added to the genomic elimination mixture (10 µl) (Table 3.1), and was mixed by pipetting, and incubated for 15 minutes at 42 ºC. The reaction was immediately stopped by incubating at 95 ºC for 5 minutes. To each reaction mixture, 91 µl of RNase-free water was added and mixed by pipetting for several times and stored at -80 ºC prior to RT² Profiler PCR Array.

Table 3.1: Genomic DNA elimination mix

Component	Amount
Total RNA	25.0 ng to 5.0 µg
GE (5X gDNA Elimination Buffer)	2.0 µl
RNase free H2O to a final volume of	10 µl

3.2.8.3 RT^2 Profiler PCR Array

The master mixture (675 µl) was prepared by mixing 337.5 µl RT^2 SYBR Green/ROX qPCR Master Mix (Qiagen) with 25.5 µl of 1 µg of cDNA synthesised from each sample. Following this, 25 µl of the aliquot mixture was loaded onto each well of a pre-designed 96-well RT² Profiler PCR Array (Qiagen) customised for 4 samples. Each well from the array plate was pre-hybridised with primer pairs for each gene as listed in Table 3.2.

The housekeeping genes, Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and Hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) were used as the internal control genes. Thermal cycling was performed using ABI-7500 (Applied Biosystems, Step One Plus Real-Time PCR System, USA) with an initial denaturation at 95 °C for 10 minutes, 40 cycles at 95 °C for 15 seconds, and 60 °C for 1 minute. A signal was acquired at 60°C during each cycle. The mRNA level for the housekeeping genes *GAPDH* and *HPRT1* were used to normalise the gene expression data. Each array contains proprietary controls to monitor genomic DNA contamination, positive PCR controls, and reverse transcription in each 96-well set on each plate.

The data were analysed using the RT² Profiler PCR Array data analysis software (https://ic.chat/rt2-pcr-array-data-analysis-center). The values of the cycle threshold (Ct) were used for the calculation of fold changes in mRNA abundance using 2-ΔΔCt method. The cut-off value of fold change 1 was considered for the gene expression analysis.

Table 3.2: List of the selected genes analysed for RT^2 Profiler PCR Array

Symbol	Description
CCND1	cyclin D1
EGFR	Epidermal growth factor receptor
PTGS2 (COX2)	Prostaglandin-endoperoxide synthase 2
RAC1	Ras-related C3 botulinum toxin substrate 1
TP53	Tumour protein p53
TWIST1	Twist homolog 1 (Drosophila)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPRT1	Hypoxanthine phosphoribosyltransferase 1

3.2.9 Reliability of histopathological evaluation on the incidence of pre-neoplasm and neoplasms in the tongue of the rats

Intra-examiner reliability was carried out by re-evaluating the same samples twice by the same examiner with one-week interval and without the knowledge of the previous reading. Cohens Kappa was carried out to determine if there was an agreement between the two readings obtained for the incidence of OSCC. There was a notable agreement between the two readings for both studies, chemo-preventive (K= 0.678, p<0.001) and chemotherapeutic (K= 0.476, p<0.001) as shown in Table 3.3 and Table 3.4 respectively.

Table 3. 3: Reliability test (Kappa test) for chemo-preventive study

Symmetric Measures					
		Value	**Asymp. Std. Error[a]**	**Approx. T[b]**	**Approx. Sig.**
Measure of Agreement	Kappa	.678	.101	5.439	<0.001
N of Valid Cases		35			

Table 3. 4: Reliability test (Kappa test) for chemotherapeutic study

Symmetric Measures					
		Value	**Asymp. Std. Error[a]**	**Approx. T[b]**	**Approx. Sig.**
Measure of Agreement	Kappa	.476	.116	4.059	<0.001
N of Valid Cases		35			

3.2.10 Statistical analysis

The data were recorded and analysed using the SPSS version 20. All assays were conducted in at least five separate experiments. The quantitative results were expressed as the mean ± SD. Data representing the IHC analysis, body weight and tumour volume, were compared using the One-way ANOVA test with post-hoc comparisons made using the Dunnetts test. The histological qualitative data were analysed using the chi-square statistical test.

The data obtained using the RT^2 Profiler PCR Array were analysed using the Gene Globe Data Analysis Center on QIAGEN's website (http://www.qiagen.com/my/shop/genes-and-pathways/data-analysis-center-overview-page/). The results were considered statistically significant at p-value < 0.05.

CHAPTER 4: RESULTS

4.1 Effect of FD extract and TH on body weight

The mean body weight for both study groups (chemo-preventive and chemotherapeutic) is shown in Figures 4.1 and 4.2 respectively. Although the rats showed signs of weight loss during the study, the weight loss was only significant ($p<0.05$) for both groups from week 17 until the end of the study period. It was observed that, at the end of the study (22 weeks), a statistically significant difference in the mean body weight was seen between the normal untreated group and the cancer-induced group using 4NQO (Table 4.1 and Table 4.2) ($p<0.05$).

In the chemo-preventive study, except for the administration of the FD extract at 250 mg/kg, a statistically significant difference was observed in the body weight for FD extract 500, TH 1000 and TH 2000 mg/kg groups when compared to the 4NQO group, ($p<0.05$). However, no significant differences were found between the 4NQO control group and all the groups of the chemotherapeutic study (Table 4.2).

Table 4.1: Mean body weights of rats during 4NQO-induced oral carcinogenesis in a chemo-preventive study. (A post hoc Dunnett test)

Groups N=7	**Untreated group (normal control)**	**4NQO group (cancer control)**	**Chemo-preventive groups**			
			FD 500 mg/kg	FD 250 mg/kg	TH 1000 mg/kg	TH 2000 mg/kg
Body weight (g)	*551±17	395±00	*479±00	455±00	*487±67	*474±17

*The mean difference is significant at the 0.05 level compared to the cancer control group

Table 4.2: Mean body weights of rats during 4NQO-induced oral carcinogenesis in a chemotherapeutic study. (A post hoc Dunnett test)

Groups N=7	**Untreated group (normal control)**	**4NQO group (cancer control)**	**Chemotherapeutic groups**			
			FD 500 mg/kg	FD 250 mg/kg	TH 1000 mg/kg	TH 2000 mg/kg
Body weight (g)	*551±17	395±00	440±00	405±00	421±67	396±67

*The mean difference is significant at the 0.05 level compared to the cancer control group

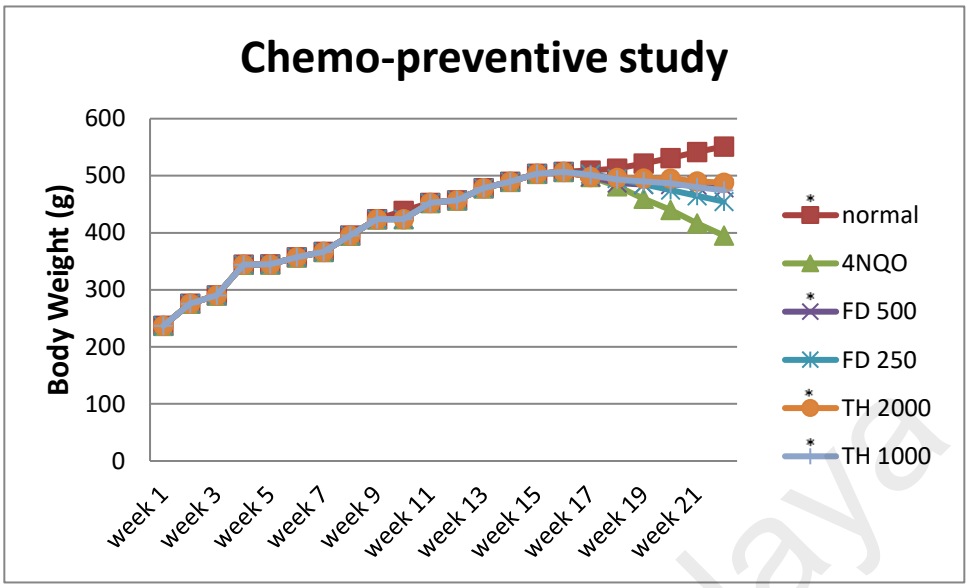


Figure 4. 1: Mean body weights of rats during 4NQO-induced oral carcinogenesis in a chemo-preventive study. *p value less than 0.05, ($p < 0.05$) significant value comparing to 4NQO control group.

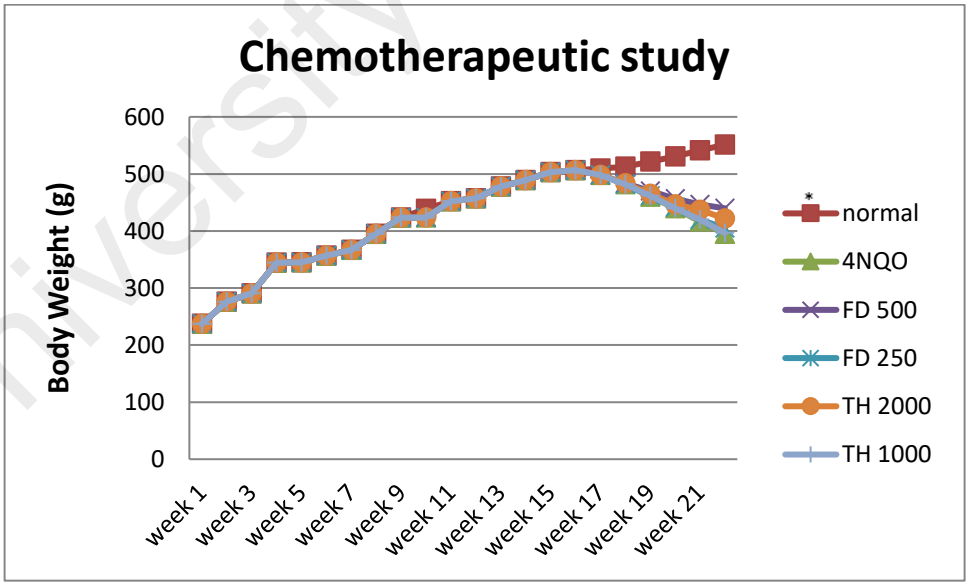


Figure 4.2: Mean body weights of rats during 4NQO-induced oral carcinogenesis in a chemotherapeutic study. *p value less than 0.05, ($p < 0.05$) significant value comparing to 4NQO control group.

4.2 Effect of FD extract and TH on tumour volume

It was observed that most of the lesions were located in the posterior third of the tongue, except for one rat in the 4NQO control group where the lesions were localised on both the anterior and posterior third of the tongue (Figure 4.3). TH and the FD extract treated groups in the chemo-preventive study showed a significant decrease ($p<0.05$) in the tongue tumour volume when compared to group 2 that was induced by 4NQO for oral carcinogenesis (Table 4.3). However, in the chemotherapeutic study, there was no significant difference between the FD extract and TH treated groups when compared to the cancer control group (Table 4.4).

Table 4.3: Effect of FD extract and TH on tongue tumour volume in 4NQO-induced oral cancer rats (chemo-preventive study).

Groups	**Tongue Tumour volume (mm^3) (Mean± SD)**	***P* value**
Cancer control group	87.03±4.73	--
TH 1000 mg/kg	58.44±5.96	0.016*
TH 2000 mg/kg	42.29±12.87	<0.001 *
FD 250 mg/kg	60.91±3.52	0.023*
FD 500 mg/kg	40.69±12.26	<0.001 *

**p* value less than 0.05, ($p< 0.05$) significant (A post hoc Dunnets test)

Table 4.4: Effect of FD extract and TH on tongue tumour volume in 4NQO-induced oral cancer rats (chemotherapeutic study).

Groups	**Tongue Tumour volume (mm^3) (Mean± SD)**	***P* value**
Cancer control group	87.03±4.73	--
TH 1000 mg/kg	63.22±13.6	0.062
TH 2000 mg/kg	76.28±29.2	0.166
FD 250 mg/kg	69.91±1.80	0.170
FD 500 mg/kg	68.21±32.46	0.114

**p* value less than 0.05, ($p < 0.05$) significant (A post hoc Dunnets test)

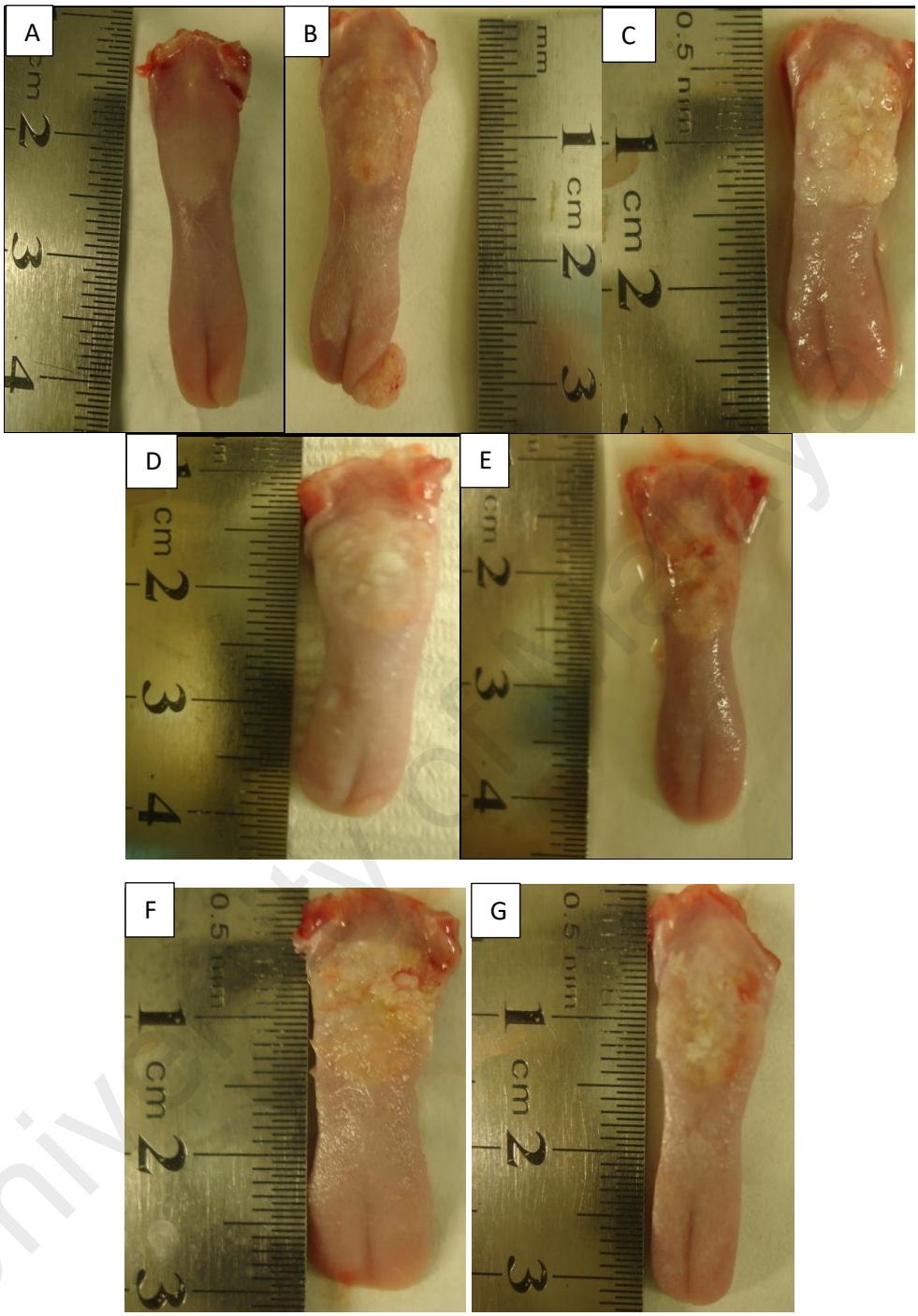


Figure 4.3: Macroscopic photos of rats tongue obtained from an animal model study for oral carcinogenesis. **A** - normal rat tongue. **B** and **C** – rat tongue induced for oral cancer using 4NQO (control cancer group). Rats treated with TH 2000 mg/kg (**D**) and FD 500 mg/kg (**E**) in a chemo-preventive study. Rats treated with TH 2000 mg/kg (**F**) and FD 500 mg/kg (**G**) in a chemotherapeutic study.

