CHEMOPREVENTIVE AND CHEMOTHERAPEUTIC ACTIVITIES OF *DRACAENA CINNABARI BALF.F* AGAINST ORAL CANCER *IN VIVO*

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FACULTY OF DENTISTRY
UNIVERSITY OF MALAYA
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2018

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF DENTISTRY
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

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Matric No: DHA130015

Name of Degree: Doctor of Philosophy

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

CHEMOPREVENTIVE AND CHEMOTHERAPEUTIC ACTIVITIES OF *DRACAENA CINNABARI BALF.F* AGAINST ORAL CANCER *IN VIVO*

Field of Study: Oral Biology

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CHEMOPREVENTIVE AND CHEMOTHERAPEUTIC ACTIVITIES OF *DRACAENA CINNABARI BALF.F* AGAINST ORAL CANCER *IN VIVO*

ABSTRACT

Dracaena cinnabari (*DC*) is a perennial tree possesses various pharmacological properties but its anticancer properties have not been clarified. **Aims:** To evaluate the toxicity, anticancer activity (chemopreventive and chemotherapeutic) and metastasis obstruction of the *DC* resin methanol extract on 4-nitroquinoline-1-oxide (4NQO)-induced oral cancer in rats. **Materials and Methods:** The powder of *DC* resin was extracted with methanol using the maceration extraction method. The toxicity profile of *DC* extract was investigated in Sprague Dawley (SD) rats using acute and sub-acute oral toxicity tests. In general, the chemopreventive study involves administration of 4NQO solution (cancer induction) to SD rats for 8 weeks alone or with *DC* extract at 100, 500 and 1000 mg/kg that started one week before the exposure until one week after the cessation of the carcinogen exposure. In the chemotherapeutic study, SD rats were given 4NQO (20 ppm) for 8 weeks followed by administration of *DC* extract at 100, 500 and 1000 mg/kg for another 10 weeks with or without Cisplatin (3 mg/kg I.P for every 3 weeks). All rats from both studies were sacrificed after 22 weeks, and histological analysis was performed to assess any incidence of pathological changes. Immunohistochemical expressions of selected tumour marker antibodies were analysed using an image analyser computer system, and the expression of selected genes involved in apoptosis and proliferative mechanism related to oral cancer were evaluated using RT^2-PCR. **Result:** Acute oral toxicity revealed that the *DC* extract could be well tolerated up to the dose of 2000 mg/kg and at the dose of 1500 mg/kg, the sub-acute test revealed no evidence of any treatment-related changes of the animals used in this study. In the chemopreventive study, the incidence of OSCC decreased with the administration of *DC* extract at 100, 500 and 1000 mg/kg compared to the induced cancer and vehicle groups.

In the chemotherapeutic study, there was no incidence of OSCC (0%) with the administration of *DC* 1000 mg/kg and Cisplatin. For both chemopreventive and chemotherapeutic studies, the survival rate increased to 100% for *DC* doses of 500 and 1000 mg/kg. The developed tumour was also observed to be smaller, and lymph node metastasis was inhibited in all *DC* treated groups with or without Cisplatin when compared to the induced cancer and vehicle groups. The *DC* 1000 mg/kg group inhibit the expression of Cyclin D1, Ki-67, Bcl-2 and p53 genes responsible for decreasing the transformation and the aggressiveness of the cancer tissue while a slight increase in the expression of β-catenin and E-cadherin genes that decrease the proliferation of cancer cells while maintaining epithelial polarity was observed. It was also observed that *DC* 1000 mg/kg induced apoptosis by upregulation of Bax and Casp3 genes and down-regulation of Tp53, Bcl-2, Cox-2, Cyclin D1 and EGFR genes when compared to the induced cancer group. **Conclusion:** This study provides scientific validation for the safety of *DC* extract up to the highest dose level used in this study. Furthermore, the data indicated that systemic administration of the *DC* extract has anticarcinogenic potency on oral carcinogenesis.

Keywords: *Dracaena cinnabari*, Oral toxicity tests, Chemoprevention, Chemotherapeutic, Oral squamous cell carcinoma.

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ABSTRAK

Dracaena cinnabari (*DC*) adalah pokok saka yang mempunyai pelbagai sifat farmakologi tetapi sifat antikansernya tidak dijelaskan. Tujuan: untuk menilai keracunan, aktiviti antikanser (kemopencegahan dan kemoterapeutik) dan halangan metastasis ekstrak metanol resin *DC* pada tikus yang diaruh kanser mulut oleh 4-nitroquinoline-1-oksida (4NQO). Bahan dan Kaedah: Serbuk resin *DC* diekstrak dengan metanol dengan menggunakan kaedah pengekstrakan makerasi. Profil toksisiti ekstrak *DC* disiasat dalam tikus Sprague Dawley (SD) yang menggunakan ujian ketoksikan akut dan sub-akut. Secara umumnya, kajian kemopencegahan melibatkan penggunaan 4NQO (aruh kanser) kepada tikus SD selama 8 minggu sahaja atau dengan ekstrak *DC* pada 100, 500 dan 1000 mg/kg yang bermula seminggu sebelum pendedahan sehingga satu minggu selepas pemberhentian pendedahan karsinogen. Dalam kajian kemoterapeutik, tikus SD diberikan 4NQO (20 ppm) selama 8 minggu diikuti dengan pemberian ekstrak *DC* pada 100, 500 dan 1000 mg/kg selama 10 minggu lagi dengan atau tanpa Cisplatin (3 mg / kg IP untuk setiap 3 minggu). Semua tikus dari kedua-dua kajian dikorbankan selepas 22 minggu, dan analisis histologi dilakukan untuk menilai sebarang kejadian perubahan patologi. Ekspresi imunohistokimia antibodi penanda tumor yang dipilih dianalisis menggunakan sistem komputer penganalisis imej, dan ungkapan gen terpilih yang terlibat dalam apoptosis dan mekanisme proliferatif yang berkaitan dengan kanser mulut dinilai menggunakan RT2-PCR. Keputusan: Ketoksikan oral akut mendedahkan bahawa ekstrak *DC* boleh diterima dengan baik sehingga dos 2000 mg/kg dan pada dos 1500 mg/kg, ujian sub-akut tidak menunjukkan bukti sebarang perubahan berkaitan dengan haiwan digunakan dalam kajian ini. Dalam kajian kemopencegahan, kejadian OSCC menurun dengan penggunaan ekstrak *DC* pada 100, 500 dan 1000 mg/kg berbanding dengan

kumpulan yang diaruh kanser dan vehicle. Dalam kajian kemoterapeutik, tidak terdapat kejadian OSCC (0%) dengan pentadbiran *DC* 1000 mg/kg dan Cisplatin. Untuk kedua-dua kajian kemopencegahan dan kemoterapeutik, kadar survival meningkat kepada 100% untuk dos *DC* 500 dan 1000 mg/kg. Pertumbuhan tumor juga diperhatikan lebih kecil, dan metastasis nodus limfa dihalang dalam semua kumpulan rawatan *DC* dengan atau tanpa Cisplatin jika dibandingkan dengan kumpulan yang diaruh kanser dan kumpulan vehicle. Kumpulan *DC* 1000 mg/kg menghalang ekspresi Cyclin D1, Ki-67, Bcl-2 dan p53 gen yang bertanggungjawab untuk mengurangkan transformasi dan agresif tisu kanser sementara sedikit peningkatan dalam ungkapan β-catenin dan E- cadherin gen yang mengurangkan sel-sel kanser disamping mengekalkan polaritas epitel dapat diperhatikan. Ia juga diperhatikan bahawa *DC* 1000 mg/kg menyebabkan apoptosis dengan peningkatan gen Bax dan Casp3 dan penurunan gen Tp53, Bcl-2, Cox-2, Cyclin D1 dan EGFR berbanding dengan kumpulan yang diaruh kanser. Kesimpulan: Kajian ini menyediakan pengesahan saintifik untuk keselamatan ekstrak *DC* hingga tahap dos tertinggi yang digunakan dalam kajian ini. Selain itu, data menunjukkan bahawa penggunaan ekstrak *DC* mempunyai potensi antikarsinogenik pada karsinogenesis mulut.

Kata kunci: *Dracaena cinnabari*, Ujian ketoksikan oral, Kemopencegahan, Kemoterapeutik, Karsinoma sel skuamosa mulut

ACKNOWLEDGEMENTS

The journey from the concept to completion of this project has been an extremely challenging and at the same time, very rewarding experience. I thank Allah first and foremost for giving me the opportunity, strength and faith to pursue my dreams and aspirations.

To my supervisors – Associate Prof. Dr. Aied Mohammed Hamood Saeed and Associate Prof. Dr. Marina Binti Mohd Bakri, throughout the process, I have strived to uphold all that you stand for academically because you exemplify what it means to me to be a researcher. You helped me become a better researcher and a person than I could have ever been on my own. This project may never have reached its full potential without you.

I extend my gratitude and blessings to all the staff of the Oral & Craniofacial Sciences Department, Faculty of Dentistry, who have supported me very much during the completion of the project. I would also like to acknowledge Dr. Anand Ramanathan, who graciously helped me when he was probably up to his neck in work.

I am indebted to my small family, my loving wife who is always by my side when I needed her most and helped me a lot in making this study. In addition, my twin daughters Aya and Alaa, and a little boy Aws who served as my inspiration to pursue this undertaking.

I dedicate this project to my parents for persistently keeping me in their prayers. I will always remain indebted to their offerings and sacrifice.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
°C	:	Degree Celsius
4NQO	:	4-nitroquinoline-1-oxide
ALP	:	Alkaline phosphatase
ALT	:	Alanine aminotransferase
ANOVA	:	Analysis of variance
AST	:	Aspartate aminotransferase
cDNA	:	Complementary DNA
DC	:	*Dracaena cinnabari*
F	:	Female
g	:	Gram
G0 phase	:	Quiescence phase
G1 phase	:	Gap 1 phase
G2 phase	:	Gap 2 phase
H&E	:	Haematoxylin and eosin
HGB	:	Haemoglobin
IHC	:	Immunohistochemical
M	:	Male
M phase	:	mitotic phase
mg/kg	:	Milligram per kilogram
nm	:	Nanometre
OECD	:	Organization for Economic Cooperation and Development
OSCC	:	Oral Squamous Cell Carcinoma
$p < 0.05$	:	p value less than 0.05
RNA	:	Ribonucleic acid
ROW	:	Relative organ's weight
S phase	:	Synthesis phase
SD	:	Sprague-Dawley
WBC	:	White blood cell

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Introduction

Cancer is a group of diseases characterised by uncontrolled growth and spread of abnormal cells. Cancer may spread in an uncontrolled manner and can cause death. The cause of cancer can be attributed to both external factors (tobacco, chemicals, radiation, and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). These factors may act together or in sequence to initiate or promote carcinogenesis (Srinivasan et al., 2008).