4.3 Histological observation of rat's tongue induced for oral cancer using 4NQO and treated with FD extract and TH

Histological evaluations were performed blindly with light microscopy by a qualified pathologist. The tongue tissue sections were assessed and graded as normal, hyperplasia, dysplasia or squamous cell carcinoma per animal.

The rat's tongue obtained from the normal untreated group (Group 1) had the histological features of normal oral mucosa with keratinized stratified squamous epithelium. The architecture of the epithelium was normal with the presence of papillae with connective tissue and skeletal muscle beneath the mucosa (Figure 4.4).

The detection of OSCC was made through the presence of the submucosal invasion of the epithelial tumour cells as shown by the presence of certain features such as islands, nests and sheets, and the discontinuation of the basement membrane. Altered nucleus in the form of cytoplasmic ratio and the presence of keratin pearls in the connective tissue were shown in the epithelial tumour cells, together with cellular and nuclear pleomorphism and hyperchromatic nuclei.

Histopathological changes such as hyperplasia and dysplasia were observed in rats that were subjected to drinking water which contained 20 ppm of 4NQO. Hyperplasia, with a clearly-defined basement membrane, was also seen in some samples in groups that were treated with TH and FD extract (Groups 3 to 10).

Dysplastic histological changes include hyperplasia of the stratum spinosum, basal cell hyperplasia, superficial mitosis, increased number and size of nucleoli with the level of atypia presence in the lower, middle or upper third of the epithelium stratification. It was also observed that irregular epithelial stratification was evident at the base of the epithelium of rats' tongues treated with the FD extract or TH (Groups 3 to 10) (Figure 4.5).

4.4 Incidence of OSCC and pre-cancerous lesions of rat's tongue induced for oral cancer using 4NQO and treated with FD extract and TH

In the 4NQO-induced cancer group, the incidence of OSCC was 85.7%. Following treatment with the FD extract at 250 and 500 mg/kg, and TH at 1000 and 2000 mg/kg, for the chemo-preventive study, the OSCC incidence were reduced significantly ($p<0.05$) at 42.9%, 14.3%, 28.6% and 14.3% respectively (Table 4.5).

The diagnosis of each tongue sample was based on the final histopathological changes. For each rat specimen that was diagnosed as having OSCC, the presence of hyperplasia and dysplasia lesions could still be detected in some parts of the same tongue.

The dysplastic lesions that developed from the chemo-preventive groups were observed to be 14.6% in the 4NQO cancer group, while the FD extract groups (treated with 250 and 500 mg/kg) were 42.9 % and 28.6%, respectively. Hyperplasia incidence in the FD extract groups treated at 250, 500 mg/kg and TH at 1000, 2000 mg/kg were 14.3%, 57.1% 14.3% and 57.1% respectively (Table 4.5).

In the chemotherapeutic groups, the incidence of OSCC in rats treated with FD extract at 250 and 500 mg/kg and TH at 1000 and 2000 mg/kg were 28.6 %, 14.3 %, 42.9 % and 28.6 % respectively. The incidence of dysplasia lesions was 14.3% in the control cancer group of 4NQO, while in the FD extract group treated with 250 and 500 mg/kg and TH with 1000, 2000 mg/kg were 71.4 %, 85.7 %, 57.1% and 71.4% and 57.1% respectively. Hyperplasia lesions in the FD, TH and 4NQO groups were 0% (Table 4.6).

Generally, it is observed that the administration of FD extract and TH especially at the highest dose (500 mg/kg and 2000 mg/kg respectively) had reduced the incidence of hyperplasia, dysplasia, and squamous cell carcinoma significantly in both chemo-preventive study ($p = 0.03$) and chemotherapeutic study ($p = 0.04$).

Table 4.5: Incidence of OSCC and pre-cancerous lesions of rat's tongue treated with FD extract and TH following induction of oral cancer using 4NQO (A chemo-preventive study)

Group	**No. of animals n (%)**	**Hyperplasia n (%)**	**Dysplasia n (%)**	**OSCC n (%)**	***P* value**
4NQO control	**7** (100)	**0** (0.0)	**1** (14.3)	**6** (85.7)	0.03*
FD 250 mg/kg	**7** (100)	**1** (14.3)	**3** (42.9)	**3** (42.9)	
FD 500 mg/kg	**7** (100)	**4** (57.1)	**2** (28.6)	**1** (14.3)	
TH 1000 mg/kg	**7** (100)	**1** (14.3)	**4** (57.1)	**2** (28.6)	
TH 2000 mg/kg	**7** (100)	**4** (57.1)	**2** (28.6)	**1** (14.3)	

* p value less than 0.05, ($p < 0.05$) significant value compared to control group (chi-square test)

Table 4.6: Incidence of OSCC and pre-cancerous lesions of rat's tongue treated with FD extract and TH following induction of oral cancer using 4NQO (A chemotherapeutic study)

Group	No. of animals n (%)	Hyperplasia n (%)	Dysplasia n (%)	OSCC n (%)	*P* value
4NQO control	**7** (100)	**0** (0.0)	**1** (14.3)	**6** (85.7)	0.04*
FD 250 mg/kg	**7** (100)	**0** (0.0)	**5** (71.4)	**2** (28.6)	
FD 500 mg/kg	**7** (100)	**0** (0.0)	**6** (85.7)	**1** (14.3)	
TH 1000 mg/kg	**7** (100)	**0** (0.0)	**4** (57.1)	**3** (42.9)	
TH 2000 mg/kg	**7** (100)	**0** (0.0)	**5** (71.4)	**2** (28.6)	

* *p* value less than 0.05, ($p < 0.05$) significant value compared to control group (chi-square test)

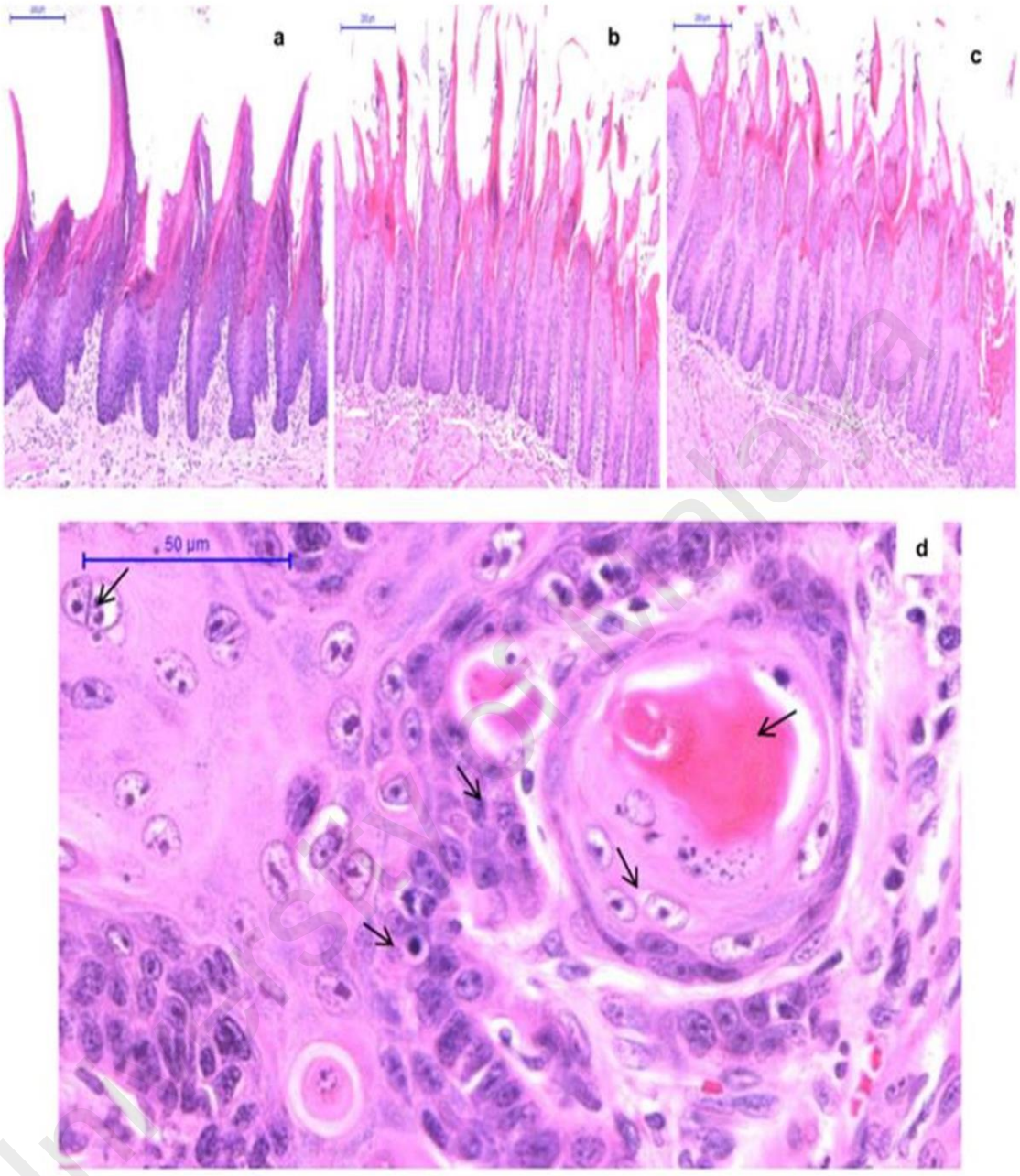


Figure 4.4: Histopathological analysis of rat's tongue stained with H & E. Rat's tongue obtained from the normal control group (a), FD extract 500 mg/kg (b) (200 µm), TH 2000 mg/kg (c) (scale bar, 200 µm). Tongue specimens of the rats induced with 4NQO showing atypical mitoses, hyperchromatic nucleus, pleomorphic cells, polygonal cells and keratin pearls (d) (scale bar, 50 µm).

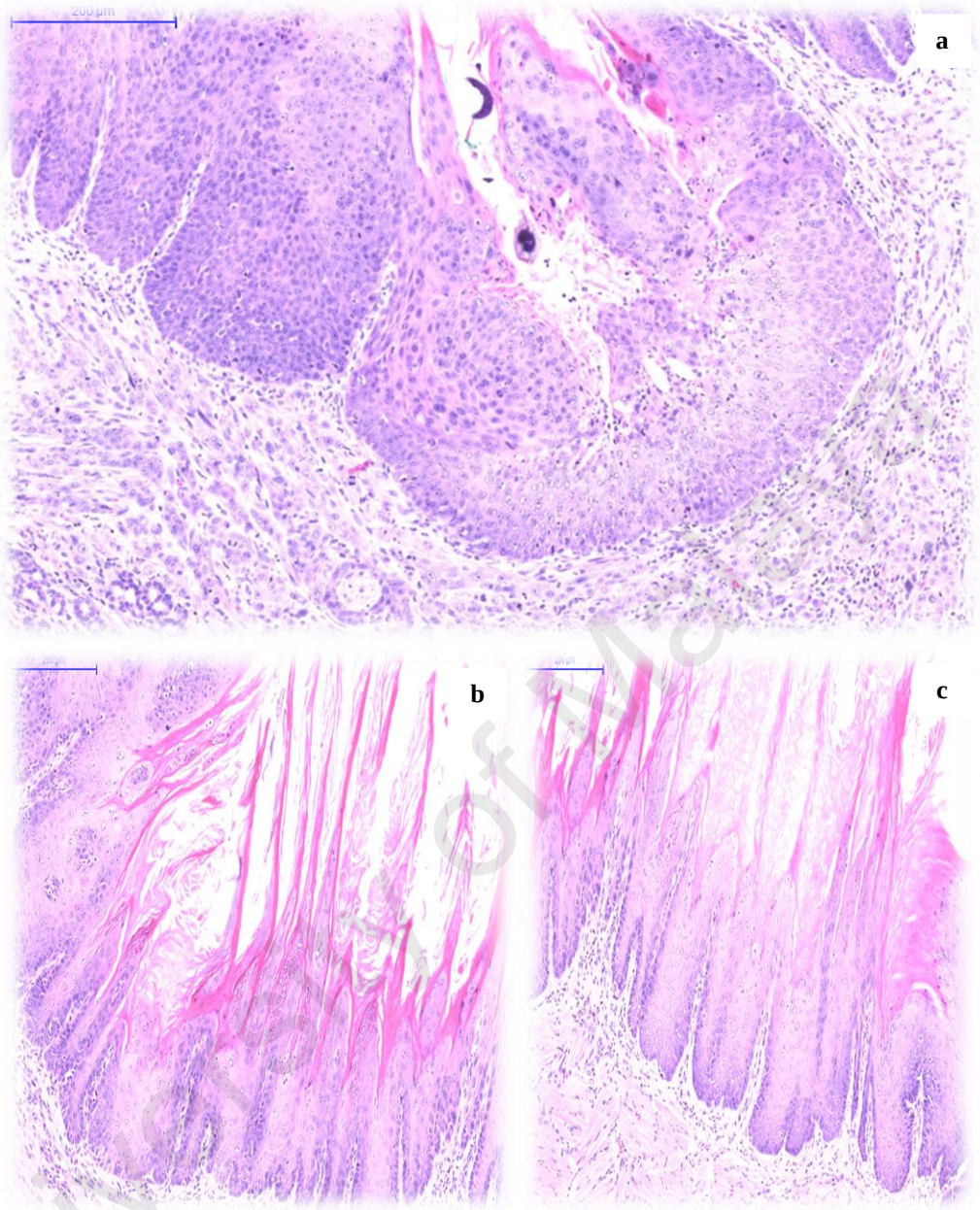
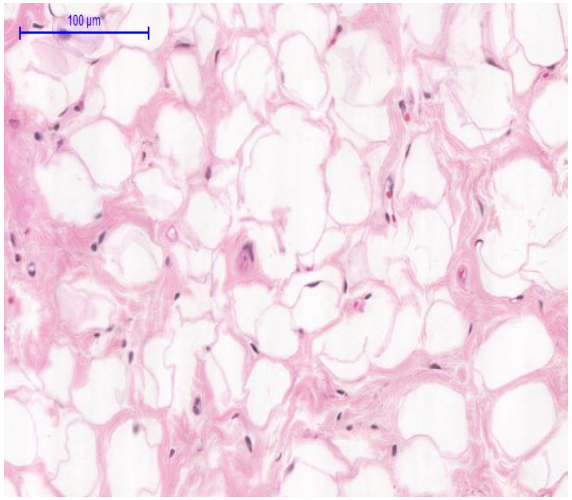
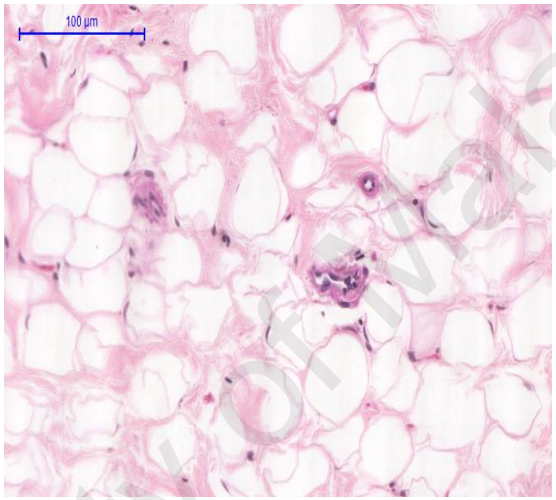
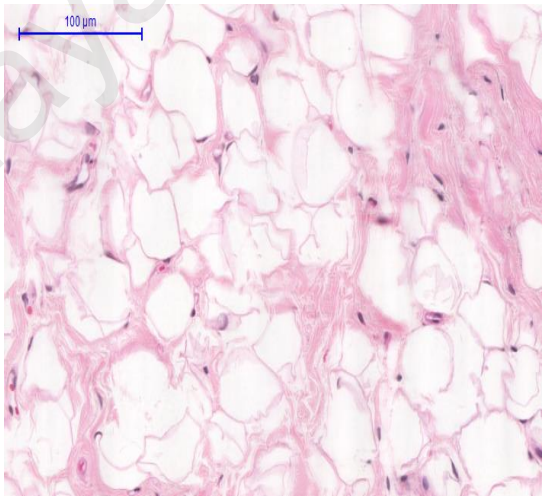
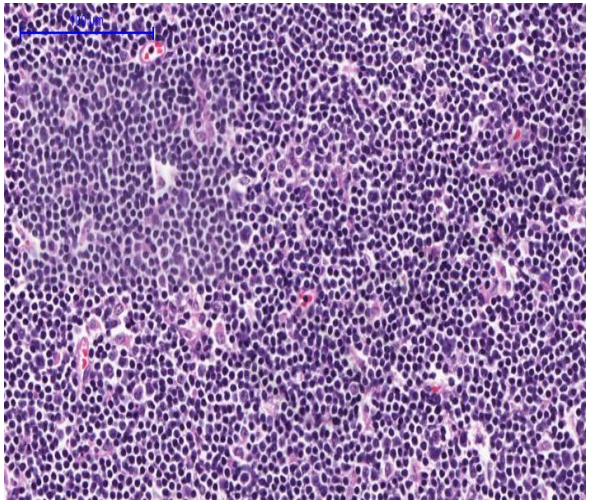
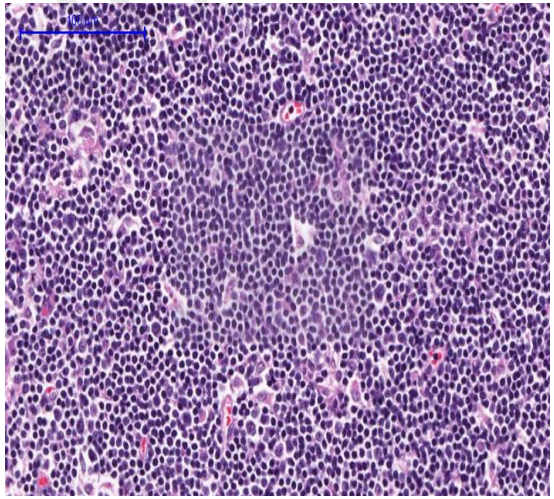
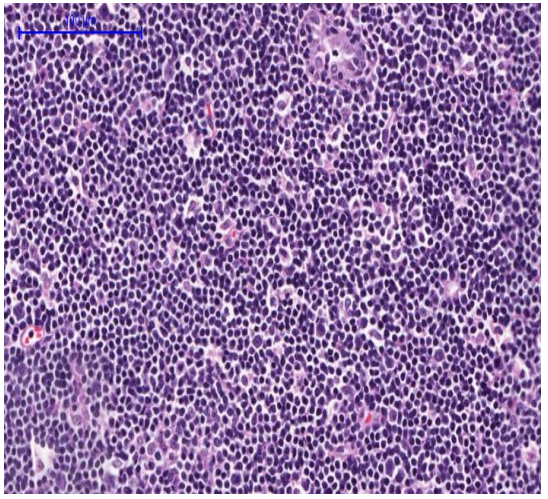


Figure 4.5: Histopathological analysis of rat's tongue obtained from a chemotherapeutic study. Rat's tongue following cancer induction with 4NQO (a), and treated with FD extract 500 mg/kg (b), and TH 2000 mg/kg (c) (scale bar, 200 µm).

4.5 Histological findings of oral cancer metastasis

Histological findings indicated that the cancer have not metastasised from the oral cavity, and that all histological features of the lymph nodes and vital organs (lung, liver and kidney) were found to be normal in both the treated groups and the cancer control group (Figure 4.6).

	Control 4NQO group	FD extract treated group	TH treated group
Lung			
Lymph Node			

Liver

Kidney

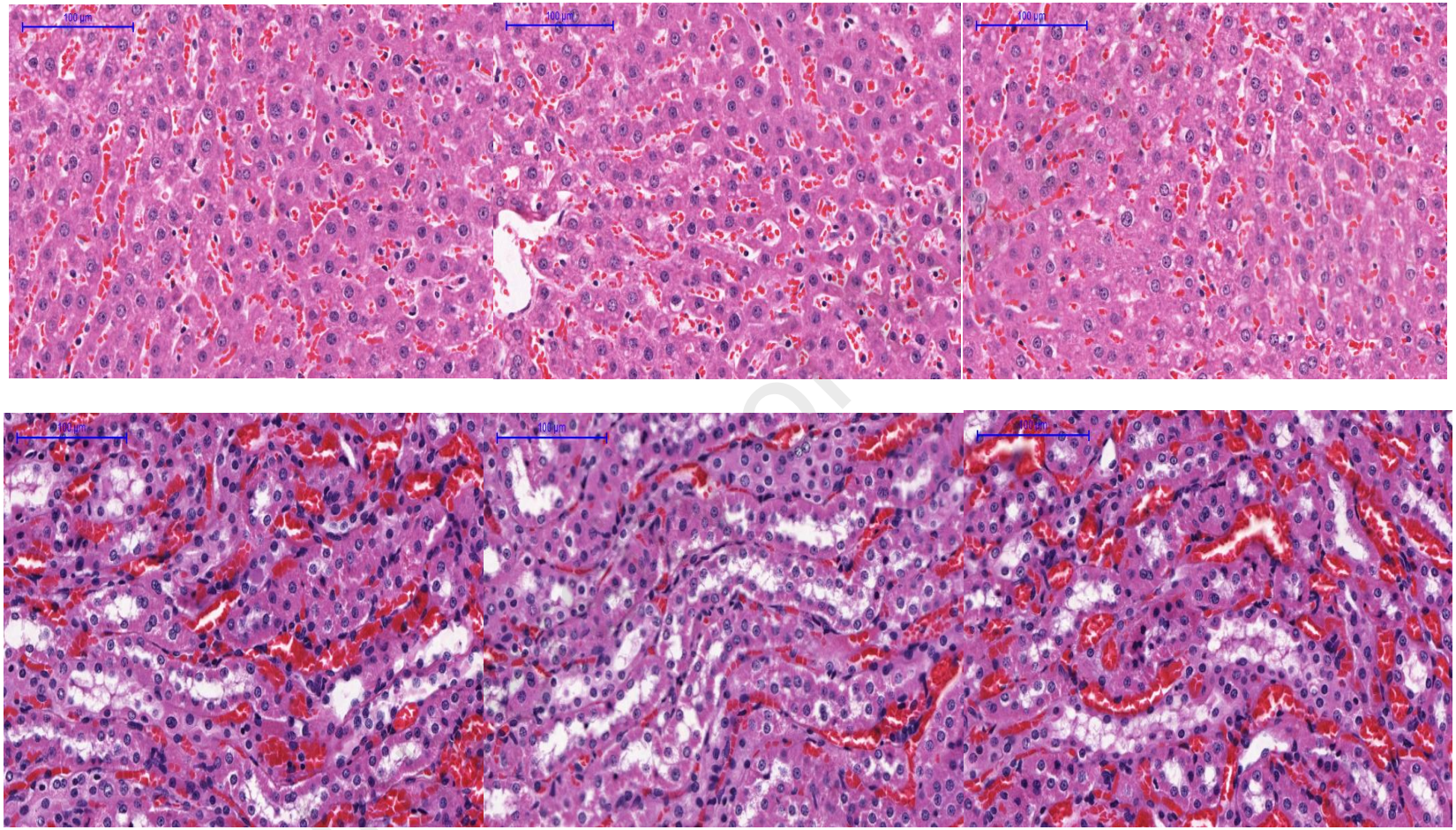


Figure 4.6: Photomicrograph of H&E obtained sections of vital organs (lung, lymph nodes, liver and kidney) of rats from the 4NQO control group and FD extract and TH treated groups. (scale bar, 100 µm)

4.6 Immunohistochemical evaluation

The IHC method allows for the detection of a specific antigen (target protein) in the cells of a tissue section. The location and expression of the antigen is based on the principle of specific binding of an antibody to its antigens in the biological tissues.

The basic protocol involves for the application of a primary antibody that is directed against a specific tissue antigen (target protein) (Figure 4.7). Following this, amplification of a signal will occur when a secondary antibody is localised to the primary antibody. A horseradish peroxidase enzyme (HRPO) molecule will be conjugated to the secondary antibody (labelled secondary antibody) producing brownish stains when diaminobenzidine (DAB) chromogen reacts with the HRPO.

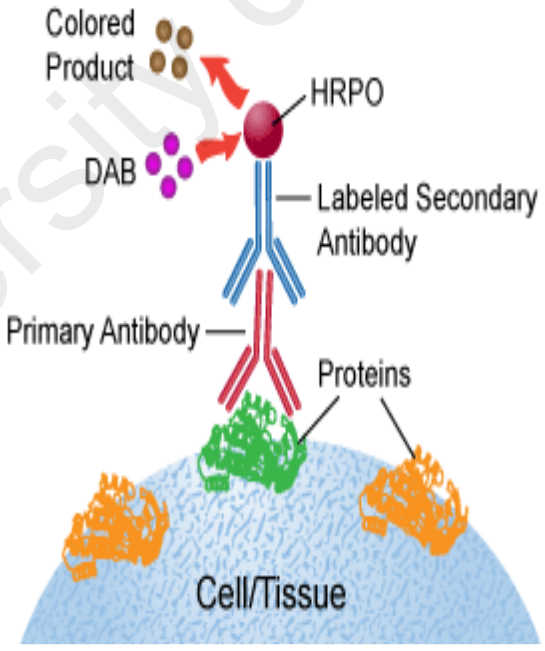


Figure 4.7: Schematic diagram showing the immunohistochemistry method for the detection of a specific antigen (www.leinco.com)

4.6.1 Immunohistochemical analysis of cyclin D1 (cell-cycle protein)

There was an increase in the cyclin D1 expression in the induced cancer group of 4NQO (Group 2), where the cyclin D1 expression was found in the parabasal and basal compartments of the keratinised stratified squamous epithelium. This then extended into the epithelial tumour cells that had invaded the underlying connective tissues (A1 of Figures 4.8, 4.10 and 4.11). Inversely, administration of the FD extract and TH at high doses in both the chemo-preventive and chemotherapeutic groups decreased the cyclin D1 expression (B1 and C1 of Figures 4.8, 4.10 and 4.11) which was restricted to the basal compartment of the keratinised stratified squamous epithelium.

4.6.2 Immunohistochemical analysis of bcl2 and p53 (apoptotic proteins)

For the bcl2 protein expression, very few cells had stained positively in the control cancer group of 4NQO, and when compared to the FD extract and TH treated groups, there was almost no staining present (Figure 4.10 and 4.11 (A2, B2 and C2)).

A characteristic cellular localisation was exhibited by the p53 protein expression. In the induced cancer group of 4NQO (Group 2), many of the cells which were positively stained for p53 were found in the basal and parabasal layers of the epithelium (Group 2). As shown in Figure 4.10 and 4.11 (A3), these cells then extended into the epithelial tumour cells that had invaded into the connective tissues (Figure 4.8). When compared to the FD extract and TH-treated groups for both chemo-preventive study and chemotherapeutic studies, very few cells had stained positively for p53 (Figure 4.10 and 4.11 (B3 and C3)).

4.6.3 Immunohistochemical analysis of β-catenin and e-cadherin (cell adhesion proteins)

A membranous pattern of the β-catenin and e-cadherin protein expressions with a strong positive expression in the parabasal layer was detected while a weak expression in most superficial layers was shown in the chemo-preventive study groups (B4 and C4 for β-catenin expression in Figures 4.10 and 4.11, and B5 and C5 for e-cadherin expression in Figures 4.10 and 4.11). The immunostaining was principally limited to the basal and spinous layers, as opposed to the induced cancer group (Group 2) where the β-catenin and e-cadherin protein expressions exhibited a partially absent membranous staining with an altered distribution in the cytoplasm with the presence of keratin pearl nest of the tumour as illustrated in Figures 4.10 and 4.11 (A4 and A5).

Based on the parametric one-way ANOVA, there was a significant difference ($p<0.05$) in the mean percentage of cyclin D1, β-catenin and e-cadherin staining in the FD extract and TH treated groups, for both the chemo-preventive and chemotherapeutic studies compared to the cancer control group (Table 4.7).

However, the bcl2 and p53 expressions showed a non-significant expression in the TH and FD extract treated groups for both chemo-preventive and chemotherapeutic studies when compared with the control cancer group (Table 4.7). A post hoc Dunnett test was used to detect the differences between the groups as presented in Table 4.8 (Figure 4.9).

Table 4.7: Immunohistochemical evaluation of the effect of FD extract and TH on rats induced for oral cancer with 4NQO (one-way ANOVA).