Among the ten most common cancer sites, oral cancer has been ranked as the sixth most common cancer worldwide and third in the developing countries (Ogbureke & Bingham, 2012). Depending on the type of tissues that are affected, the development of cancer involves several phases; dysplasia, cancer in situ, localised invasive cancer, regional lymph node involvement, and distant metastases (WHO, 2002). The six hallmark features of cancer cells that provide a logical framework for understanding the cell biology and survival of cancer cells are: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan & Weinberg, 2000).

Oral squamous cell carcinoma (OSCC) has been considered as one of the most common malignancies of the oral cavity. The most important predisposing risk factors of OSCC include tobacco use and excessive alcohol consumption while other factors such as viral infections (e.g. human papillomavirus and herpes simplex viruses) and diet deficient in antioxidants have also implicated (Yedida et al., 2013). Although the development of OSCC progresses sequentially from hyperplasia through to dysplasia and

carcinomas, in most cases, patients are often presented with the advanced stage of the disease at the time of diagnosis (Yedida et al., 2013).

The ultimate goal of oral cancer treatment is to eradicate cancer, preserve or restore form and function, minimise the complication of treatment and finally to prevent any subsequent new primary types of cancer. To achieve these goals, the currently available treatment pattern includes surgery, radiotherapy, chemotherapy, combined treatments and primary and secondary prevention strategies including lifestyle changes as well as chemoprevention (Shah & Gil, 2009). Radical removal of a tumour and surrounding tissues results in significant tissue defect. Thus, tissue and organ preservation would be a major aim of alternative therapeutic strategies (Kreimann et al., 2001). Cancer treatment with radiation therapy can result in oral mucositis and xerostomia due to patients with squamous cell carcinoma of the head and neck receiving radiation at the oral and pharyngeal regions (Silverman, 2007).

Recently, chemotherapeutic agents have been clinically used in combined treatments, and this has been reported to have a synergistic effect. Although the patient response in combined treatments is improved, it is often associated with toxicity, and there is very little change in the general survival rate of the patients (Ho & Slevin, 2008; Vermorken & Specenier, 2010). Depending on the location of the cancer tissue, the survival rate of the patient may vary. The survival rate for 50% of patient with cancers of the tongue is about five-years while the survival rate for 90% of patient with cancers of the lip is also about five years (Warnakulasuriya, 2009b). There are many complications associated with cancer treatments, and very often, patients have to overcome the devastating consequences of their treatment where the patient's appearance and function, e.g. eating, drinking, swallowing and speaking may be affected. These residual defects can lead to

other issues like depression and nutritional deficiency, and therefore the quality of life in this patients is also affected (Warnakulasuriya, 2009b).

Medicinal plants have played an important role in ancient medicine for preventive and curative treatments in many parts of the world population such as the Chinese, Ayurvedic, Unani and their secondaries in many Asian countries (Vasisht & Kumar, 2003). These medicinal plants have attracted much attention not only for their health benefits and disease treatment but also in drug discovery and development (Jiao et al., 2013).

Dracaena cinnabari (*DC*) (Agavaceae) is a perennial tree that is native to Socotra Island located on the Southern coast of Yemen. The tree produces a deep red resin called the Dragon's blood and is classified under the family of Dracaenaceae, a genus of Dracaena and species of cinnabari (Gupta et al., 2008). The dry powdered resin is often used in the Arabian Peninsula, as well as in other countries as a herbal remedy for many ailments including analgesic, astringent, antiseptic, haemostatic, antiulcer and as an abortifacient if taken during the first trimester of pregnancy (Al-Awthan et al., 2010). Although *DC* has been used as a herbal remedy for many ailments, the anticancer activity of the *DC* and its effect on oral cancer is not known.

1.2 Significance of the Study

Cancer of the oral cavity is one of the most common neoplasms worldwide, and the increased incidence of oral cancer in many countries is associated with the prevalence of risk-associated behaviours (e.g. smoking and alcohol consumption). Oral Squamous cell carcinoma (OSCC) represent over 90% of oral cancer which arises in the mucosal lining of the oral cavity. Due to the increased incidence of oral cancer worldwide, new approaches such as the use of medicinal plants for the treatment of oral cancer have been emerging. This study was carried out to investigate the anticancer effect of *DC* and the role of *DC* as an anticancer drug for the treatment of oral cancer.

1.3 Null Hypothesis

DC resin methanol extract is not safe to be used on animals and does not have anticancer activities towards oral cancer. The extract cannot be developed as a chemopreventive or chemotherapeutic agent for oral cancer prevention and treatment.

1.4 Purpose of the Study

1.4.1 Aim of Study

To evaluate the toxicity of *DC* resin methanol extract using an animal model, and to evaluate the anticancer activity (chemopreventive and chemotherapeutic) and metastasis obstruction of *DC* resin methanol extract on 4NQO-induced oral cancer in rats.

1.4.2 Objectives of Study

1. To evaluate the acute and sub-acute oral toxicities of *DC* resin methanol extract in rats.
2. To evaluate the chemopreventive and chemotherapeutic effects of *DC* resin methanol extract at different concentrations (100, 500 and 1000 mg) on oral carcinogenesis *in vivo* with respect to food consumption, water intake, body weight gain, haematological and biochemical parameters, tongue tumour size and histopathological changes in rat tongue epithelium.
3. To study the effect of *DC* resin methanol extract on oral cancer regional lymph node metastasis.
4. To investigate the expression of selected tumour markers; Cyclin D1, Ki-67, Bcl-2, p53, β-catenin and E-cadherin on oral cancer *in vivo* using immunohistochemistry.
5. To investigate the expression of selected apoptosis and proliferative genes related to oral cancer *in vivo* using RT^2-PCR.

CHAPTER 2: LITERATURE REVIEW

2.1 Overview of Cancer

The aetiology of cancer is multifactorial involving both genetic and environmental factors. Cancer cells are associated with abnormal cellular proliferation and exhibit prevalent features that distinguish them from normal cells (Stathopoulou et al., 2012). The abnormal cellular proliferation interrupts with the normal processes of cell division which are controlled by the genetic material (DNA) of the cells (Reddy et al., 2003). Several factors that can act together or in sequence to initiate or promote carcinogenesis include external factors (chemicals, tobacco, radiation, and infectious organisms) and internal factors (hormones, inherited mutations, immune conditions and metabolic mutations) (Srinivasan et al., 2008). Cancer can also be associated with improper diet, genetic predisposition and environmental factors. Current evaluations from the American Cancer Society and the International Union Against Cancer indicate that 12 million cases of cancer were diagnosed in the year 2008 with 7 million deaths worldwide. It is predicted that these numbers will be doubled by 2030 (27 million cases with 17 million deaths) (Bhanot et al., 2011).

2.2 Oral Cancer

The oral cavity is lined by a mucous membrane consisting of stratified squamous epithelium and lamina propria which is made up of dense connective tissue. The squamous epithelium of the hard palate, gingiva and the dorsum of the tongue is entirely keratinized with a superficial horny layer, whereas the epithelium of the cheek, lip, vestibular fornix, alveolar mucosa, the floor of the mouth and soft palate is non-keratinized (Syrjänen, 2003).

Squamous cell carcinoma of the oral cavity is one of the most common human cancers in the world. Jafari et al. (2013), stated that the successful treatment of oral cancer could

be achieved up to 70% to 90% when the tumours are diagnosed in the early clinical stage. However, 60–70% of cancers of the oral cavity are diagnosed only after the tumours have become locally progressive (Tang et al., 2014). OSCC represent over 90% of this group of oral cancer which arise in the mucosal (epithelium) lining of the oral cavity, pharynx and larynx (Ragin & Taioli, 2007; Valente et al., 2016). The World Health Organization (WHO) stated that the oral cavity cancer has one of the highest mortality ratios of all malignancies because of extensive local invasion and distant metastasis even at the stage when initial diagnosis was being made (Koh et al., 2011). The common site for intraoral carcinoma is mostly the tongue, which accounts for around 40% of all cases in the oral cavity proper. The tongue cancers frequently occur on the posterior-lateral border and ventral surfaces of the tongue. The second most common intraoral location of oral cancer is the floor of the mouth. Less common sites of oral cancer include the gingival, buccal mucosa, labial mucosa, and hard plate (Tanaka et al., 2011).

Cancer of the oral cavity is one amongst the most well-known neoplasms worldwide with up to 263,900 new cases occurred in 2008 including lip cancer (Jemal et al., 2011). It is frightening to note that the estimated number of deaths from oral cancer was 145,000 in 2012 and this is expected to increase in the next 15 years despite easy accessibility to this site of the body for diagnosis (Ferlay et al., 2015). The incidence of oral cancer varies worldwide with India and South and Southeast Asia showing the highest rates (Rodrigues et al., 2014). The incidence of oral cancer is increasing in many countries mainly because of the prevalence of risk-associated behaviours (e.g. varying patterns of use of tobacco and alcohol consumption). In many Asian countries including Malaysia, the consumption of betel quid chewing plays a vital role in the development of oral cancer (Saleh et al., 2014). In Malaysia, the incidence of oral cancer has been shown to be the tenth most common cancer among both the male and female Indian ethnic group, with the highest incidence reported in Indian females (Omar & Ibrahim Tamin, 2011). Furthermore, in

Malaysia, oral cancer has been ranked as the second most common cause of cancer-related deaths among males and this could be partially contributed to the late disease presentation as 70% of patients with oral cancer often presented with cancers of stage III/IV when initial diagnosis was being made (Saleh et al., 2016).

2.3 Carcinogenesis

Oral carcinogenesis is a multistep and complex process related to the serial incidence of modifications in genetic structures, promoting inhibitory or excitatory effects of the tumour oncogenes and gene suppressors, compromising the histophysiology of the cell division, differentiation and cell death (Shah & Kaur, 2014).

Carcinogenesis involves initiation, promotion and tumour progression in which a genetic damage may occur at any point of the disease (Macluskey & Ogden, 2000; Oliveira et al., 2007) (Figure 2.1). The first cellular alterations occur at the molecular level, such as alterations in the genetic material which are not detectable by conventional histopathology (Martínez & Rivera, 2012). Initiation comprises mutational events in unknown genes that may be caused by chemical carcinogens, radiation or viruses and is thought to be irreversible (Macluskey & Ogden, 2000). During this stage, the initiated cells can stay in a latent phase for several weeks, months or years, or they can grow in an autonomous and clonal pattern. This initiation process ensures that cellular division remains symmetrical by creating two new initiated cells (Trosko, 2003). Cell proliferation is essential for this stage, if cellular division occurs before DNA repair systems take place then the injury becomes permanent and irreversible (Oliveira et al., 2007).