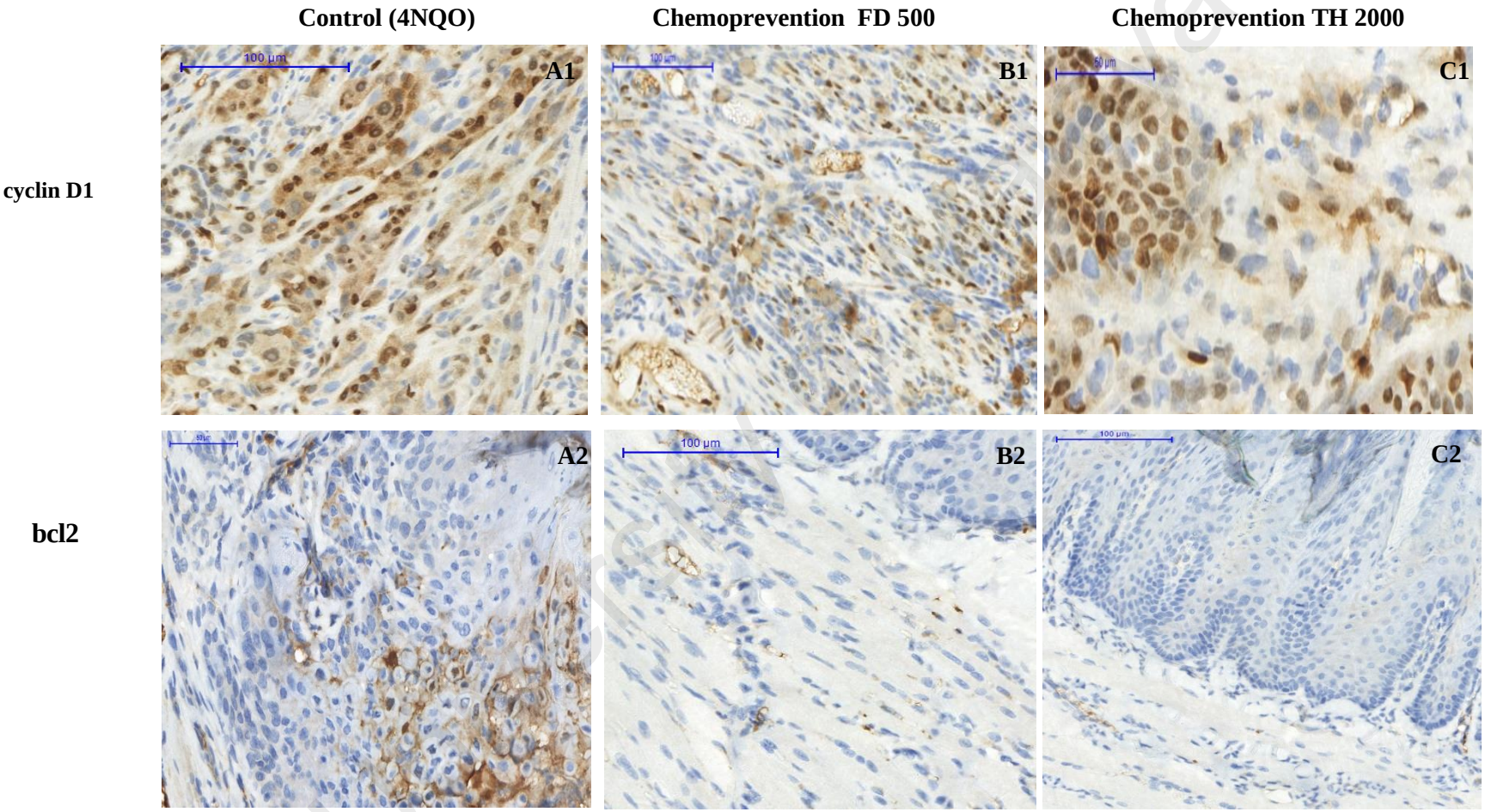
IHC Stain	Groups	Mean± SD	*P* value
cyclin D1	4NQO control	48.54±13.4	.000*
	FD prevention	7.556±8.1	
	FD therapeutic	26.44±8.7	
	TH prevention	19.608±10.0	
	TH therapeutic	28.334±10.0	
bcl2	4NQO control	6.113±1.1	.579
	FD prevention	3.8664±0.9	
	FD therapeutic	6.113±4.1	
	TH prevention	4.0664±10.9	
	TH therapeutic	3.8664±9.9	
e-cadherin	4NQO control	13.9482±6.15	.005*
	FD prevention	30.5272±4.02	
	FD therapeutic	22.0900±5.44	
	TH prevention	26.3272±18.10	
	TH therapeutic	22.1720±17.48	
β-catenin	4NQO control	33.6676±9.47	.000*
	FD prevention	62.3252±6.24	
	FD therapeutic	48.4482±2.96	
	TH prevention	54.2470±5.05	
	TH therapeutic	46.1554±4.41	
p53	4NQO control	50.1804±4.16	.221
	FD prevention	40.593±0.70	
	FD therapeutic	39.1852±0.66	
	TH prevention	40.593±0.70	
	TH therapeutic	39.1852±0.66	

*p value less than 0.05, ($p < 0.05$) significant value compared to control group

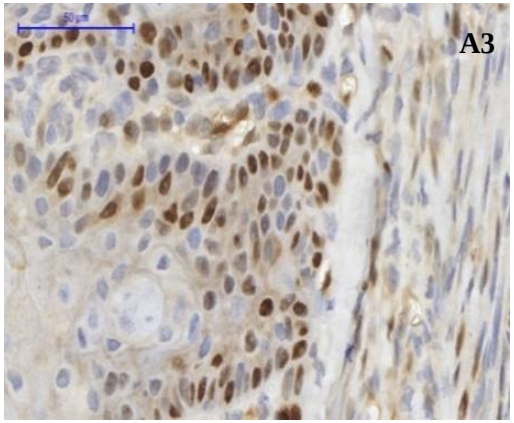
Table 4.8: Immunohistochemical evaluation of the FD extract and TH effect on rats induced for oral cancer with 4NQO (A post hoc Dunnett test).

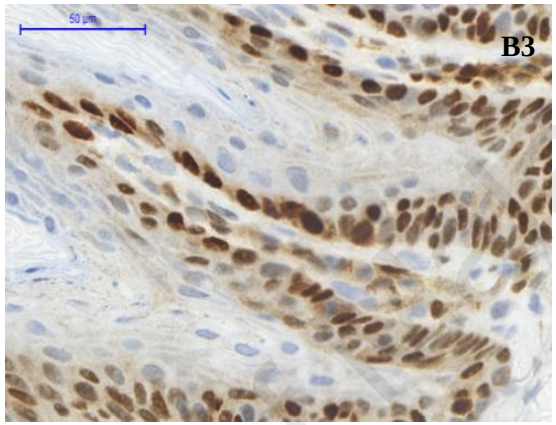
IHC Stain	Treated groups	Control group	*P* value
cyclin D1	FD prevention	4NQO (control)	.000*
	FD therapeutic		.020*
	TH prevention		.002*
	TH therapeutic		.035*
bcl2	FD prevention		.622
	FD therapeutic		1.000
	TH prevention		.690
	TH therapeutic		.622
e-cadherin	FD prevention		.000*
	FD therapeutic		.040*
	TH prevention		.002*
	TH therapeutic		.037*
β -catenin	FD prevention		.000*
	FD therapeutic		.017*
	TH prevention		.001*
	TH therapeutic		.048*
p53	FD prevention		.237
	FD therapeutic		.149
	TH prevention		.237
	TH therapeutic		.149

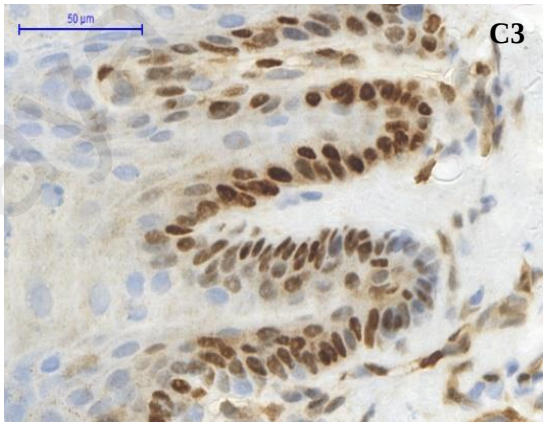
**p* value less than 0.05, ($p < 0.05$) significant value compared to control group


Control (4NQO)
Chemoprevention FD 500
Chemoprevention TH 2000
cyclin D1
bcl2
A1
B1
C1
A2
B2
C2
100 µm

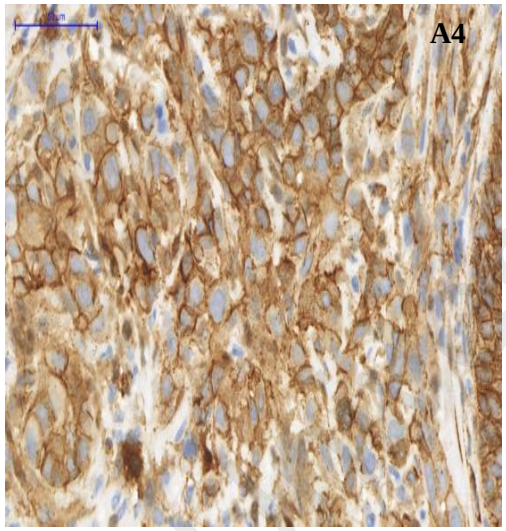
P53

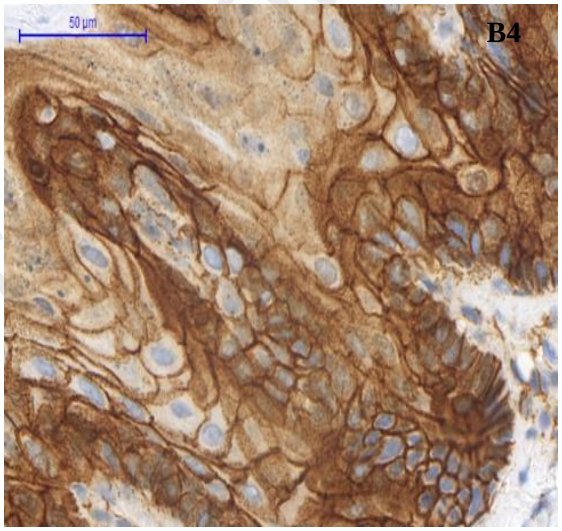


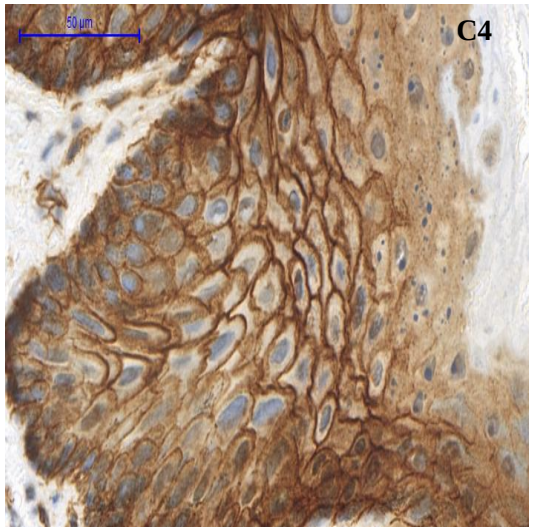




β-catenin







e-cadherin

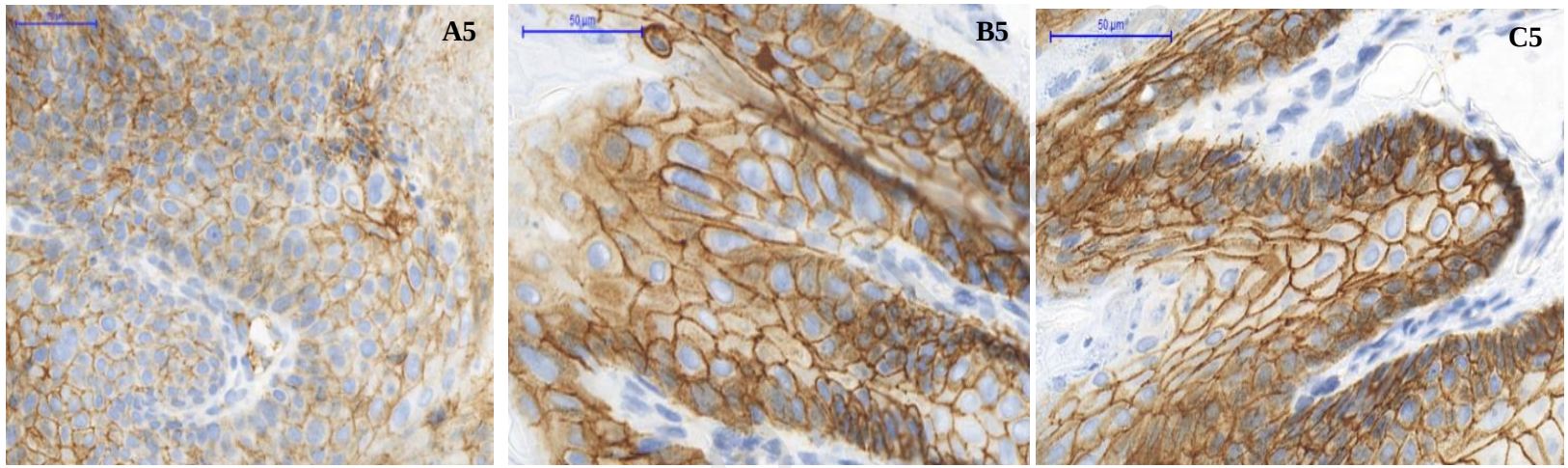


Figure 4.8: Photomicrographs of a chemo-preventive study showing immunohistochemistry stains for cyclin D1, bcl2, p53, β-catenin and e-cadherin from cancer control group (A1, A2, A3, A4 and A5 respectively) and groups treated with FD extracts 500 mg/kg (B1, B2, B3, B4 and B5 respectively) and TH 2000 mg/kg (C1, C2, C3, C4 and C5 respectively). Scale bars represent 50 and 100 µm.