In the later stages of promotion further genetic alterations may occur leading to malignancy (Macluskey & Ogden, 2000). Over time, these molecular changes accumulate causing massive individual cell changes that may be detected histologically and when the number of altered cells increases to a substantial amount, it is possible to observe

macroscopic changes of the tissues in the affected areas (Martínez & Rivera, 2012). The expansion of a malignant clone ultimately leads to local invasion and possible distant metastasis (Macluskey & Ogden, 2000). During carcinogenesis, chromosomal damage can lead to the activation of cell-cycle checkpoint controls that regulate cell progression and proliferation. The progression towards malignancy can range from hyperplasia through dysplasia to carcinoma in situ and invasive carcinoma, which are determined by the accumulation of sequences in genetic events (Ye et al., 2008).

Another explanation of carcinogenesis involves the process of initiation, promotion and progression that has been described by Surh (2003). Initiation is a rapid and irreversible process which involves a chain of extracellular and intracellular events. These include the initial uptake of or exposure to a carcinogenic agent, its distribution and transport to organs and tissues where metabolic activation and detoxification can occur, and the covalent interaction of reactive species with target cell DNA, leading to genotoxic damage. In contrast to initiation, tumour promotion is considered to be a relatively lengthy and reversible process in which actively proliferating preneoplastic cells accumulate. Progression, the final stage of neoplastic transformation, involves the growth of a tumour with invasive and metastatic potential.

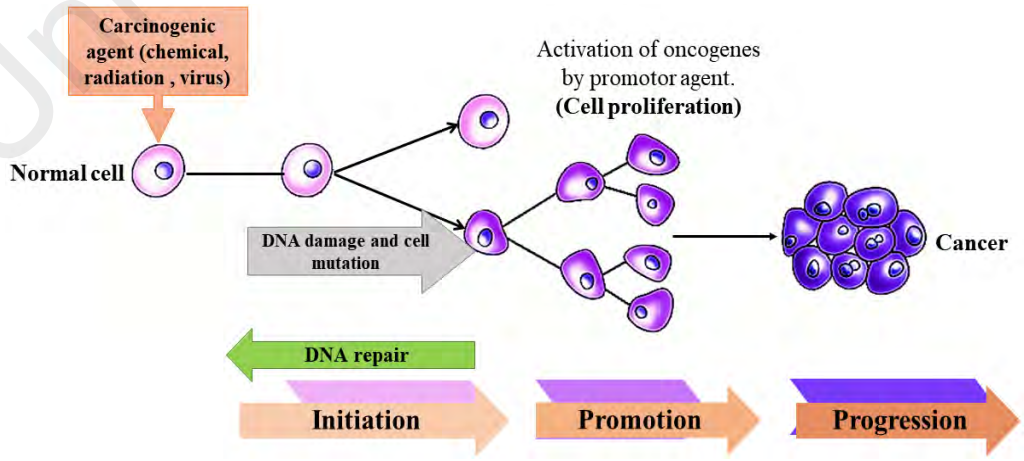

Figure 2. 1: The process of carcinogenesis involves initiation, promotion and tumour progression. Adopted from (Oliveira et al., 2007).

2.4 Clinical Characteristics and Histological Appearance of Oral Cancer

OSCC may appear in any location in the oral cavity. However, there are certain areas in which it is more commonly found. The most common locations for oral cancer with poor prognosis are the tongue and the floor of the mouth (Brandizzi et al., 2008).

The clinical presentation for oral cancer may vary as follows (Zain et al., 2002):

1. White lesion: This lesion may develop as a white area but is indurated. The surface may be nodular or ulcerated. There may be fixation if the cancer tissue is located on a movable part of the mucosa.
2. Red lesion: This lesion may develop as a red area with induration. The tissue is firm and thickened throughout the lesion or at the margins if ulcerated.
3. Ulcerated lesion: This lesion is ulcerated with indurations at the ulcer margins. The ulcer may have a raised, rolled border and may develop as a white ulcerated mass. It should be distinguished from a large solitary major aphthous ulcer, traumatic ulcer or infectious ulcer.
4. Exophytic mucosal swelling: The lesion may appear as a fungating exophytic mass which could easily bleed in the later stages. It may also appear as a painless lesion with a warty or white nodular surface as in verrucous carcinoma.

OSSC clinically can be differentiated in the advanced stages because there is usually a clear suspicion of malignancy. In contrast, it is relatively possible in the early stages to make the wrong diagnosis. Due to the insufficient clinical characteristics, a biopsy and histopathological examination will be crucial for the diagnosis of OSCC (Bagan et al., 2010).

Although this disease can cause destruction to the oral tissues and spread to regional lymph nodes, the metastases at distant sites for oral cancer are relatively small compared with other malignancies such as cancers of the stomach, pancreas, lung, breast or kidney.

However, when distant metastasizes to other organs occur, the chance for a cure from oral cancer becomes very low and the survival rate decreases (Garavello et al., 2006).

OSCC can be defined as a malignant epithelial neoplasm exhibiting squamous differentiation as characterised by the formation of keratin and the presence of intercellular bridges (Pindborg et al., 1997). Over 90% of OSCC is derived from the surface epithelium and extends from the basement membrane which would expand upward and laterally to replace the normal epithelium (Nagpal & Das, 2003). Based on the histologic resemblance of the cancer lesion to its original normal tissue, the WHO grading system (Barnes et al., 2005) classifies OSCC into well, moderately and poorly differentiated tumours as follows:

I. Well-differentiated SCC: The tumour tissue (and individual tumour cells) is similar to the squamous epithelial cells of normal oral mucosa. The tumour contains foci of extra- or intracellular keratinization. The cells have few mitotic figures and rarely show nuclear pleomorphism or variation in cell size and shape. Atypical mitoses are rare or absent.

II. Moderately-differentiated SCC: Tumour cells are less keratinized, and mitotic figures are present. Nuclear pleomorphism or variation of cell size and shape can be present. A few atypical mitotic figures may be seen.

III. Poorly-differentiated SCC: Tumour cells are rarely keratinized, and mitotic figures are more frequent. Nuclear pleomorphism and variation in cell size and shape are commonly seen. Atypical mitoses are common.

The presence of dysplasia in the epithelial tissue is believed to be associated with a possible progression to cancer. The term dysplasia is applied when a disturbance in its architecture is accompanied by cytological atypia (variations in the size and shape of the keratinocytes) (Warnakulasuriya et al., 2008).

There is evidence that the severity of the dysplasia is related to the progression of malignancy. Infrequently, non-dysplastic lesions may also show malignant development (Warnakulasuriya et al., 2008). The presence of dysplasia is more important in predicting malignant development than its clinical presentation such as leukoplakia, erythroplakia or leuko-erythroplakia (Reibel, 2003). Based on the abnormality of cell proliferation and disorderly maturation as seen in epithelial tissue, WHO classifies epithelial dysplasia as mild, moderate and severe where dysplastic changes are associated with the basal third of the epithelium or affecting up to two-thirds of the epithelium or more than two-thirds of the epithelium respectively (Barnes et al., 2005).

2.5 Risk Factors for Oral Cancer

The main factors attributed to the development of oral cancer are tobacco, consumption of alcohol or betel quid usage and that these factors may act separately or synergistically (Warnakulasuriya, 2009b). Both tobacco and alcohol have been shown to increase the risk of oral cancer by more than 80%. Heavy drinkers and smokers are 38 times more prone than the abstainers of alcohol and tobacco smoking (Blot et al., 1988). There are different forms of tobacco smoking such as oral and nasal smokeless tobacco and evidence for smokeless tobacco causing oral and pharyngeal cancer have been confirmed (Boffetta et al., 2008). Other factors such as HPV infection may also be involved in the cancer of the oral cavity and oropharynx (Herrero et al., 2003).

2.5.1 Alcohol and Tobacco

The consumption of alcohol and tobacco smoking are lifestyle risk factors which play a major role in oral cancer development and are considered as important etiological factors. Since the 70's, there are consistent observational studies which have reported that these lifestyle risk factors are significantly associated with oral cancer (IARC, 2004b, 2010). Factors such as alcohol consumption and tobacco smoking may act separately or

synergistically and are associated with an increased risk of various types of cancer, including those of the upper aerodigestive tract and liver. Both alcohol and tobacco use can increase the danger of cancer of the oral cavity and throat (pharynx), and their combined use has an increasing effect on the risk of oral cancer development. Compared to other regions, areas of the oral cavity and throat that are directly exposed to alcohol or tobacco are more prone towards cancer development (Pelucchi et al., 2008). In studies where smoking is controlled, moderate-to-heavy drinkers have been shown to have three to nine times greater risk of developing oral cancer (Lewin et al., 1998).

The risk of developing oral and oropharyngeal cancer has been shown to increase up to 30 times among heavy drinkers (more than 100 gm of alcohol per day) (Blot et al., 1988). As mentioned elsewhere, the effect of alcohol abuse on other types of carcinogens, particularly tobacco, has been shown as in heavy smokers who are also heavy drinkers, the risk of oral cancer increases up to 100 times (Andre et al., 1995; Blot et al., 1988). Alcohol can directly act on cell membranes and alters the mucosal permeability resulting in increasing penetration of carcinogens across the oral mucosa (Wright & Morgan, 2013). Moreover, during alcohol metabolism, acetaldehyde which is a cytotoxic compound is produced which results in the production of free radicals and hydroxylation of DNA bases which further causes cellular DNA damage (Scully et al., 2000). As stated by Ministry of Health Malaysia in 2006, the prevalence of alcohol users in the Malaysian population was around 12.2% where 1.5 million drinkers were between 25-64 years old, and when compared to women, men were found to be the most frequent alcohol drinkers in Malaysia (Tan et al., 2009).

The risk of the developing oral cancer has shown to be five to nine times greater in smokers compared to non-smokers. This risk may increase to as much as 17 times higher for heavy smokers who smoke 80 or more cigarettes per day (Andre et al., 1995; Blot et

al., 1988). Tobacco products contain a diverse array of chemicals, including nicotine and carcinogens. The effect of tobacco on human health varies depending on the types of tobacco products used and the duration of exposure (Xue et al., 2014). Nicotine is the principal component of all kinds of tobacco products and smoke. Nicotine is addictive and noncarcinogenic (Hukkanen et al., 2005). Most type of carcinogens, when exposed to organisms, are enzymatically transformed to other forms of metabolites that are more readily excreted by the organisms. The initial steps in this process involve cytochrome P450, and if the oxygenated intermediates are formed in these initial reactions, they may react with DNA or other macromolecules to form covalent binding products known as adducts (IARC, 2004b).

2.5.2 Betel Quid Use

In 1996, a consensus from a workshop held in Kuala Lumpur, Malaysia recommended that the term "quid" should be defined as "a substance or mixture of substances, placed in the mouth, usually containing one or both of the two basic ingredients, tobacco or areca nut, in a raw or manufactured or processed form" which remains in contact with the oral mucosa over an extended period (Zain et al., 1999). The composition of the quid can vary and in many countries, ready-made, mass-produced packets are available as proprietary mixtures known as pan masala or gutka (IARC, 2004a). In Malaysia, the major constituents of the quid are areca nut (taken either fresh or dried), betel leaf and slaked lime where they are occasionally folded in betel leaves like little parcels and chewed. However, the quid from different ethnic groups may also vary, and additional ingredients such as tobacco and spices may be added and this may be consumed using different chewing methods (Zain et al., 1999).

Tobacco, when added to the component of the quid, is associated with a significant risk of oral cancer. Another component of the quid, the lime, has been shown to release

reactive oxygen species from extracts of areca nut, which might contribute to the cytogenetic damage involved in oral cancer (Nair et al., 1992). Sharan et al. (2012) performed a systematic review and concluded that betel quid seems to be significantly associated with susceptibility to oral and oropharyngeal cancers. Despite the strong association between betel quid and human susceptibility to oral cancer, the habit of consuming betel quid is still taking place in many parts of the world.

2.5.3 Human Papillomavirus Infection

Human papillomavirus (HPV) infection is the most common sexually transmitted viral infection worldwide (IARC, 2007). The oral mucosa is lined by a mucous membrane, and its histology is similar to that of the uterine cervix or the lower genital tract. The histology of the oral mucosa consists of stratified squamous epithelium and lamina propria made up of dense connective tissue. Based on these similarities, one can anticipate that both the mucosal and cutaneous types of human papillomavirus (HPV) can also be present in the different types of squamous cell lesions of the oral mucosa (Syrjänen, 2003).

According to their clinical behaviour, HPVs are divided into two main groups. Low-risk HPVs cause wart-like lesions of the skin, anogenital region and the oral mucosa while high-risk HPVs are aetiologically associated with cervical and anogenital cancers (IARC, 2007; Syrjänen et al., 2011). HPVs are epitheliotropic DNA viruses that can induce hyperplastic, papillomatous and verrucous squamous cell lesions in the stratified squamous epithelia of skin and mucosa (Varnai et al., 2009).

The most frequently detected oncogenic HPV in OSCC is HPV16 and is present in up to 22% of cases, either alone or in combination with other HPV types e.g. HPV18 which may be present in up to 14% of cases (Sugerman & Shillitoe, 1997). Recently, HPV infection especially HPV16 has been identified as an etiologic agent for a subset of OSCC, particularly those that arise from the base of the tongue and tonsil (Gillison et al.,

2000). Patients with HPV DNA-positive OSCC have been shown to be younger and are less likely to have a history of tobacco or alcohol abuse compared to patients with HPV DNA-negative OSCC (Gillison, 2007). Interestingly, it has been shown that patients with HPV-positive SCC have a better outcome and were estimated to have up to 80% reduction in risk of disease failure compared to HPV-negative patients (Boscolo-Rizzo et al., 2013; Goldenberg et al., 2008).

2.5.4 Poor Diet and Nutritional Deficiencies

Poor diet, or a diet lacking in fresh fruit and vegetables, has appeared to be a significant risk factor for head and neck cancer, independent of tobacco, alcohol, betel nut consumption and HPV infection (Bradshaw et al., 2012; Veer & kampmann, 2007). A diet high in consumption of fruits and vegetables seems to reduce the risk of oral cancer in epidemiologic studies where tobacco and alcohol use are controlled (Pavia et al., 2006; Winn et al., 1984). More investigation about this phenomenon has shown that β-carotene and vitamin A supplement has significantly lead to regression of some of the oral potentially malignant disorders (Stich, Hornby, et al., 1988; Stich, Rosin, et al., 1988).

An animal study has shown that the occurrence of tumour in iron-deficient animals was significantly earlier (mean 183 days) when compared to the controls of non-iron-deficient animals (mean 229 days) (Prime et al., 1983). The hypothesis for this is that iron deficiency may increase one's susceptibility towards oral cancer as the epithelium becomes thinner, atrophic and more permeable epithelium towards chemical carcinogens (Rennie & MacDonald, 1982; Rennie et al., 1984).

It has also been reported that drinks and foods should not be consumed when they are scalding hot as this may increase the risk for cancers of the oral cavity, pharynx and oesophagus (Key et al., 2004). The consumption of meat, especially red and processed

meats, may also increase the risk of several types of cancer and that vegetarian diets are reported to be a useful strategy in reducing the risk of cancer (Lanou & Svenson, 2011).

2.5.5 Oral Hygiene and Chronic Inflammation

Poor oral hygiene and the status of an individual dentition (e.g. faulty restorations, sharp teeth, and ill-fitting dentures) have also been associated with oral cancer in a few epidemiological type of studies, but it is not clear whether confounding factors such as tobacco smoking and alcohol consumption were taken into consideration (Marshall et al., 1992; Warnakulasuriya, 2009a).

Periodontal disease has been shown to increase the risk of oral cancer. Periodontitis, characterised by epithelial proliferation and migration often results in the chronic release of inflammatory cytokines, chemokines, growth factors, prostaglandins and enzymes, all of which are associated with cancer development (Rajesh et al., 2013). This may explain why poor oral hygiene is linked to oral cancer. Some microorganisms facilitate the metabolism of ethanol to acetaldehyde in the oral cavity. Acetaldehyde, a known carcinogen, has been considered as a major factor in oral carcinogenesis (Kocaelli et al., 2014). The production of acetaldehyde by the microbes of the oral cavity, including *Candida albicans*, has been reported (Bakri et al., 2015). *Candida albicans* have the ability to invade the oral epithelium and cause the tissue to undergo dysplastic change (Bakri et al., 2014).

Evidence suggests that other factors such as inflammatory response and secretion of nitrosamines as generated by the fungus may also activate proto-oncogenes which are related to the development of oral cancer (Scully, 2011). A case-control study from Japan reported that frequent tooth brushing could reduce the risk of cancer of the upper aerodigestive tract, especially in the high-risk group of heavy tobacco and alcohol consumers (Sato et al., 2011).

2.5.6 Others

The predisposition to head and neck SCC in an individual with a family history of cancer is thought to be based on or at least in part, on the genetic variability in the metabolic activation and detoxification of the environmental procarcinogens, although it is noted that there is variability in individual susceptibility to chemically induced carcinomas (Bongers et al., 1996). The relative risk of oral cancer was between 1.2% and 3.8% for those who had a family history (first-degree relative) of head and neck cancer when compared with those with no such family history (Radoi & Luce, 2013).

Other factors such as immunosuppression has been reported to increase the incidence of lip cancer following kidney transplantation and is significantly related to use of immunosuppressive drugs such as azathioprine and cyclosporine (van Leeuwen et al., 2009). Other controversial risk factors with limited evidence include HIV infection, immunosuppression resulting from HIV infection, cannabis smoking, indoor air pollution and alcohol containing mouthwash (Warnakulasuriya, 2009a). The evidence that the use of mouthwashes increases the risk of oral cavity cancer remains controversial (Radoi & Luce, 2013). An in vitro study performed by McCullough and Farah (2008), reported that alcohol-containing mouth-washes have been shown to penetrate the oral microbial biofilms efficiently and thus reducing the oral bacterial load. However, as alcohol can be converted to acetaldehyde, a pro-carcinogen, by the oral microorganisms, it would be wise to restrict the use of an alcoholic mouthwash to short-term therapeutic situations like the use of alcohol-free mouth-washes may also be equally effective (McCullough & Farah, 2008). Similarly, it has been found that the use of alcohol-free chlorhexidine solution has been associated with a lower incidence of adverse events (e.g. staining of teeth and tongue, taste disturbances and reversible swelling of the lips) and that this mouthwash was better accepted by trial participants than the alcohol-containing chlorhexidine mouthwash (Santos et al., 2017).

2.6 General Treatment of OSCC

The goal of oral cancer treatment is to eradicate cancer, preserve or restore form and function, minimise the complications of treatment and finally prevent any subsequent new primary cancers. To achieve these goals, the currently available treatment modalities include surgery, radiotherapy, chemotherapy, combined modality treatments and primary and secondary prevention strategies including lifestyle changes as well as chemoprevention (Shah & Gil, 2009). The selection of treatment modalities depends on a wide range of factors including the site and stage of the lesion, preservation of the underlying anatomical structures and their function, patient and institutional preference, patient age, general health condition and risk of tumour recurrence (second primary tumour) (Fung & Grandis, 2010).

2.6.1 Surgery and /or Radiation Therapy

Surgery has been considered as one of the most well established primary treatment for oral cancers (Shah & Gil, 2009). Generally, in the early-stages of head and neck SCC (stage I and II), the treatment could consist of a single modality treatment of either surgery or radiation or a combination of both types of treatments. The success rate for both types of treatment has been reported to be 90% for stage I and 70% for stage II patients (Fung & Grandis, 2010). Radiation therapy is usually given as an adjunct treatment following surgery to prevent lymph node involvement (Cooper et al., 2004). In the advanced stages of SCC, the treatment and management are more complicated, and patients could be treated with a combination of chemotherapy, radiation, or surgery (Haddad & Shin, 2008).

2.6.2 Chemotherapy

Cancer continues to be one of the main causes of death in humans, and thus continuous efforts are being undertaken to develop cancer treatments (Leaf, 2004). Chemotherapeutic

agents can be used either as a single modality intervention or in combination with surgery or radiation in patients with recurrent and/or metastatic tumour (Vermorken & Specenier, 2010). Unfortunately, the commonly used cancer treatment of chemotherapy is detrimental to the patient's health, and moreover, the patients become more susceptible to other diseases, and this may often cause the death of the patient as the immune system of the patient's body is weakened (Carelle et al., 2002). In addition, the drug-resistant cancer cells that survived the chemotherapy treatment are responsible for the recurrence of tumours and is often associated with poor prognosis for the recovery of the cancer patients. The occurrence of drug resistance which may occur during treatment may also lead to the failure of tumour treatment (Gottesman, 2002). Among the challenges associated with chemotherapy treatment are the occurrence of toxicity on the remaining healthy cells and development of multi-drug resistance against the chemotherapeutic agents. The lack of tumour cell specificity towards chemotherapeutic agents has often restricted the strategy of delivering the systemic chemotherapeutic agents at a high dose which in turn, can prevent the life-threatening side effects of the chemotherapy agent (Carelle et al., 2002).