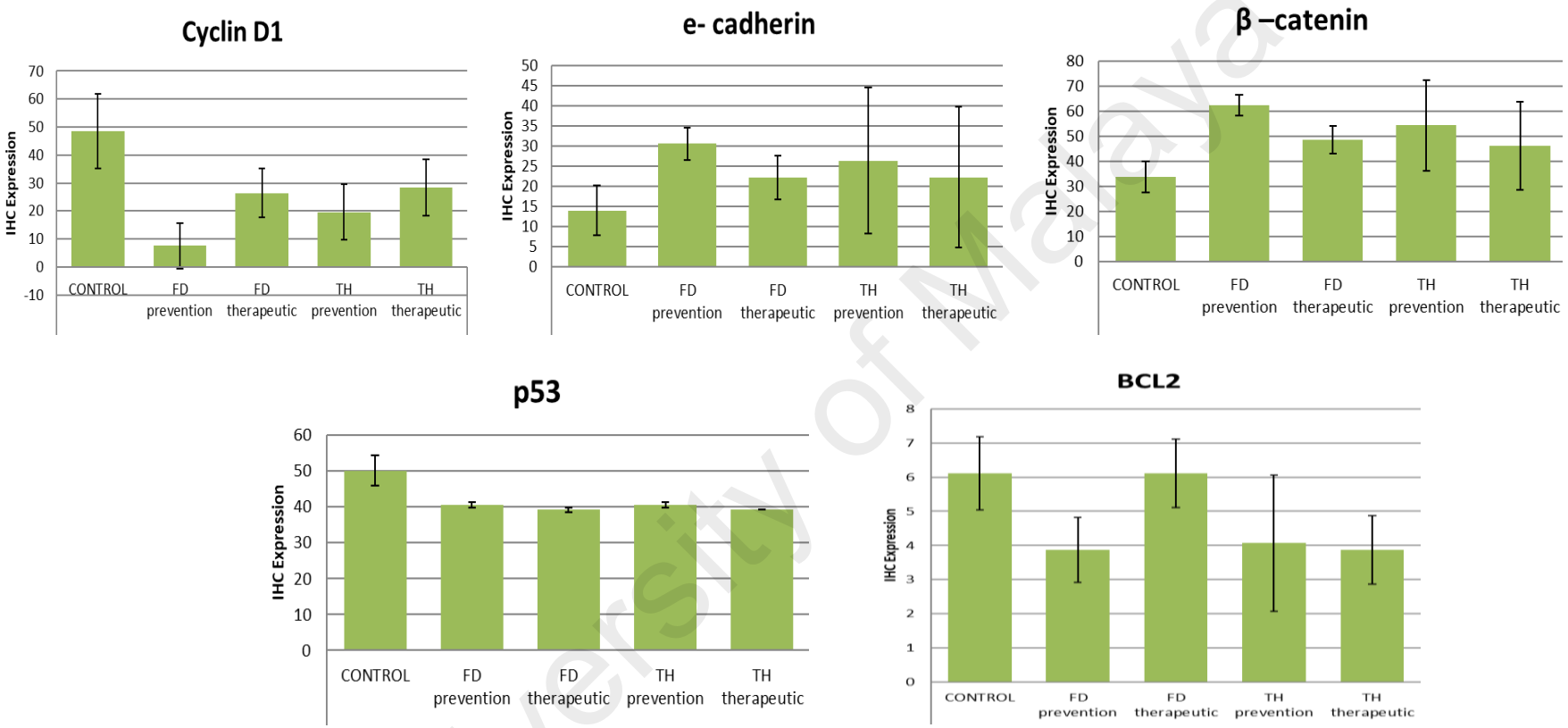
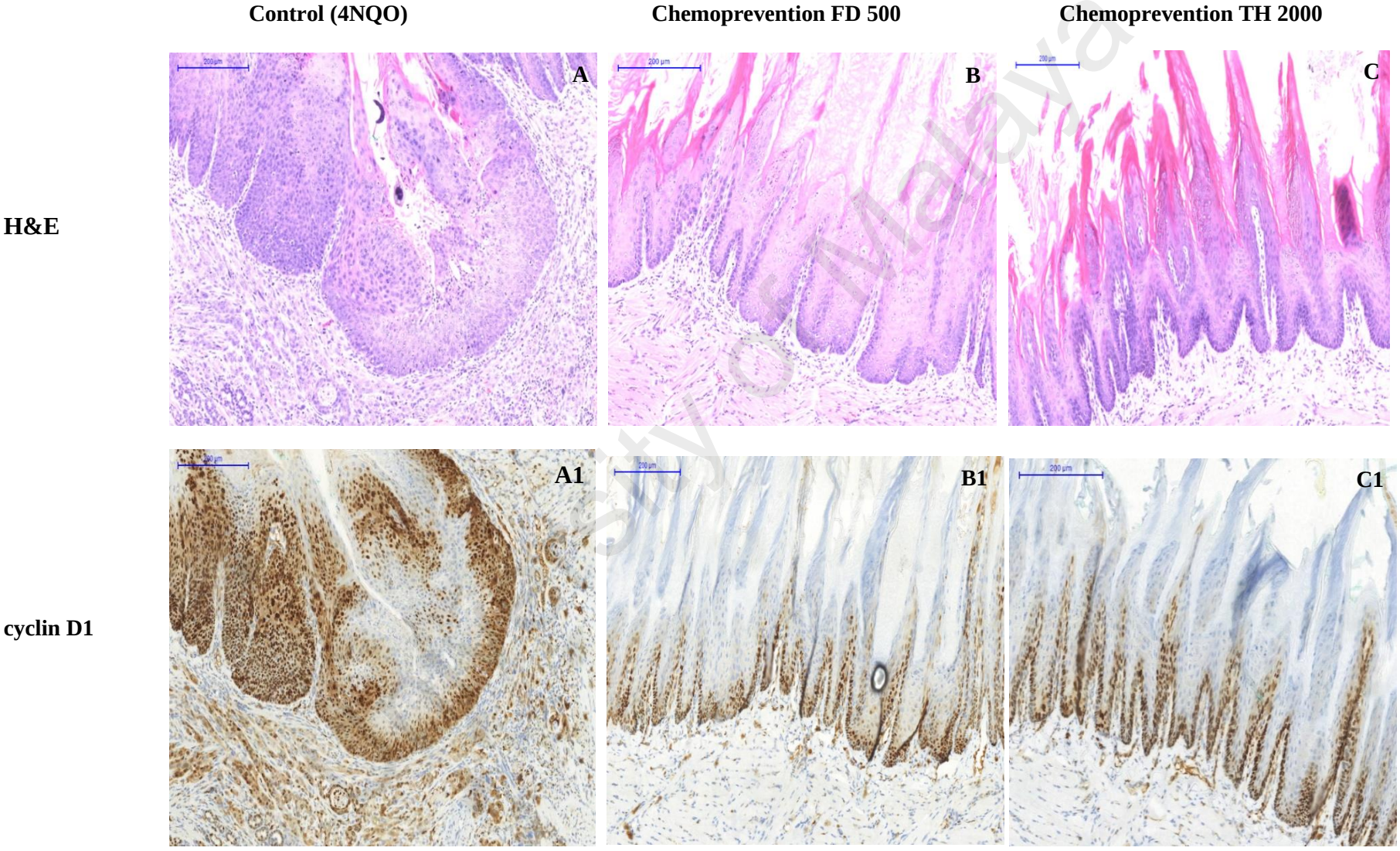
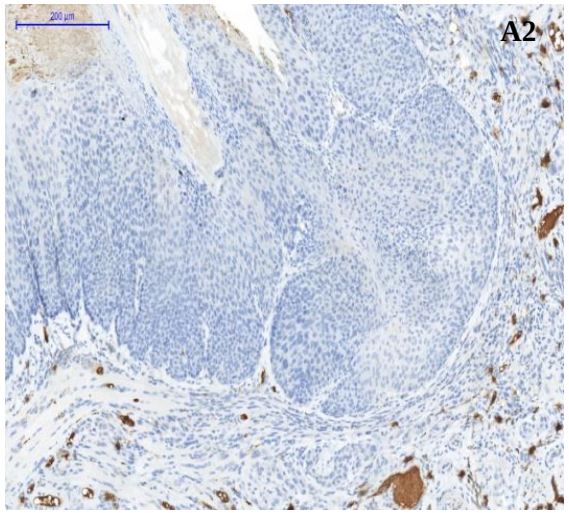
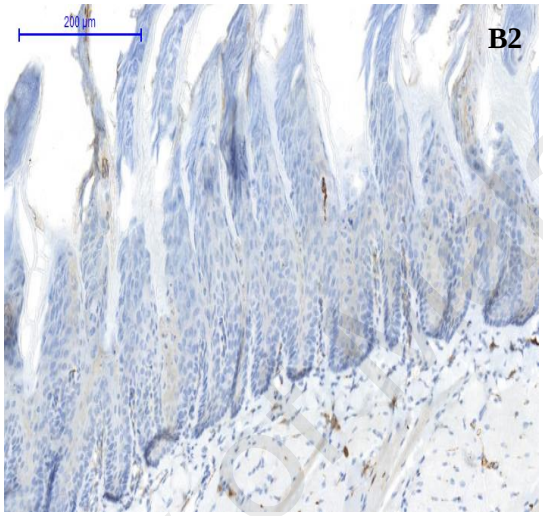


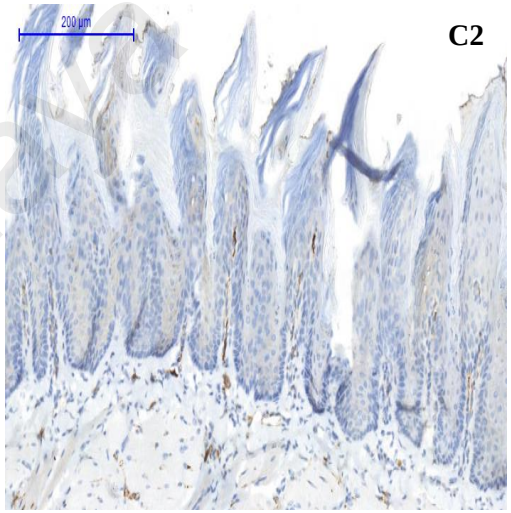
Figure 4.9: Immunohistochemistry analysis of rat tongues treated with TH (1000 and 2000 mg/kg) and FD extracts (250 and 500 mg/kg) obtained from both chemo-preventive and chemotherapeutic studies. Bar charts show the expression levels of β –catenin, e- cadherin, cyclin D1, p53 and bcl2 compared to the cancer control group. **p* value less than 0.05, ($p < 0.05$) significant value



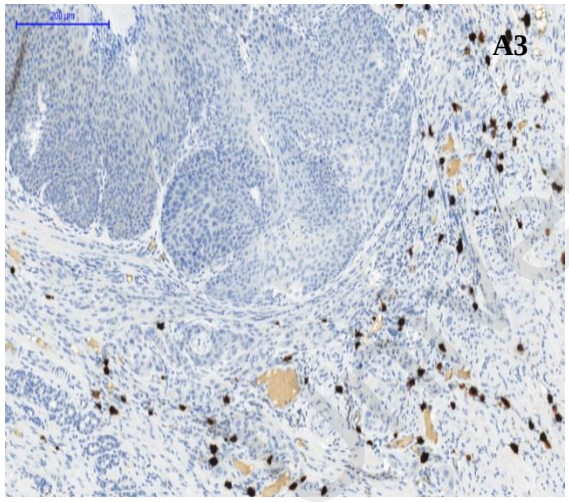
bcl2

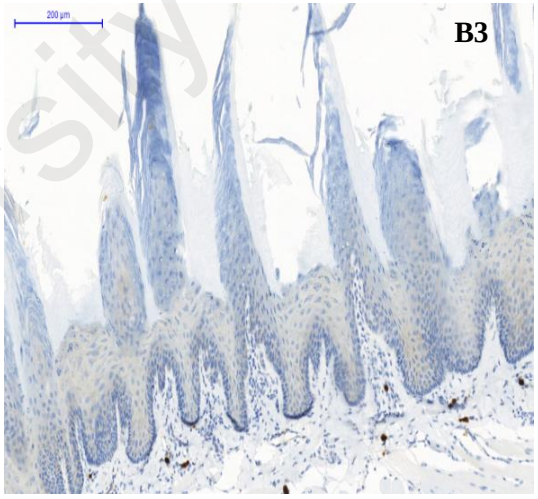


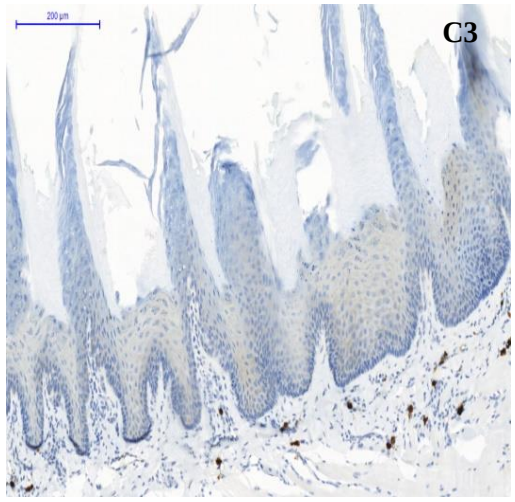




P53







β-catenin

e-cadherin

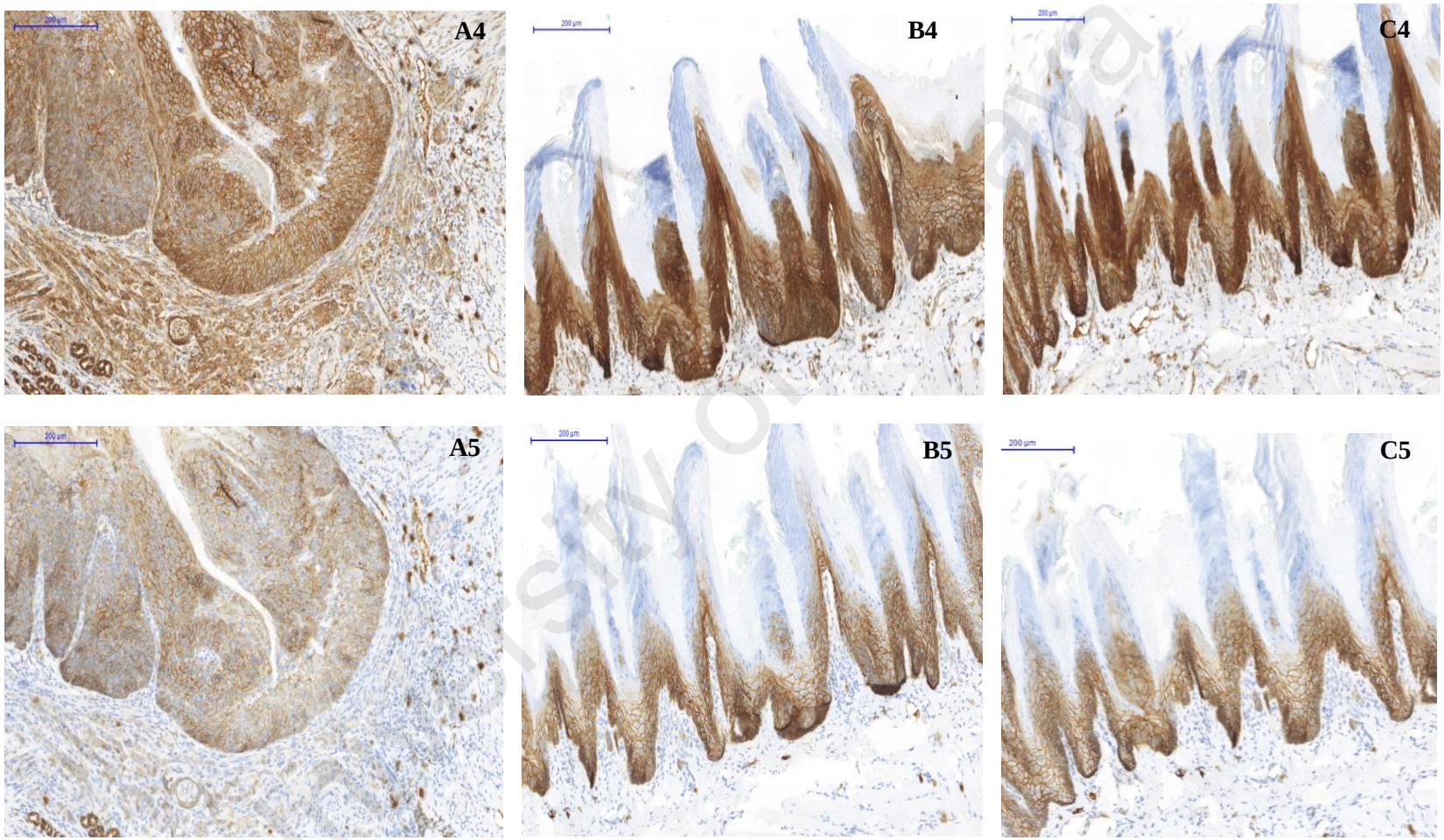
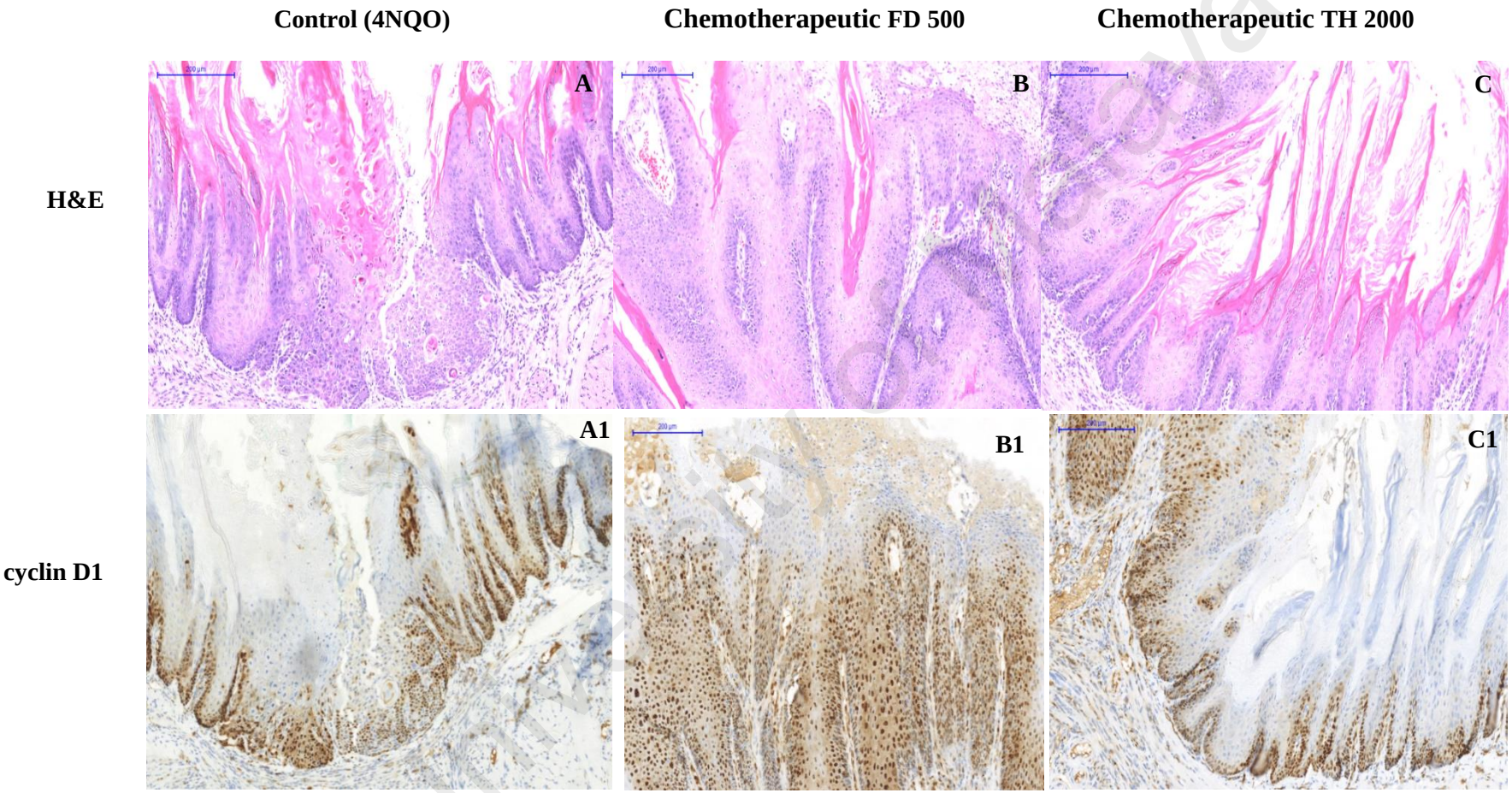
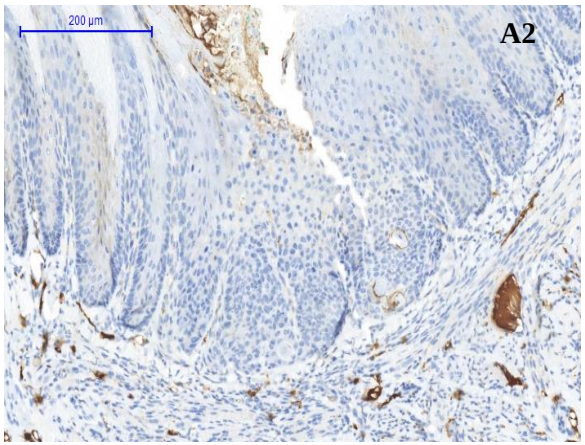
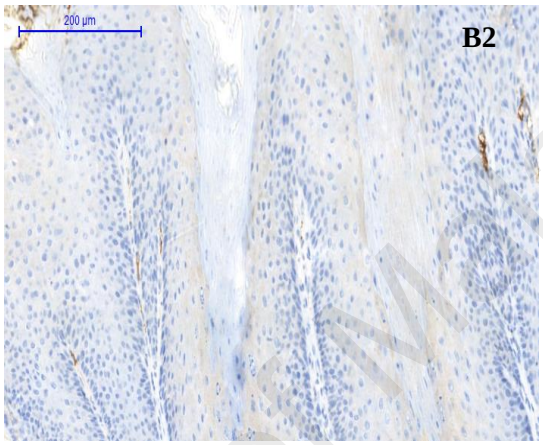