The most common component of chemotherapy for the treatment of solid organ cancers is Cisplatin that was first described by Michele Peyrone as early as in 1845 (Kim et al., 2015). Cisplatin and other platinum drugs are the common drugs in contemporary medical oncology and are considered to have a major impact in the management of ovary, testes, head and neck and other types of cancers (Kelland, 2007). However, the dose toxicities associated with platinum therapy has presented a serious concern as it has been shown that Cisplatin can affect certain organs such as the kidneys and also the nervous system (Hartmann et al., 1999; Hartmann & Lipp, 2003). Several forms of the chemotherapy treatment are targeted at the process of cell division as the proliferation rate for cancer cells are more likely to be higher than normal cells. Unfortunately, their

mode of action is not specific and are associated with significant toxicity side effects (Payne & Miles, 2008). As a result, the search for less toxic platinum compounds and treatment regimens or delivery methods that can help improve the anticancer efficacy effects of the compounds with minimum side effects still continues to this day (Amin & Buratovich, 2009; Cossa et al., 2009). However, chemoradiotherapy treatment which involves administration of chemotherapeutic agent with radiation therapy has been used in an effort to treat tumours without previous surgical intervention (Haddad & Shin, 2008).

2.6.2.1 Mechanisms of Anticancer Drugs

Currently, chemotherapeutic agents used for cancer treatment consist of around 60% small molecule compounds or natural products (Cragg et al., 1997). Small molecule compounds play a major role in anti-cancer medicine and are major players in anti-cancer drug discovery. The small molecule compounds are involved in several processes such as apoptosis, angiogenesis, cell cycle, macromolecule synthesis, mitochondrial respiration, mitosis, multidrug efflux and signal transduction (Nagle et al., 2004).

Most cancer therapeutic approaches, such as radiation and chemotherapy, inhibit cancer by activating cancer cell apoptosis (Marsoni & Damia, 2004). The apoptotic mechanism by using apoptotic inducers or modulators can be targeted for therapeutic benefits through the targeting of protein kinases, phosphatases, and transcription factors (Luo et al., 2008). The effective anticancer drug should be able to eliminate cancer cells without producing excessive damages to normal cells. However, this ideal situation can be reached successfully by inducing apoptosis in cancer cells (Taraphdar et al., 2001).

The cytotoxic process following exposure to chemotherapeutic agents can be described as follow; the chemotherapeutic agents disrupt cellular homeostasis which results in dysfunction of the target structures such as DNA, RNA or microtubules. Following the

disruption of the target structures, p53, bcl-2 and presumably other types of proteins activate the family of cysteine proteases known as caspases. Depending on the severity of the injury, the cells will then decide to undergo repair or proceed for apoptotic cell death, in which case activation of the caspases are initiated causing cell death (Bold et al., 1997).

2.7 Medicinal Plant as Anticancer Drugs

Although the present chemotherapeutic drugs improved the overall survival rate of patients, the outcome of treatment remains poor due to the development of drug resistance (Lee et al., 2013) and the presence of severe side effects of chemotherapy and radiation therapy (Madhuri & Pandey, 2009). Thus, alternative treatments which are 'back-to-nature' might yield improved treatment possibilities with fewer or no undesirable effects (Lee et al., 2013).

Medicinal plant formed the source of traditional medicine systems that have been used for centuries in many countries such as China, Egypt and India (Balandrin et al., 1993). However, preventive measures such as abstinence from the use of tobacco products, dietary changes, treatment of inflammatory diseases and consumption of nutritional supplements that support the immune functions are also being practised. Based on new discoveries in cell biology, researchers around the world are still continuing to further develop the use of the chemotherapy method for the treatment of cancer with minimum toxic side effects (Madhuri & Pandey, 2009).

On another hand, the term natural products refer to small molecules that are produced from biological sources (Krause & Tobin, 2013). Natural products have attracted wide attention not only in health promotion and disease treatment but also in drug discovery and development. The natural product discovery and development plays a major role in

the development of therapeutic agents for many types of diseases as well as for cancer treatment (Jiao et al., 2013).

WHO (2000) has characterised herbs to comprise of crude (rough and ready) plant materials such as leaves, flowers, fruits, seeds, stems, wood, barks, root, rhizomes or other plant parts which may be in its entire form, fragmented or powdered. Herbal products may also consist of herbal preparations made from one or more herbs and may contain excipients in addition to the active components (WHO, 2000). Herbal medicine or medicinal plants have been defined as the use of crude drugs of plant source to treat illnesses or to compliment and support the health system (Archer & Boyle, 2008). In the developing countries of Asia, medicinal plants play a major role in preventive and curative treatments (Vasisht & Kumar, 2003). The WHO has estimated that 80% of the population of the world depend on traditional medicines to help meet their essential health care needs (Gopal et al., 2014) and that 60% of the current drugs employed in the treatment of cancer are derived from sources found in nature (Gordaliza, 2007). In fact, more than 60% of cancer patients use vitamins or herbs in the management and treatment of cancer (Madhuri & Pandey, 2008).

The search for anticancer agents from plant-derived compounds started around the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine and the isolation of the cytotoxic podophyllotoxins. Between the 1960 and 1982, the National Cancer Institute screened around 114,000 extracts from an estimated 35,000 plant samples for anticancer activity (Cragg & Newman, 2005). The use of plant extracts has provided an option for the search for drugs with cytotoxic activity in inhibiting tumour growth (Júnior et al., 2010; Lee et al., 2010).

There are several steps involved in the discovery and evaluation of plant-derived anticancer agents; the initial step is the authentication and extraction of the plant material,

followed by the separation and isolation of the components of interest, characterization of the isolated compounds and quantitative evaluation (Evans, 2002).

An example of a plant-derived agent that has been successfully used as an anticancer drug is paclitaxel (Taxol®) which was prepared from the bark of the Pacific Yew, Taxus brevifolia Nutt. (Taxaceae) (Cragg & Newman, 2005). Other examples are topotecan and irinotecan which are semi-synthetic derivatives of camptothecin and are used to treat cancer of ovarian and colorectal cancers, respectively (Creemers et al., 1996; Fuchs et al., 2006).

2.7.1 Dragon's Blood

Dragon's blood is a term that refers to reddish resinous products, and this deep red resin has been used as a traditional medicine since ancient times by many cultures. Dragon's blood can be found in several forms; granules, powder, lumps or sticks. The term "Dragon's blood" is used for all kinds of resins and saps isolated from four distinct plant genera endemic to various regions worldwide; *Croton* (Euphorbiaceae) from the tropics and subtropics countries around the world, *Dracaena* (Dracaenaceae) Socotra of Canary Islands, Madeira and East Africa, *Daemonorops* (Palmaceae) from South East Asia, and *Pterocarpus* (Fabaceae) of West Indies and South America. The origin of the Dragon's blood is believed to be from Socotra Island which is located on the southern coast of Yemen (Alexander & Miller, 1996; Edwards et al., 1997; Gupta et al., 2008).

Many years ago, Dragon's blood was used by the Greeks, Romans and Arabs due to its medicinal properties. The Dragon's blood (*Dracaena*) was used by residents of Moomy city on Socotra island and is known as a "cure-all" as it is used for wound healing, diarrhea, fevers, dysentery diseases, internal ulcers of the mouth, throat, intestines, stomach, as a coagulant, as an antiviral for respiratory and stomach viruses and skin disorders such as eczema. In general, it used for curing dysentery, diarrhoea, haemorrhage

and external ulcers in Yemeni folk medicine (Milburn, 1984). In addition, a resin of Dragon's Blood in Unani medicine is commonly known as Dammul-akhwain. It has also been widely used as an anti-inflammatory, antioxidant and antitumor agent (Milburn, 1984).

A resin of Dragon's blood obtained from *Croton urucurana* has been evaluated and reported to exhibit antibacterial activity against *Staphylococcus aureus* and *Salmonella typhimurium* (Peres et al., 1997). Other studies reviewed by Jones (2003) and Gonzales and Valerio (2006) presented that dragon's blood resin (*Croton spp*.) possesses an anti-diarrheic, anticancer, anti-inflammatory and antirheumatic activity. In addition, dragon's blood resin obtained from *Daemonorops spp.* is a popular traditional Chinese medicine used to stimulate circulation, promote tissue regeneration and was also use for healing of fractures, sprains, ulcers and for the control of bleeding and pain sensation (Bensky et al., 1993).

2.7.2 *Dracaena cinnabari Balf.f*

Dracaena cinnabari (*DC*) (Dragon's Blood Tree) is an endemic species and the most famous plant of the Socotra Island, Republic of Yemen and it is classified under the family of *Dracaenaceae*, genus of *Dracaena* and species of *cinnabari* (Gupta et al., 2008; Hubálková et al., 2015). It is a large, single-trunked tree with a height of up to 10 m with smooth grey barks. Its branches, which are sausage-shaped, form an umbrella-shaped crown (Hubálková et al., 2015) (Figure 2.2). The genus *Dracaena* includes 60 to 100 species and has been taxonomically classified as three families, i.e. *Agavaceae*, *Liliaceae* and *Dracaenaceae*, the latter is a family formerly encompassing the other two (Adolt & Pavlis, 2004). Initially, *DC* was first described by Lieutenant Wellsted of the East India Company in 1835 during a survey of Socotra Island and was named *Pterocarpus draco*.

However, in 1880 the Scottish botanist Isaac Bayley Balfour made a formal description of the species and renamed it as *Dracaena cinnabari* (Balfour, 1888).

Figure 2. 2: *Dracaena cinnabari* (Yehia et al., 2013)

Locals in Socotra used the resin from *DC* for many purposes; pottery and house decorations, textile dye (wool), glue pottery, breath freshener and even used as a lipstick (Alexander & Miller, 1996). In addition, *DC* resin has been used as a famous traditional medicine since ancient times in many cultures. It is used as an astringent for treating diarrhoea and dysentery as well as an antiseptic, haemostatic and as an antiulcer remedy (Gupta et al., 2008; Gupta et al., 2009; Mothana et al., 2007; Yehia et al., 2013).

Several active compounds have been subsequently isolated and identified from the *DC* resin. These compounds mainly consist of homoisoflavans, along with flavans, flavanones, flavones, chalcones, dihydrochalcones, di- and triflavonoids (Himmelreich et al., 1995; Machala et al., 2001; Suchý et al., 1991). The dominant compound in the extracts of fresh material of *DC* resin was 7-hydroxy-3-(4- hydroxybenzyl)-8-methoxy-chroman (Baumer & Dietemann, 2010). In addition, (2S)-7-hydroxyflavan-4-one, 4,4'-

Dihydroxy-2'-methoxychalcone, Cinnabarone 7,4'-Dihydroxyflavone, Damalachawin and Dracophane have been identified in previous studies (Himmelreich et al., 1995; Masaoud, Ripperger, Himmelreich, et al., 1995; Masaoud, Ripperger, Porzel, et al., 1995; Veselá et al., 2002).