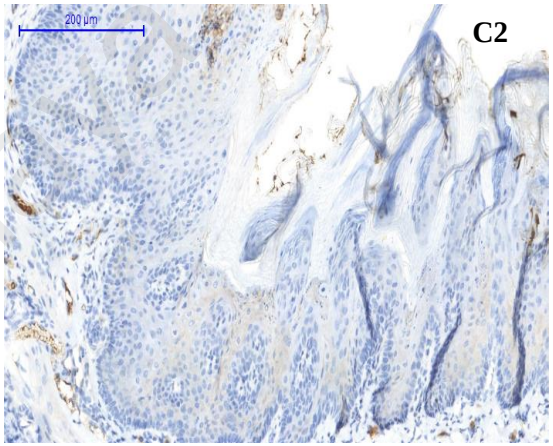
Figure 4.10: Photomicrographs of rat tongues obtained from a chemo-prevention study. Expression of cyclin D1, bcl2, p53, β-catenin and e-cadherin of rat tongues treated with FD 500 mg/kg (B1, B2, B3, B4 and B5 respectively) and TH 2000 mg/kg (C1, C2, C3, C4 and C5 respectively) as compared to cancer control group (A1, A2, A3, A4 and A5 respectively). (Scale bar, 200 µm)


Control (4NQO)
Chemotherapeutic FD 500
Chemotherapeutic TH 2000
H&E
cyclin D1
A
B
C
A1
B1
C1

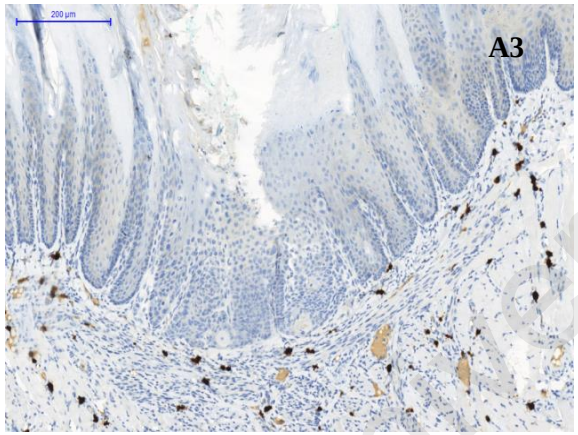
bcl2

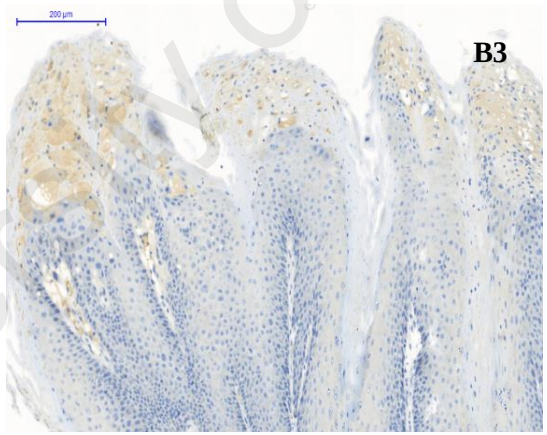


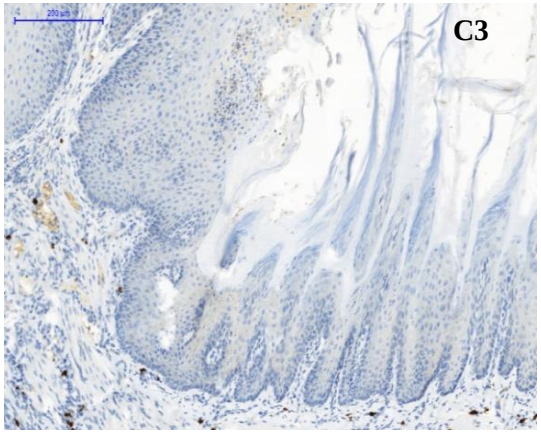




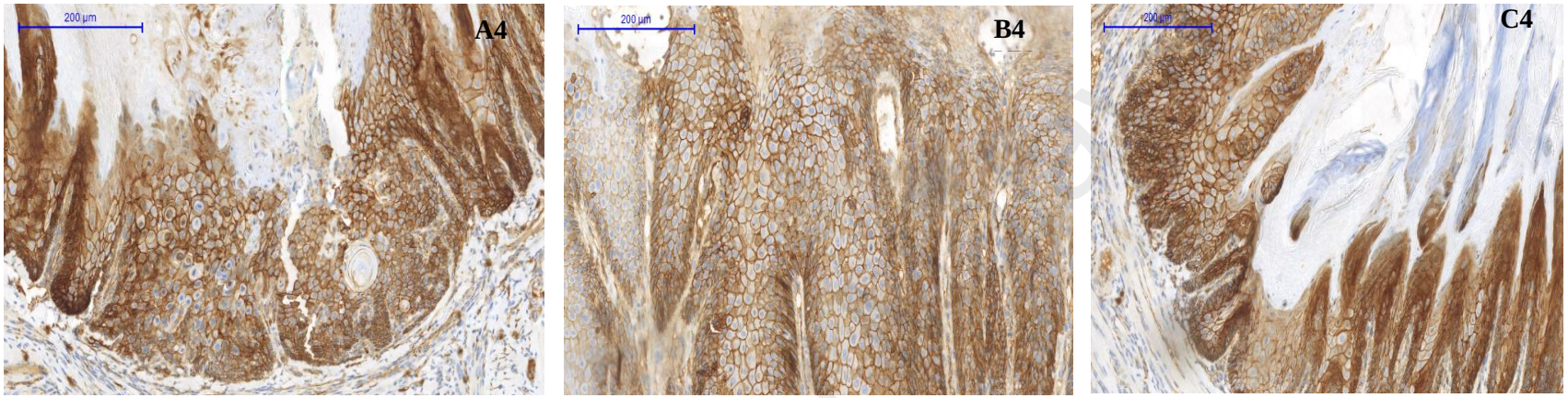
p53







β-catenin



e-cadherin

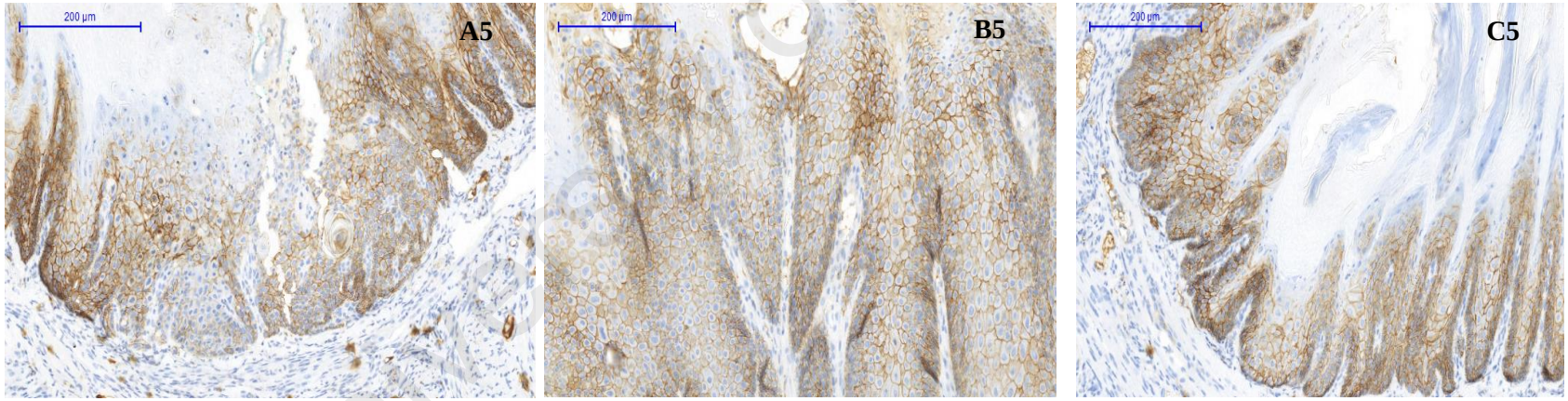


Figure 4.11: Photomicrographs of rat tongues obtained from a chemotherapeutic study. Expression of cyclin D1, bcl2, p53, β-catenin and e-cadherin of rat tongues treated with FD 500 mg/kg (B1, B2, B3, B4 and B5 respectively) and TH 2000 mg/kg (C1, C2, C3, C4 and C5 respectively) as compared to cancer control group (A1, A2, A3, A4 and A5 respectively). (Scale bar, 200 µm)

4.7 Gene expression evaluation

A custom RT^2 Profiler PCR Array was designed to determine the pattern of gene expression between two groups, the induced cancer group that was given 4NQO alone (Group 2), as well as the FD extract and TH treated groups for both the chemo-preventive and chemotherapeutic studies (Figure 4.12).

In general*, TP53*, *RAC1*, *COX-2, TWIST 1, CCND1* and *EGFR* gene expressions for both the chemo-preventive and chemotherapeutic studies were down-regulated in both the TH and FD extract treated groups (Groups 4, 6, 8 and 10) when compared with the cancer control group (Group 2) (Table 4.9 and Table 4.10).

Of these genes, *CCND1, COX-2* and *EGFR* expressions were significantly down-regulated ($p< 0.05$) in all groups whereas a non-statistically significant trend for *TP53* expression was observed to have decreased following the FD and TH treatment ($P>0.05$) in all groups.

For both chemo-preventive and chemotherapeutic groups, there was a non-significant decrease in the *RAC1* expression in the TH groups when compared to the cancer control group, while a significant decrease in *RAC1* expression ($p< 0.05$) was only observed in the FD extract treated groups.

As for the *TWIST1* gene, its expression was significantly down-regulated ($P>0.05$) in all groups except for the FD extract chemotherapeutic group (Table 4.10).