The *DC* extract has also been shown to possess numerous therapeutic activities: the analgesic and anti-inflammatory effects of the ethanolic extract of *DC* balf resin have been investigated and shown to have a significant peripheral analgesic effect when tested using the acetic acid-induced writhing response test. It was then suggested that the release of endogenous mediators of pain (such as prostaglandin, kinin, histamine) stimulate the nociceptive neurones, which are sensitive to non-steroidal anti-inflammatory drugs and opioïds (Alwashli et al., 2012).

The presence of flavonoids in the methanolic extract of *DC* may contribute to anti-inflammatory activities as flavonoids are recognised to target prostaglandins which are involved in the late phase of acute inflammation (Gupta et al., 2014). The *DC* resin has also been shown to exert a relaxant effect on rat isolated ileum, uterus and urinary bladder and this effect may again be attributed to the presence of flavonoids in the *DC* resin (Al-Awthan et al., 2010). Another possible attribute of flavonoids in *DC* is that *DC* also increases heart contractility (Al-Awthan et al., 2010). Furthermore, in anaesthetized rats, the aqueous extract of *DC* has been reported to reduce both the systolic and diastolic blood pressure, and this could have been due to vasodilatation of the blood vessels of the smooth muscles which were relaxed. Due to the hypotensive effect related to the vasodilation or increased glomerular filtration rate, this can also result in increased urinary excretion in conscious rats (Al-Awthan et al., 2010).

A study performed by Mothana and Lindequist (2005) reported an antimicrobial activity of *DC* resin (chloroform and methanol extract) against *Staphylococcus*

aureus, *Bacillus subtilis*, *Micrococcus flavus* and *Escherichia coli*. The methanol extract of *DC* has also been reported to exert an antiviral activity against herpes simplex and human influenza viruses (Mothana et al., 2006). Recently, Ansari et al. (2016) also reported antimicrobial activity of the ethanolic extract of *DC* against Gram-positive, Gram-negative pathogens and fungi and concluded that *DC* could play a major role in controlling microbial infections in human. Due to the antimicrobial and antioxidants properties of *DC* resin (Gupta & Gupta, 2011; Yehia et al., 2013), *DC* resin could be considered as a potential food preservative agent due to its inhibitory effect on various foodborne pathogens (Gupta & Gupta, 2011). Due to its high cytotoxicity, the methanolic extract of *DC* also exhibited a non-specific inhibition of the parasites (Mothana et al., 2012) while the anti-diabetic activity of the ethyl acetate extract of *DC* tested using glucose uptake inducing activity (98.86%) indicate that this extract has the potential to be utilized as an antidiabetic drug (Mohammed & Khanum, 2016).

DC has also been investigated for its antitumor and cytotoxicity effect and was found to be very effective. In 1995 Vachálková and his colleges studied the carcinogenicity of three homo-isoflavonoids and four flavonoids isolated from the resin of *DC* (Vachálková et al., 1995). The cytotoxic activity of *DC* resin collected from Yemen was investigated against human ECV-304 cells and displayed marked toxicity (IC_{50} of 8.9 µg/ml) (Al-Fatimi et al., 2005).

A study on the effect of *DC* on five human cancer cell lines: two lung cancers (A-427 and LCLC-103H), two urinary bladder carcinomas (5637 and RT-112) and one breast cancer (MCF-7) have been conducted. It was reported that the methanol extract of *DC* demonstrated the highest toxicity on all tumour cell lines with IC_{50} values ranging between 0.29 and 5.54 µg/ml (Mothana et al., 2007). Recently, the methanol extract of the *DC* crude extract was studied by Alabsi et al. (2016) where the cytotoxic effects of

DC when observed in a panel of OSCC cell lines were most pronounced in H400 cells, grown in a time-dependent manner with an IC_{50} value of 5.9 μg/ml. It was reported that the mode of cell death was achieved through the process of apoptosis (Alabsi et al., 2016).

2.7.3 Extraction of Plants

Plants have often been considered as the source for different drugs groups such as anti-cancer, antimicrobial, antispasmodics, etc. All over the world, the tribal people have always depended on many types of plants for curing several types of diseases. Based on traditional claims many researchers have continued to evaluate the relationship between different types of plants and their ability in treating diseases (Srivastav & Das, 2014; Tiwari et al., 2011).

Extraction is a term used to describe the process of separating active components of the plant from the inert plant's constituents by using specific solvents through standard procedures. Following extraction, the extracted products may be kept in several forms; liquid state, semi-solid state, dry powder or in relatively complex mixtures of metabolites and can be intended for external or oral use (Tiwari et al., 2011).

Following treatment of the plants with a selective solvent (menstruum), extraction of the plant extracts can be performed using basic techniques such as maceration, infusion and percolation. There are a few factors to be considered when the extraction process is completed; the transport rate of solvent into the plant mass, the solubility rate of the soluble plant components by the solvent and the transport rate of solution out of the insoluble plant mass (Singh, 2008). As stated by Tiwari et al. (2011), the quality and quantity of an extract depend on many basic parameters: parts of the plant used for extraction, properties of the solvent selected for the extraction process such as its concentration, especially when extracting the therapeutic components (obtaining) from the inert unwanted plant's components (eliminating) following treatment with a selective

solvent (menstruum). Several factors may affect the extraction process such as the polarity of the extract as this may affect the quantity as well as the presence of a secondary metabolite of an extract. Finally, the extraction methods can also play a significant role as the type of extraction process, the extent of extraction period and temperature at which the process of extraction was carried out can affect the quantity and quality of the obtained extract.

In general, the plant's materials should be grounded into finer portions as this will increase the surface area of the plant's materials, and in turn, this will also increase the contact area of the plant material with the selected solvent chosen for the extraction process. Consequently, increasing the surface area of the plant material will increase the rate of the extraction process. It is advocated that the ideal ratio used for mixing the plant materials to the solvent be 10:1 (selected solvent volume: weight of plants materials) (Das et al., 2010).

2.7.3.1 Solvents Used in Extraction of Plants

Many types of solvents have been used in the process of plant extraction. The most commonly used solvents are as follow:

1. Water: Water has been considered as the universal solvent. It is inexpensive and used to extract plant products with antimicrobial activity. Investigators have been using water extracts for the initial screening of plants for possible antimicrobial activity (Cowan, 1999). However, plant extracts obtained using other organic solvents (e.g. alcohols) have been found to give better and more consistent antimicrobial activity than plants extracts obtained using water as the solvent (Das et al., 2010).
2. Acetone: It is used to dissolve many types of hydrophilic and lipophilic components of the plants. It is volatile, miscible with water and has a low toxicity. It is the solvent of choice when more phenolic compounds with anti-microbial activity are required to

be extracted (Eloff, 1998). Earlier studies stated that extraction of tannins and other phenolic components was better in aqueous acetone than in aqueous methanol (Das et al., 2010; Eloff, 1998).

3. Alcohol: Two of the most common types of alcohol used as a solvent for plant extraction are ethanol and methanol (Bimakr et al., 2011; Wang et al., 2010). They have been extensively used to extract antioxidant compounds from various types of plants and plant-based foods (fruits, vegetables, etc.) (Peschel et al., 2006). Using alcohol as a solvent can produce plant extracts with higher antimicrobial activity when compared to the use of water as the solvent (Lapornik et al., 2005).

The combined use of water and organic solvent may facilitate the extraction of chemicals that are soluble in water and/or organic solvent. This may explain the reason why the yield of plant extracts using solvents such as aqueous methanol, ethanol, and acetone extracts are higher than the yield of plant extracts using water, methanol, ethanol, and acetone solvents (Do et al., 2014). However, there has been a report showing that plant extracts obtained using aqueous methanol as the solvent were more effective in recovering the highest amounts of phenolic compounds from rice bran (Shahid Chatha et al., 2006). When flavonoid compounds were extracted from the leaves of spearmint using different types of solvent, the use of methanol produced the highest yield of plant extracts (Bimakr et al., 2011). In general, the methanol solvent has more polarity than ethanol, and this could have attributed to the extraction process being more efficient and enabling the extraction of more plant constituents (Bimakr et al., 2011; Wang et al., 2010).

2.7.3.2 Extraction Methods

Many extraction methods have been used for extracting plant extracts. The most commonly used techniques are maceration, infusion and percolation. These methods can

be utilized for the initial plant extraction process and can also be employed for bulk extraction of plant constituents (Handa et al., 2008).

(a) ***Maceration***

Maceration is one of the basic techniques for extraction in medicinal plant research. The general procedure of maceration includes soaking a suitable ground plant material into a selected solvent (menstruum) in a closed vessel, and following this, the mixture can stand at room temperature with constant shaking for at least three days. This step is intended to soften and break the plant's cell wall to enable the release of soluble phytochemicals. Following this, the mixture is then strained by filtration leaving behind a solid residue of the mixture (marc) which is then pressed to obtain as much occluded liquid as possible from the marc. Then, the pressed and strained liquid is mixed and filtered to separate it from the coarse plant materials.

The general principle of maceration, also referred to as leaching, can be affected by several factors; the transport rate of the solvent into the plant materials, the solubilizing rate of the plant's soluble constituents into the solvent and the transport rate of the solution out of the insoluble plant materials. Thus, grinding the plant material to minimise its size is essential to increase the contact surface area between the plant material and the solvent. By decreasing the radial distances of the plant solid materials, the transport rate of the solvent into the plant materials as well as transport of the solution from the insoluble plant materials will improve. Frequent shaking during maceration is a vital step which supports the diffusion process and ensures dispersal of the concentrated solution around the plant's material surface and a continuous flow of fresh solvent to the plant's material surface for further extraction (Handa et al., 2008).

(b) ***Infusion***

Fresh infusions are produced by macerating the plant materials for a short period using either cold water or boiling water. The solution produced is the soluble constituents of the plant materials (Handa et al., 2008).

(c) ***Percolation***

This procedure is the most frequently used method for extracting active constituents in the preparation of tinctures and fluid extracts. A narrow, cone-shaped vessel is opened from each end of the percolator, and the plant material is moistened with an appropriate amount of solvent and allowed to stand for approximately 4 hours in a well-closed container. Following this, the mass is packed, and the top end of the percolator is closed. Additional solvent is added until it forms a shallow layer above the mass and the mixture is left to macerate in the closed percolator for 24 hours. The outlet of the percolator then is opened, and this will allow the liquid to drip down slowly. The plant material is then pressed and the expressed liquid is added to the percolator. Sufficient solvent is then added to produce the required volume which is then clarified by filtration (Handa et al., 2008).

2.8 Chemopreventive Agents

Cancer chemoprevention was first defined by Sporn (1976). This term refers to the use of natural, synthetic, or biologic chemical agents in an attempt to reverse, suppress or prevent carcinogenic progression. Chemopreventive agents consist of materials of natural or synthetic origin. The difference between these agents and other drugs (which do not prevent disease) is that the incidence of diseases such as cancer is reduced before the onset of clinical symptoms (Tanaka et al., 2011). It can also suppress the elevation of cancer and prevent the transformation of premalignant cells by modifying the cell differentiation process (Schwartz, 2000). The mechanism of cancer prevention is targeted

at any of the six principles of essential alterations in cell physiology that is required for tumour growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan & Weinberg, 2000).

The strategy for chemoprevention treatment is often classified into three broad categories; primary, secondary or tertiary strategy. By exerting a barrier, the primary prevention approach (blocking agents) prevents healthy people who are at risk (e.g. smokers) from developing cancer, and this involves preventing the carcinogenic agents from reaching or reacting with the important target site (Shukla & Pal, 2004; Soria et al., 2003). Secondary prevention involves patients who are already diagnosed with premalignant lesions (e.g. oral leukoplakia). Prevention of the progression of the premalignant lesions into cancer involves decreasing the susceptibility of the target tissue to carcinogenic stimuli using a chemopreventive agent. Tertiary prevention approach is aimed at patients who are cured of their primary cancer or patients who have been treated for their premalignant lesions and to prevent the recurrence or development of secondary-primary tumours (Shukla & Pal, 2004; Soria et al., 2003). The requirements for a chemopreventive agents are; (1) minimum toxicity or none at all, (2) high efficacy in several sites, (3) proficiency for oral consumption, (4) known mechanism of action, (5) low cost, and (6) human acceptance (Lee & Choi, 2011).

There are several examples of chemopreventive agents that have been used to treat head and neck cancers that were induced in animals (*in vivo*); the benefits of using apple extract has been reported to suppress rat tongue carcinogenesis due to its anti-inflammatory activity and ability to induce apoptosis through the intrinsic mitochondrial pathway, particularly by downregulation of COX- 2 and TNF-alpha expression and

increased expression of Cytochrome C and caspase 3 (Ribeiro et al., 2014). Extraction of green tea polyphenols inhibits Phase I enzymes (high in rat treated with 4NQO) that deactivate the carcinogen, and this plant extract can also induce the expression of Phase II enzymes that detoxify 4NQO. It was then suggested that green tea polyphenols play a role as a detoxifying agent by preventing/inhibiting the formation of cancer (Srinivasan et al., 2008).

2.9 4NQO Carcinogenesis in Animal Models

Numerous animal models (such as a hamster, rats and mouse) are currently being utilised for research in oral cancer development, and these animal models have been established for the evaluation of therapeutic agents (synthetic or natural) (Suzuki et al., 2006). Many carcinogenic agents such as coal tar, 20 methyl cholanthrene (20MC), 9,10-dimethyl-1,2-benzanthracene (DMBA) and 4-nitroquinoline-1-oxide (4NQO) have been used to induce oral cancer, but 4NQO is the preferred carcinogen in the development of experimental oral carcinogenesis (Kanojia & Vaidya, 2006).

4NQO is a water soluble quinoline derivative (a highly carcinogenic chemical), which stimulates tumours development mainly in the oral cavity (Kanojia & Vaidya, 2006) especially on the tongue of rats following consumption of 4NQO in drinking water or by topical application of 4NQO to the oral mucosa (Long et al., 2007). As a result, the development of oral carcinogenesis all stages are produced; hyperplasia, dysplasia, severe dysplasia, carcinoma in situ and squamous cell carcinoma (Kanojia & Vaidya, 2006). 4NQO induces the formation of DNA adducts; whereby adenosine is substituted for guanosine and induces intracellular oxidative stress resulting in mutations and DNA strand breaks. These genetic alterations are similar to that as provoked by the tobacco carcinogens and therefore, 4NQO has been associated as a substitute for tobacco exposure (Vitale-Cross et al., 2009).

As the 4NQO carcinogenesis in animal models can result in a spectrum of preneoplastic and neoplastic lesions, it is the suitable model for studying oral squamous cell carcinoma (OSCC) as well as to evaluate the efficiency of chemopreventive and chemotherapeutic agents and screening for biomarkers for both tumour progression and treatment (Peng & McCormick, 2016). In fact, this model has the advantage as the development of the neoplastic lesions was found to be parallel the development of OSCC in humans, i.e., the dysplastic lesions are produced by long-term ingestion of small amounts of carcinogen (Vered et al., 2005). The 4NQO induced tongue carcinogenesis model has been considered quite useful for investigating oral carcinogenesis and identification of cancer chemopreventive agents because the most common site for oral carcinoma is the tongue and the administration of drinking water containing 4NQO is a straightforward and easy method (Tanaka et al., 2011). The oral lesions produced by 4NQO are similar to human lesions due to the development of dysplasia and the presence of many ulcerated, endophytic or exophytic tongue tumours (Yoshida et al., 2005).

Long et al. (2007) studied the chemopreventive effect of fermented brown rice and rice bran on 4NQO induced tongue neoplasms in rats. When 4NQO was given at a concentration of 20 ppm in their drinking water for eight weeks, endophytic or exophytic tumours developed in the dorsal site of the rat's tongues. In a related chemopreventive study, oral tongue carcinogenesis in rats was also induced following eight weeks of 4NQO administration in their drinking water (Ribeiro et al., 2014). The primary histopathological change for all groups exposed to 4NQO was hyperplasia, hyperkeratosis with the spinous cell layer gradually thickened and epithelial dysplasia (Ribeiro et al., 2014). The use of 4NQO to induce oral cancer *in vivo* have been used in many animal studies as shown in Table 2.1.

Table 2. 1: 4NQO carcinogenesis in animal models

Animal	Route of administration and concentration of 4NQO	Duration of Administration	Duration of experiment	Product used as treatment	Tissue affected in the oral cavity	References
Male Wistar Rats	0.5% 4NQO in propylene glycol applied to tongue by a paint brush	Thrice a week for 8 weeks	22 weeks	Green tea polyphenols	Tongue	(Srinivasan et al., 2008)
F344 Rats	DW of 4NQO (20 ppm)	8 weeks	32 weeks	Auraptene	Tongue	(Tanaka et al., 1998)
Male Albino Rats	DW of 4NQO (50 ppm)	8 or 12 weeks	12 weeks	Curcumin	Tongue	(de Paiva Gonçalves et al., 2015)
Male Sprague-Dawley Rats	DW of 4NQO (20 ppm) for 4 weeks followed by (30 ppm)	8 weeks	18 weeks	Zn gluconate	Tongue	(Fong et al., 2011)
	DW of 4NQO (10 ppm)		21 weeks			
Female C57BL/6 Mice	DW of 4NQO (100 µg/ml)	16 weeks	16 weeks	Grape seed extract and resveratrol	Tongue	(Shrotriya et al., 2013)
Female Wild-Type C57BL/6 Mice	DW of 4NQO (100 µg/ml)	10 weeks	27 weeks	Bexarotene and the retinoid CD1530	Tongue	(Tang et al., 2014)
Male F344 Rats	DW of 4NQO (20 ppm)	8 weeks	32 weeks	Lactobacillus Salivarius REN	Tongue	(Zhang et al., 2013)
Male Wistar Rats	DW of 4NQO (20 ppm)	8 weeks	8 weeks	Grape juice	Tongue	(de Jesus et al., 2014)

Table 2.1 continued

Male Fisher Rats	DW of 4NQO (20 ppm)	14 weeks	32 weeks	Polyethylene glycol (PEG)	Tongue	(Wali et al., 2012)
Male Wistar Rats	DW of 4NQO (20ppm)	8 weeks	8 weeks	Apple extract	Tongue	(Ribeiro et al., 2014)
Male Wistar Rats	0.5% 4NQO in propylene glycol applied to tongue by a paint brush	Thrice a week for 8 weeks	24 weeks	Cisplatin and Telmisartan	Tongue	(Patel & Damle, 2013)
Male Sprague-Dawley Rats	DW of 4NQO (50 ppm)	12 weeks	24 weeks and 36 weeks	Green tea catechin	Tongue	(Kuroda & Hara, 1999)
Male Sprague-Dawley Rats	4NQO dissolved in acetone then given in DW (30 ppm)	120, 160 and 200 days	15 days after completion of 4NQO	-	Tongue	(Soni et al., 2014)

DW= Drinking water, ppm (parts per million) = milligrammes per litre.

2.10 Molecular and Genetic Changes during Carcinogenesis

Molecular biology can be defined as the study of biology at a molecular level and involves understanding the interactions between DNA, RNA, and protein biosynthesis and how these interactions are controlled (Ahmed & Jigna, 2009). The ability to the identify genes involved in human disease has led to a revolutionary increase in the scientific knowledge of genetic disorders, its pathological process and advancement in the treatment of genetic disorders. Once a diagnosis of a genetic disorder has been made, the clinical application, based on the background knowledge of the disease, will be applied for the treatment of the disease. In fact, molecular techniques have provided a strong tool for genetic diagnosis in many cases allowing accurate identification of individuals who carry a mutation by using any readily accessible source of tissue for analysis of DNA (Harada & Korf, 2013). The use of biomarkers in cancer screening prevention as well as for detection of the earliest stages of oral mucosal malignant transformation is gaining importance. It reveals the genetic and molecular changes related to early, intermediate and late end-points processes of oral carcinogenesis. These biomarkers will refine the ability to enhance the prognosis, diagnosis, and treatment of oral carcinomas. Genetic and molecular biomarkers have also used in studies to determine the efficacy and safety of the chemopreventive agents (Tanaka & Ishigamori, 2011).

Some examples of the molecular analytical tools that have been used for determining the diagnosis and treatment of oral cancers are chromosome in situ hybridization, cytomorphometry, immunohistochemistry, polymerase chain reaction (PCR), DNA image cytometry, DNA microarrays, proteomics and gene therapy (Ahmed & Jigna, 2009). Tissue biopsies taken on patients is a common procedure used for diagnosis of oral cancer although it may be uncomfortable for the patient and time-consuming. Apart from tissue biopsy, non-invasive oral screening methods such as brush biopsy, autofluorescence imaging and toluidine blue staining have also been used to diagnose oral

cancer (Lingen et al., 2008), however, it has been reported that the experience of the physician is critical when using those methods (Huang et al., 2017).