Table 4.9: Gene expression analysis on the effect of the FD extract and TH on rats induced for oral cancer with 4NQO (chemo-preventive study)

	CCND1	***EGFR***	***COX2***	***RAC1***	***TP53***	***TWIST 1***
4NQO (2^-ΔCt)	0.062	0.080	0.080	0.076	0.038	0.004
FD 500 (2^-ΔCt)	0.022	0.009	0.008	0.020	0.011	0.001
Fold change	0.358	0.114	0.113	0.262	0.287	0.272
***P* value**	0.036 *	0.037 *	0.038 *	0.016 *	0.067	0.039 *
4NQO (2^-ΔCt)	0.062	0.080	0.080	0.076	0.038	0.004
TH 2000 (2^-ΔCt)	0.018	0.009	0.009	0.019	0.009	0.001
Fold change	0.296	0.119	0.118	0.229	0.251	0.306
***P* value**	0.029 *	0.037 *	0.038 *	0.053	0.064	0.044 *

**p* value less than 0.05, ($p < 0.05$) significant value comparing to 4NQO group.

Table 4.10: Gene expression analysis on the effect of the FD extract and TH on rats induced for oral cancer with 4NQO (chemotherapeutic study)

	CCND1	***EGFR***	***COX2***	***RAC1***	***TP53***	***TWIST 1***
4NQO (2^-ΔCt)	0.062	0.080	0.080	0.076	0.038	0.004
FD 500 (2^-ΔCt)	0.018	0.018	0.015	0.0188	0.011	0.002
Fold change	0.292	0.225	0.196	0.244	0.290	0.437
***P* value**	0.028 *	0.049 *	0.046 *	0.015 *	0.068	0.063
4NQO (2^-ΔCt)	0.062	0.080	0.080	0.076	0.038	0.004
TH 2000 (2^-ΔCt)	0.019	0.015	0.013	0.019	0.011	0.001
Fold change	0.307	0.190	0.165	0.221	0.290	0.338
***P* value**	0.030*	0.046*	0.043*	0.053	0.064	0.049*

**p* value less than 0.05, (p< 0.05) significant value comparing to 4NQO group.

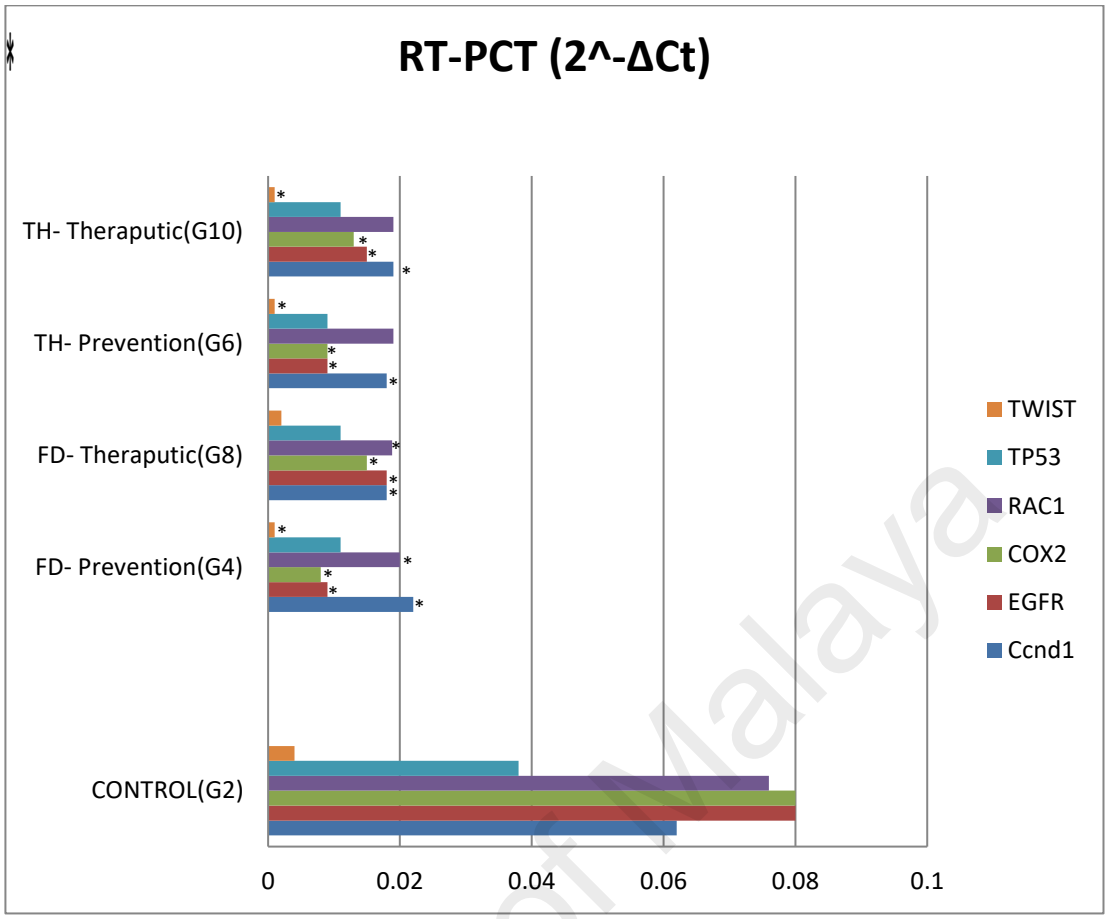


Figure 4.12: Bar chart showing gene expression in rats induced for oral cancer with 4NQO and treated with FD extract and TH. The bars indicate the 2^-ΔCt value of *TP53*, *RAC1*, *COX-2*, *TWIST*, *CCND1* and *EGFR* genes in G2, G4, G8, G6 and G10 groups. **p* value less than 0.05, ($p < 0.05$) significant value comparing to 4NQO control group.

CHAPTER 5: DISCUSSION

5.1 Introduction

There are three phases in cancer development; initiation, promotion, and progression (Aggarwal & Shishodia, 2006; Hahn & Weinberg, 2002). The present study was performed to evaluate the null hypothesis that carcinogenesis in the oral cavity cannot be inhibited or delayed through pharmacological activation of medicinal plant or natural products. To address this null hypothesis, an *in vivo* study was performed to determine the chemo-preventive and chemotherapeutic activities of the FD extract and TH.

In this study, 4-Nitroquinoline-1-oxide (4NQO) was used to induce oral cancer in a rat model. 4NQO, a quinoline derivative, is a potent chemical carcinogen. Quinoline and isoquinoline are major industrial chemicals which have been detected as components of the cigarette smoke (LaVoie et al., 1983).

4NQO administration to rodents form DNA adducts, and the damage induced by 4NQO to the DNA structure may be similar to that caused by tobacco smoke in humans. The molecular events induced by 4NQO have been shown to mimic the multistage progression of human oral cancer, thereby confirming the applicability of this model to our study (Kanojia & Vaidya, 2006).

The tongue is the most common site for oral cancer and therefore, the induction of oral cancer on the tongue using 4NQO in an animal model has been used extensively for oral cancer research (El-Rouby, 2011; Ribeiro et al., 2014). This method of inducing oral cancer has been employed in many studies in determining the effect of using chemo-preventive agents against oral cancer (De Paiva Gonçalves et al., 2015; El-Rouby, 2011; Ribeiro et al., 2014).

In addition, the administration of 4NQO in drinking water is a simple and straightforward method that can be easily applied on the animals (Tanaka & Ishigamori, 2011). Lesions that develop from the application of 4NQO usually do not produce nonspecific inflammatory changes when compared to other carcinogenic substances such as 7, 12-dimethylbenz (a) anthracene which can cause necrosis, sloughing of the tissue and produce lesions that are cytologically and morphologically not similar to the human lesions (Hasina et al., 2009). The method for delivering 4NQO in two different types of mouse strain has been studied. The method of painting the tongues with 4NQO has been compared with the delivery of 4NQO in the drinking water and it has been revealed that the latter method would result in a much higher incidence of oral cancer (Tang et al., 2004).

4NQO has been used in the previous studies to induce oral cancer in rat tongues (Lee & Choi, 2011; McCormick et al., 2015; Patel & Damle, 2013; Ribeiro et al., 2009). Clinical and histopathological studies have shown that the incidence of OSCC induced by 4NQO is considered equivalent to the human OSCC (Arima et al., 2006). In many oral cancer chemo-prevention studies, the 4NQO rat model has been performed using many types of rat strains. In a study involving seven strains of rats (Dark-Agouti, Long-Evans, Sprague-Dawley, ACI/Ms, F344, Donryu, and Wistar/Furth), Kitano and colleagues reported that there had been considerable inter-strain differences in the development of OSCC following exposure to 4NQO; Dark-Agouti rats were the most sensitive strain for the induction of oral carcinogenesis using 4NQO, while Wistar/Furth rats were found to be the least sensitive (Kitano et al., 1992).

5.2 Body weight

It has been reported that body weight loss may provide a useful indication of the clinical progression of OSCC (McCormick et al., 2015). 4NQO administration in animals for cancer induction can also result in substantial body weight loss due to the occurrence of oral cancer, as well as the lack of appetite, the inability to eat and an increase in metabolic rate (Thandavamoorthy et al., 2014). Patients with an advanced stage of cancer undergo a wasting syndrome characterised by the loss of weight with a poor prognosis, referred to as the cancer anorexia/cachexia syndrome (Fearon & Preston, 1990). Similar to the previous reports (Al-Afifi et al., 2018; McCormick et al., 2015), the use of 4NQO in this study has produced a significant reduction in body weight among the cancer-induced animals.

In this study, weight loss was only significant ($p<0.05$) after week 17 of 4NQO administration until the end of the study. The result of this study is in line with the previous studies as it was found that the body weight loss of the 4NQO-induced rats was significant only after 16 (Al-Afifi et al., 2018) and 14 weeks (Dayan et al., 1997) of 4NQO application.

In the chemo-preventive part of this study, it was observed that the reduction in body weight was established following the treatment with the FD extract and TH, especially when administrated at a higher dose. In a similar manner, the administration of rosiglitazone (800 mg/kg diet) after the cessation of 4NQO (10th week) had also increased the mean body weight (McCormick et al., 2015). In a related chemo- preventive study, Al-Afifi et al. (2018) found that the body weight reduction caused by 4NQO was also controlled by the extract of a plant, Dracaena cinnabari, when administrated at 100, 500, and 1000 mg/kg.

On the other hand, Bothwell (2015) reported that the body weight measurement of the rats throughout the 4NQO exposure period was found to be non-significant (Bothwell, 2015). Similar observation was also reported by Khiavi et al. (2015) in a chemotherapeutic study testing the effects of doxorubicin plus methotrexate in rats induced for cancer using 4NQO (Khiavi et al., 2015). It was also observed in this study that there had been no significant difference in the rats' body weight for the 4NQO control group and the chemotherapeutic groups.

5.3 Tongue tumour volume

In this study, the tumour volume was significantly (p<0.05) increased in the induced cancer group compared to the FD extract and TH treated groups in the chemo-preventive study following the 4NQO administration. The result obtained is in line with a previous study, where Wali et al. (2012) reported that the administration of PEG-8000, a chemo-preventive agent, following cancer induction for 14 weeks had significantly induced tumours of smaller volume than the 4NQO control group. Al-Afifi et al. (2018) reported that the administration of the DC extract at 500 and 1000 mg/kg, had significantly decreased the tongue tumour size induced by 4NQO. However, there was no significant difference in the tumour volume of rats treated with the FD extract and TH in the chemotherapeutic study when compared with the cancer control group.

5.4 Histopathological changes in the tongue of the rats administrated with 4NQO

In this study, oral cancer was successfully induced in the Sprague Dawley rat model using 20 ppm of 4NQO agent dissolved in drinking water for 8 weeks. From the normal histology of the rats' tongue epithelium, the 4NQO administration was shown to produce histopathological changes involving pre-malignant lesions such as dysplasia, and SCC.

This cancerous progression may be similar in humans as it has been reported that oral carcinogenesis may progress from mild dysplasia to moderate or severe dysplasia and then to OSSC (Lindenblatt et al., 2012; Scully et al., 2003; Ribeiro et al., 2004). The use of animal models for cancer induction has been regarded as essential when investigating the progression of diseases or for the development of diagnostic or therapeutic protocols. In this study, it was observed that none of the animals from the untreated control group developed any visible tongue epithelia lesions or underwent any histopathological changes. However, the administration of 4NQO for 8 weeks in drinking water at 20 ppm had produced precancerous and cancerous lesions on the tongue epithelium by the end of the experiment (22 weeks). This is in line with the previous reports which use 4NQO in animal models for oral carcinogenesis. The administration of 4NQO for 8 weeks at 20-30 ppm showed that the incidence of developing SCC and dysplasia at 28 weeks of the study was 100% and 21.4%, respectively (Yamamoto et al., 2004). In a related study, when 4NQO was administrated to the rats at 20 ppm for 10 weeks, the prevalence of OSCC development at the end of the study (26 weeks) was found to be highly reproducible at 83% (Peng et al., 2015).

The histological examination of the cancerous lesions found in the rats' tongues is in agreement with the previous reports. Zhao et al. (2014) reported that the histopathological grade of the SCC in the 4NQO animal model usually involves well-differentiated type of SCC and that the tumours may spread into the submucosa and underlying muscle layer, forming small nests of cells with the typical keratin pearl formation (Zhao et al., 2014). In like manner, Yamamoto et al. (2004) and Al-Afifi et al. (2018) have also reported that the cancerous lesions consisted of a well-differentiated type of SCC in the rats' tongues induced for oral cancer using 4NQO.

Depending on the dose and duration of the carcinogen being administered, 4NQO may trigger different types of dysplastic and neoplastic lesions with molecular and morphological alterations that may be related to the human carcinogenesis. This has been reported by Tank et al. (2004) where he observed that the protein expressions for the pre-neoplastic and neoplastic lesions in the oro-esophageal epithelium of mice administrated with 4NQO were similar for the same proteins associated with the human carcinogenesis (Tang et al., 2004).

5.5 Oral cancer metastasis

One of the greatest challenges associated with oral cancer is to block the metastatic spread of the cancer lesion. Most of the current anticancer treatments have been developed to treat the primary disease, yet they often fail in blocking the spread of the disease (Desmet et al., 2013). The prognosis of cancer patients or the success or failure of cancer treatment is often associated with the presence or absence of metastasis (Kawashiri et al., 2001).

The detection of metastatic foci, whether in the lymph nodes or the distant organs, can present a significant challenge. Classically, the presence of metastasis is evaluated in euthanised mice after the completion of a study. In OSCC, most investigators resect both the draining lymph node basins and the lungs for pathological evaluation (Milas et al., 2009).

There are many types of cancer treatment such as surgery, radiotherapy, chemotherapy and so forth. The effect of cancer treatment on metastasis, for example chemotherapy treatment, may be improved when applied in combination with either surgery and/or radiotherapy. However, there are very few reports investigating the inhibition of metastasis involving chemotherapy treatment.

Kawashiri et al. (2009) reported that neoadjuvant chemotherapy (administration of a therapeutic agent before the main treatment of choice) is effective for inhibiting metastasis of SCC in a mouse model and recommended that chemotherapy treatment should begin as early as possible to prevent metastasis (Kawashiri et al., 2009).

In a related study, the chemo-preventive effect of the olive tree leaf extract, oleuropein-rich extract (ORE), on 4NQO-induced rat tongue carcinogenesis using 20 ppm 4NQO in drinking water for 8 weeks was evaluated. It was reported that no metastasis was noted in any of the rats that were sacrificed after 37 weeks (Grawish et al., 2011). In a previous study done by Ohne et al. (1985), oral cancer was induced in rats using 10 ppm of 4NQO for 28 weeks and it was also observed that none of the lymph nodes showed any signs of metastasis in any of the sacrificed rats. Similarly, in the present study, no metastases were found in any of the vital organs (lung, lymph node, liver and kidney) of the rats induced for oral cancer using 4NQO. It is not known if higher doses of 4NQO (exceeding 20 ppm) with longer experimental period could cause the metastatic spread of the cancer.

5.6 Molecular analysis (Immunohistochemistry and RT^2 profiler PCR Array)

Substrate DAB (3,3' – diaminobenzidine) and HRP (horseradish peroxidase) have been widely used in immunohistochemistry. The reaction of the substrate produces a brown stain, indicating protein expression (Helps et al., 2012). However, the colour intensity of the stain may vary, thus resulting in discrepancies when interpreting the histological slides and this can lead to inter-observer variations. The colour intensity of the stain may also affect the rate of reproducibility when calculating the percentage of the stained area during IHC analysis of the histological slide, even when the inter-observer reproducibility was found to range from good to excellent (Jonmarker et al., 2009).

Due to the constraints mentioned above involving visual IHC evaluation, the quantification of IHC images using automated methods as an alternative will offer the required details necessary for enhancing the quality of the IHC data. Recently, the use of advanced digital image processing system has been introduced to enable the histological slides to be scored for IHC analysis (Varghese et al., 2014).

ImageJ and it's public domain, the Java-based image processing program, was developed at the National Institute of Mental Health, Bethesda, MD, USA (Schneider et al., 2012). ImageJ, released in 1997, involves colour deconvolution and immunoratio plugins and are freely available through web-based applications, thus enabling their routine use for quantitative IHC analysis (Sysel et al., 2013).

This technique generates an automated digital image that can be used for IHC analysis. A visual illustration of the statistical model is generated and the automatic detection of a specified colour could be performed by the ImageJ software with an IHC-toolbox plugin (Shu et al., 2016). Hence, the use of ImageJ software with an IHC-toolbox plugin to assess the histological slides was applied in this study.

Apart from IHC, another method used in this study for the analysis of tumour biomarkers is the RT2 profiler PCR Array. This method has been used to analyse the expression of many genes at the same time in just one reaction. Among the major advantages of this method over single gene analysis are the lower reagent costs and the ability to provide internal controls (Bernard & Wittwer, 2002).

To obtain further insight into the molecular mechanisms involved in the antitumour activity of TH and FD extract in the 4NQO oral cancer animal model, we assessed the expression of selected genes and proteins associated with tumour growth activities such as cellular proliferation and cancer progression (*CCND1*, *EGFR* and *COX-2*), cellular adhesion and epithelial-mesenchymal transition (EMT) process (β–catenin, e-cadherin, *TWIST1* and *RAC1*) and apoptosis (bcl2 and p53).

Cyclin D1 is a protein required for the progression of the G1 phase of the cell cycle. Cyclin D1 overexpression promotes transformation of a malignant phenotype that is associated with the progression of various types of cancer (Shan et al., 2009). The overexpression of cyclin D1 has been reported to be directly linked to the histopathological differentiation of OSCC (Saawarn et al., 2012) and also serves as an independent prognostic factor for oral cancer aggressiveness (Huang et al., 2012).

This feature is also evident in this study where in the control group of rats induced for oral cancer, the cyclin D1 expression by immunohostochemistry was found in the parabasal and basal compartments of the keratinised stratified squamous epithelium. The expression of cyclin D1 was also found in the connective tissues, indicating invasion of the tumour cells into the underlying tissues. Inversely, administration of the FD extract and TH at high doses in both the chemo-preventive and chemotherapeutic groups decreased the cyclin D1 expression which was restricted to the basal compartment of the keratinised stratified squamous epithelium. This could indicate that the FD extract and TH at high doses could have played a role in decreasing tumour agresiveness.

CCND1 gene is a positive regulator of cell cycle (Huang et al., 2012) and the overexpression of *CCND1* in OSCC has been associated with a shorter survival rate of cancer patients (Kaminagakura et al., 2011; Perez-Ordoñez et al., 2006). In addition, Wilkey et al. (2009) reported in an animal study that *CCND1* overexpression increases the susceptibility of the mice towards oral carcinogenesis induced by 4NQO.

Following induction of oral cancer in an animal 4NQO model, it was reported that the expression of *CCND1* decreased when the animals were exposed to substances with antitumour activity. Yoshida and his colleagues found that the administration of troglitazone (used to suppress the growth of tumours) had decreased the expression of *CCND1* in the 4NQO animal model (Yoshida et al., 2003). Similarly, Naoi et al. (2010) found that nimesulide (shown to inhibit the proliferation of cancer cells) had significantly reduced the expression of *CCND1* and *COX-2* in the 4NQO animal model induced for oral cancer. In the present study, the effects of the FD extract and TH on *CCND1* expression were investigated using IHC and RT^2 Profiler PCR Array for both chemo-preventive and chemotherapeutic studies. In line with the previous studies using substances with antitumour activity, *CCND1* expression was also found to have decreased in the FD extract and TH treated groups following cancer induction using 4NQO. The results obtained were validated by both the IHC and RT^2 Profiler PCR Array methods.

The overexpression of the *COX-2* gene has been associated with oral cancer and oral pre-malignant lesions. It was reported that cancer lesions at the later stage have a higher expression of the *COX-2* gene, as well as in patients with poor prognosis (Wang, 2005). Ribeiro et al. (2009) also concluded that the expression of *COX-2* is linked with the later stage of oral carcinogenesis; where the expression of *COX-2* has been found to be up-regulated.

In a related study, the mean expression of *COX-2* in OSCC was found to be 15.9 ± 6.7-fold greater than that in the adjacent tissues which consist of normal oral tissues (McCormick et al., 2010). A previous study assessing the antitumour effect of the apple extract reported that the *COX-2* expression had decreased in the apple extract treated groups following cancer induction using 4NQO, in comparison to the control group of 4NQO alone (Ribeiro et al., 2014).

Similarly, the *COX-2* expression by the RT^2 Profiler PCR Array in this study was found to have decreased when treated with the FD extract and TH when compared to the cancer control group for both types of experiment; chemo-preventive and chemotherapeutic.

The epidermal growth factor receptor (*EGFR*) has been reported to be overexpressed in > 80% of head and neck SCC (Taoudi et al., 2010). The *EGFR* gene overexpression has also been correlated to several other types of cancer, including anal and lung cancers (Khan et al., 2006). The *EGFR* pathway when blocked, has been reported to have an effect on the signalling pathways and can lead the cancer cells towards the apoptosis stage (Khan et al., 2006).

As pointed out by Singh et al. (2011), the potential for these cancer cells to become invasive is eradicated through the inhibition of *EGFR*. Wali et al. (2012) have shown that the painting of the rat's oral cavity with polyethylene glycol-8000 using a sable brush for up to 3–4 minutes has significantly lowered the expression of *EGFR* in the 4NQO treated rats. As for the present study, the results obtained are in line with that of the previous studies. It was found that there was a reduction in the expression of *EGFR* by the RT^2 Profiler PCR Array in the FD extract and TH treated groups for both chemo-preventive and chemotherapeutic experiments when compared to the 4NQO control group.

Ravi et al. (1996) stated that the expression of the antiapoptotic bcl2 protein is closely correlated to the expression of p53. The overexpression of p53 has been reported in 55% and 75% of the North Indian patients with oral dysplasia and OSCC respectively (Kaur et al., 1994). Scrobota et al. (2016) discovered that the expressions of ki-67, cyclin D1, p63, bcl2 and p53 were found to have increased in line with the severity of the rats' dysplastic lesion when 4NQO was topically applied on the rats' tongues for 12 weeks.

On the other hand, other studies have reported the lack of expression or a sporadic bcl2 expression in oral dysplasia (McAlinden et al., 2000; Ohlsson et al., 2002). Ribeiro et al. (2005), have shown that bcl2 has an important role in the initiation of SCC in the rats' tongues mucosa but in well-differentiated SCC, induced by 4NQO for 20 weeks, the bcl2 expression was sometimes absent or reduced (Ribeiro et al., 2005).

Other studies have also reported that bcl2 was overexpressed in poorly differentiated carcinomas (Chen et al., 2000; Jordan et al., 1996). In the present study, the bcl2 expression between the FD extract and the TH groups and the cancer control group was observed to be not significant. It could be that the development of oral cancer in this study is associated with the well-differentiated type of OSCC, and hence this could have contributed to the none significant expression of bcl2.

The p53 protein is generated by the *P53* tumour suppressor gene, which functions during the G1-S phase of the cell cycle that involves the repair of damaged DNAs, direct damaged cells to enter apoptosis or to prevent the cells from entering the S phase (Patil et al., 2016). From a clinical perspective, patients with an increased expression of p53 may have a poor prognosis compared to cancer patients with a low expression of p53 (Xie et al.,1999).

Fong et al. (2011) conducted a research investigating the effect of consuming zinc as a supplement for the 4NQO-induced oral carcinogenesis. They reported that zinc had decreased the expression of tumour markers such as p53, cox-2 and cyclin D1 when compared to the 4NQO control group.

In this study, the FD extract and the TH treated groups for both the chemo-preventive and chemotherapeutic experiments have shown an overexpression of p53 in the induced cancer group (4NQO-treated rats). However, the results were not significant for both the IHC and the RT^2 Profiler PCR molecular analysis methods. It has been reported that the *P53* gene is mutated in about 50% of all types of human cancer, including oral cancer (Greenblatt et al., 1994) and this could have contributed to the non-significant expression of p53 in this study.

The epithelial calcium dependent adhesion molecules e-cadherin and β-catenin are cell adhesion proteins. A reduction in the cell adhesive proteins has been reported in tumour development (Hung et al., 2006), and in the progression of the head and neck SCC (Andrews et al., 1997). A decreased expression of β-catenin, associated with poor prognosis of breast cancer has also been reported (Dolled-Filhart et al., 2006). El-Rouby (2011) reported an increase in β-catenin and e-cadherin expressions when lycopene or tomato carotenoids was used to treat oral cancer in 4NQO-induced rats. Similarly, when the FD extract and TH were used to treat oral cancer induced by 4NQO, it was observed that the expressions of the β-catenin and e-cadherin had increased.

Our study demonstrated that the TH and FD extract had significantly reduced the *TWIST1* expression ($p<0.05$) when compared to the control 4NQO group. *TWIST1* can act as a predictor of distant metastasis, plays an important role in the progression of OSCC during the EMT process, and is also involved in the later stage of cancer development (Ou et al., 2008).

Similar to our study, De Paiva Gonçalves et al. (2015) reported that the *TWIST1* expression was found to have decreased, although non-significantly in the curcumin treatment group when compared with the control 4NQO rat group (De Paiva Gonçalves et al., 2015).

Rac1, a key protein that is involved in the transduction signalling pathway, belongs to the Ras superfamily of GTP-binding proteins (Hunter, 1997). The role of Rac1 in Ras transformation has been analysed by introducing constitutively active mutants (Anand-Apte et al., 1997; Keely et al., 1997; Qiu et al., 1995). It has been reported in lung cancer that *RAC1* overexpression is related to the EMT process with poor prognosis (Zhou et al., 2016). The overexpression of the *RAC1* gene in the breast cancer tissues has also been reported (Schnelzer et al., 2000). In this study the *RAC1* gene was down-regulated in both groups, the FD extract and TH, but was only significant ($p<0.05$) in the FD extract treated group when compared to the control 4NQO group.

CHAPTER 6: CONCLUSION

General conclusion

The current study provides an insight into the chemo-preventive and chemotherapeutic effects of the FD extract and TH in an animal model induced for oral cancer using 4NQO. Body weight gain/loss, tumour tongue volume and the incidence of OSCC were assessed in the SD rats. To obtain further insight into the molecular mechanisms involved in the chemo-preventive and chemotherapeutic effects of the FD extract and TH in the animal model induced for oral cancer, the expression of selected genes and proteins associated with the tumour and its growth activities, such as cellular proliferation and cancer progression (*CCND1*, *EGFR* and *COX-2*), cellular adhesion and epithelial-mesenchymal transition (EMT) process (β –catenin, e-cadherin, *TWIST1* and *RAC1*) and apoptosis (bcl2 and p53) have been assessed.

In this study, the tumour volume was significantly increased in the control cancer group compared to the FD extract and TH treated groups in the chemo-preventive study following 4NQO administration. In addition, it was observed that the reduction in body weight was stabilised following the treatment with the FD extract and TH, especially when administrated at a higher dose (500 mg/kg and 2000 mg/kg respectively). Moreover, the incidence of hyperplasia, dysplasia, and squamous cell carcinoma were reduced significantly in the FD extract and TH treated groups compared to the control cancer group. Thus, the FD extract and TH confer statistically significant protection against carcinogenesis in the rat model induced for oral cancer.

Cyclin D1, a protein that is associated with the progression of various types of cancer and may also serves as an independent prognostic factor for oral cancer aggressiveness was found to be down-regulated in this study when FD extract and TH was administered at high doses in both the chemo-preventive and chemotherapeutic groups. This could indicate that the FD extract and TH at high doses could reduce the aggressive nature of the tumour cells.

In rats treated with the FD extract and TH following cancer induction using 4NQO, the expression of *CCND1* and *COX-2* and *EFGR* genes, a positive regulator of cell cycle and associated with many types of cancer, were found to have been underexpressed in both the chemo-preventive and chemotherapeutic studies. This would suggest that the FD extract and TH had significantly inhibited the proliferation of cancer cells and thus, may have hindered tumour development and progression. On the other hand, the overexpression of cell adhesion proteins such as β-catenin and e-cadherin, which have been shown to be underexpressed in tumour development, could further support the tumour suppressive actions of the FD extract and TH.

The *TWIST1* gene plays an important role in the progression of OSCC during the EMT process and can also act as a predictor of distant metastasis. In this study, the expression of the *TWIST1* gene was found to have been underexpressed in both the chemo-preventive and chemotherapeutic groups treated with the FD extract and TH. A key protein involved in the transduction signalling pathway, *RAC1*, was also shown to be under- expressed in both groups, but was only significant in the FD extract treated group. *RAC1* plays an important role in cancer cell motility and since one of the hallmarks of cancer is cell invasion and metastasis, it could be inferred that the FD extract and TH may inhibit cancer development through the deregulation of cell motility and cell growth.

The bcl2 protein, associated with apoptosis, is reported to have been overexpressed in poorly differentiated carcinomas. In the present study, the bcl2 expression between the FD extract and TH groups and the cancer control group was observed to be not significant. It could be that the development of oral cancer in this study is associated with the well-differentiated type of OSCC. Similarly, the expression of p53 in the induced cancer groups treated with the FD extract and TH for both the chemo-preventive and chemotherapeutic experiments have also showed no significant results when compared to the control cancer group.

Based on the results obtained from this study, it is concluded that the FD extract and TH have the potential in being developed as a chemo-preventive and chemotherapeutic agent for oral cancer therapy. This study strongly supports the benefits for FD and TH to be consumed as daily supplements to increase the quality of life in cancer patients.

Limitations

In the present study, time and/or cost are major contributors for the limitations. The limitations in the present study are as follows:

1) The small sample size of rats per group (7 rats per group) had possibly contributed to the none significant findings in the statistical analysis of some of the important parameters.

2) The short experimental period (22 weeks) may be expanded for thorough investigation of cancer metastasis.

3) The exact molecular pathway involved in the anticancer activity of the FD extract and TH in oral cancer was not evaluated.

Recommendation for future study

The identification of pure compounds and chemical characterisation of the cytotoxically active compounds of the FD extract and TH in the future would be beneficial. Further studies would also be required to further elucidate the molecular pathway involved in the anticancer activity of the FD extract and TH in oral cancer. Moreover, an animal model that can produce a high incidence of cancer metastasis as well as longer experimental duration is necessary to verify the metastasis obstruction effect of the FD extract and TH.

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LIST OF PAPERS AND PRESENTATION

Papers:

1) May Al-koshab, Alabsi, Aied, Bakri, Marina, Subramaniam, Manimalar, (2019). Antitumor activity of Ficus Deltoidea extract on oral cancer -An In Vivo study. Journal of Oncology (ISI). (Accepted)

2) May Al-koshab, Alabsi, Aied, Bakri, Marina, Subramaniam, Manimalar, Seyedan, Atefehalsadat, (2019). Chemopreventive activity of Tualang Honey against oral cancer –*in vivo*. Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology (ISI). (Under Review)

Presentations:

1) Oral presentation: May Al-koshab. Therapeutic role of honey in oral health and its anticancer activity. Paper presented at the International Conference on Oral Immunology & Oral Microbiology (ICOIOM). University of Malaya, Malaysia 4th Aug 2018.