There are various types of mutagenic agents and depending on the type or nature of stimuli; chemical (tobacco), physical (radiation) or infection (*Human papillomavirus, Candida*), these mutagenic agents that may cause changes in gene and chromosome structure by point mutations, gene amplifications and chromosomal rearrangements. Some of these changes may occur spontaneously and these genetic alterations, which can occur during carcinogenesis, may be used as targets when detecting cancer cells in clinical samples (Todd et al., 1997; Williams, 2000).

Oral carcinogenesis is a complex, multistep process in which specific genetic events within a signal transduction pathways that control the normal cellular physiology are changed either quantitatively or qualitatively (Sidransky, 1995; Todd et al., 1997). In normal conditions, the basic cellular functions such as cell division, differentiation and senescence which are under control in normal tissues are affected in cancer tissue. The regulation of oral epithelium involves tightly controlled excitatory and inhibitory pathways. The excitatory or inhibitory signals are generated when growth factors bind to a cell surface receptor forming a receptor-ligand complex, and the signals are sent through intracellular and nuclear messengers that can either directly alter cell function or can stimulate the transcription of genes. However, oral cancer may occur when there is an accumulation of changes in the excitatory and inhibitory cellular pathways that can occur at any level of a given pathway. As the cell collects these alterations or mutations, it becomes functionally independent from the surrounding oral epithelium, the normal oral keratinocyte neighbours (Sidransky, 1995). The normal cellular functions which are strongly controlled by the excitatory and inhibitory pathways are destabilised in a cancer cell, allowing it to divide more rapidly, sequester blood vessels to feed that growth, delete

or amplify signals to produce abnormal structural or functional changes and invade normal tissue at local or distant sites. Accumulation of these changes is believed to generate the histologic progression of oral carcinogenesis from hyperplasia to dysplasia, followed by severe dysplasia and eventually invasion and metastases (Todd et al., 1997; Vokes et al., 1993).

Genetic damage to cancer cells can be divided into two categories: "dominant" or alteration of genes in the excitatory pathways involving proto-oncogenes and "recessive" or mutation of growth inhibitory pathways involving tumour suppressor genes. "Dominant" genetic damage results in a gain of function, whereas "recessive" damage causes loss of function" (Bishop, 1991). In neoplasms, cell proliferation is excessive and autonomous, uncoordinated and despite DNA damage due to loss of cell cycle check-points, the process of cell division would continue (Nagpal & Das, 2003).

Proto-oncogenes are known as a group of genes that cause normal cells to become cancerous when they are mutated (Weinstein & Joe, 2006). When proto-oncogenes are mutated, the mutated version of proto-oncogenes is called oncogenes. Proto-oncogenes often encode proteins that function to stimulate cell division, inhibit cell differentiation and halt cell death. These processes are necessary for normal human development and the maintenance of tissues and organs. However, oncogenes exhibit increased production of these proteins, thus leading to increased cell division, decreased cell differentiation and inhibition of cell death. Thus, oncogenes are currently a major molecular target for anti-cancer drug design (Chial, 2008). They were originally described in relation to RNA viruses (retrovirus) containing nucleic acid sequences capable of inducing oncogenic properties in host cells (Weiner & Cance, 1994). These genes are described as the fundamental components of a multistep tumorigenesis. The mechanism by which oncogenes are activated includes point mutations, gene amplification and gene

overexpression. Accurate regulation of all this positive and negative signalling is essential to preserve normal cell growth, and disturbance of such regulation can lead to neoplasia (Khan et al., 2010). Epidermal growth factor receptor (EGFR) (a tyrosine kinase receptor from the ErbB family) plays a key role in many of the cellular processes involved in cancer development and has proven to be a promising target for cancer therapy (Oliveira et al., 2006). It is an oncogene that has been overexpressed in numerous malignancies, including cancer of the prostate, breast, bladder, pulmonary system and head and neck (Oliveira et al., 2006). EGFR appears to be the dominant controlling factor underlying the malignant phenotype in squamous cell carcinomas of head and neck, via adjustment of the molecules involved in invasive angiogenic and lymphangiogenic processes (Pornchai et al., 2002).

Tumour suppressor genes normally act by inhibiting cell proliferation and tumour development. In several tumours, these genes are lost or inactivated resulting in negative regulators of cell proliferation and contribute to the abnormal proliferation of tumour cells (Cooper & Hausman, 2013b). Many tumour suppressor genes, such as the retinoblastoma (Rb) gene, were initially identified in paediatric tumours that are formed early in life and the loss of this tumour suppressor activity was reported to be necessary for oral carcinogenesis to take place (Todd et al., 1997).

In approximately 70% of all known tumours presented in the adult, the tumour suppressor gene p53 was reported to be mutated (Todd et al., 1997). However, in SCC of the head and neck region, 40-50% of the tumours studied have shown mutation of the p53 gene (Nylander et al., 2000). The name p53 gene is used because it produces a 53 kDa nuclear phosphoprotein (wild type or normal p53 protein) which is located on the short arm (p) of chromosome17 (Scully et al., 2000).

In normal physiological conditions, the p53 gene is expressed at low levels and has a short half-life due to the rapid turnover of the p53 protein mediated by ubiquitination and proteolysis. p53 is stabilised and activated when responding to some stressful stimuli such as exposure of cells to hypoxia, DNA damaging agents, nucleotide depletion or oncogene activation. Once p53 is activated, it performs its function as a tumour suppressor through some growth controlling endpoints which include: cell cycle arrest, apoptosis, senescence, differentiation and anti-angiogenesis. Therefore, p53 has been considered as a 'guardian of the genome' or 'gatekeeper for growth and division' when regulating critical checkpoints in response to distinct stresses (El-Deiry, 1998).

2.11 Cell Cycle

The cell cycle (cell-division cycle) is a series of events which lead to cell division and duplication (Alberts et al., 2008; MacLachlan et al., 1995). The human cells cell cycle as demonstrated by tissue culture divides approximately every 24 hours. The cell cycle begins with interphase as the first phase, in which the chromosomes amplify and is distributed throughout the nucleus. However, during interphase, both cell growth and DNA replication occur in order to prepare the cell for the next phase of cell division (Cooper & Hausman, 2013a). Interphase consists of three distinct phases: G1 phase (Gap 1), S phase (synthesis) and G2 (Gap 2) phase, followed by the mitotic phase (M phase) (Kleinsmith, 2006). Mitosis (nuclear division) is the most dramatic stage of the cell cycle and usually, ends with cell division (cytokinesis) where chromosomes and cytoplasm separate into two new daughter cells. Interphase occupies about 95 % of a typical cell cycle, whereas the actual process of cell division (M phase) only takes about 5 % (Cooper & Hausman, 2013a).

The cell grows at a steady rate throughout the interphase, with most dividing cells doubling in size between one mitosis and the next. It also during a part of interphase that

the DNA is synthesised. The M phase of the cycle that corresponds to mitosis is usually followed by cytokinesis. The next phase is the G1 phase (gap 1) which corresponds to the interval (gap) between mitosis and initiation of DNA replication. During the G1 phase, the cell is metabolically active and continuous to grow but does not replicate its DNA. G1 is then followed by the S phase (synthesis), during which DNA replication occurs. The completion of DNA synthesis is followed by the G2 phase (gap 2), during which cell growth continues, and proteins are synthesised in preparation for mitosis (Cooper & Hausman, 2013a; Kleinsmith, 2006). When the cell undergoes differentiation, it exits from the G1 phase of the cell cycle to enter into an inactive state referred to as the G0 phase (Pucci et al., 2000).

The relationship between cell cycle and cancer is understandable: while cell cycle is involved in regulating cell proliferation, cancer is a disease involving inappropriate cell proliferation (Collins et al., 1997). Therefore, alteration in the expression and/or activity of cell cycle related proteins permit cancer cell to survive beyond its normal life span and to proliferate abnormally (Feitelson et al., 2015).

2.12 Cell Death

In the last two decades, the field of cell death has attracted much attention (Lockshin & Zakeri, 2001). Cells respond to stress in different ways ranging from the activation of the survival pathways to the initiation of cell death that eliminates damaged cells (Fulda et al., 2010). According to the level and mode of stress, different defence mechanisms and pro-survival strategies can be triggered; however, if these are ineffective, then the cell death programs are activated to eliminate these damaged cells from the organism. The mechanism of cell death depends on its ability to deal with the conditions to which it is exposed and involve apoptosis, necrosis, pyroptosis, or autophagic cell death (Fulda et

al., 2010). There are many morphological differences between necrosis and apoptosis (Figure 2.3).

2.12.1 Apoptosis

Apoptosis (cell suicide pathway) also known as programmed cell death is essential for the elimination of cells that are no longer needed or are damaged (Sayers, 2011) (Hongmei, 2012). Apoptosis occurs during normal cell development and turnover, as well as in a variety of pathological conditions. However, inappropriate regulation of apoptosis can contribute to certain disorders such as viral infection, autoimmune diseases, AIDS, neurodegenerative disorders, stroke, anaemia and cancer (Pucci et al., 2000). Apoptosis is characterised by specific morphological alterations, including chromatin condensation, cell shrinkage, phosphatidylserine translocation, the formation of apoptotic bodies and plasma membrane blebbing before cell lysis (Hsu et al., 2004). At the molecular level, apoptosis occurs through two distinct pathways. The intrinsic or mitochondrial pathway is activated by intracellular events and depends on the release of proapoptotic factors from the mitochondria. The extrinsic apoptosis pathway receives signals through the binding of extracellular protein death ligands to proapoptotic death receptors (Sayers, 2011). Some key proteins linked to the process of apoptosis are Bcl-2 family protein, caspase-3, caspase-8, caspase-9, Bid and Bax (Hongmei, 2012).

2.12.2 Necrosis

The alternative to apoptotic cell death is necrosis. Certain types of injury/stimuli such as heat, radiation, hypoxia or exposure to cytotoxic anticancer drugs at low doses can induce apoptosis, but when the types of injury/stimuli become more severe it can result in necrosis (Elmore, 2007). Necrosis may occur when cells are exposed to conditions that are not related to physiological conditions (e.g., hypothermia) or to compounds that cause damage to the cell membrane. Necrosis is initiated when the cell cannot maintain

homeostasis and this will lead to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell undergoes swelling and may rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, *in vivo* necrotic cell death is frequently associated with extensive tissue damage resulting in an intense inflammatory response (Miret et al., 2006; Yanina et al., 2011). The morphological changes in necrotic cells are cell swelling, the formation of cytoplasmic vacuoles and cytoplasmic blebs, distended endoplasmic reticulum, swollen or ruptured mitochondria and lysosomes and accompanied with disrupted organelle or cell membranes (Trump et al., 1997).

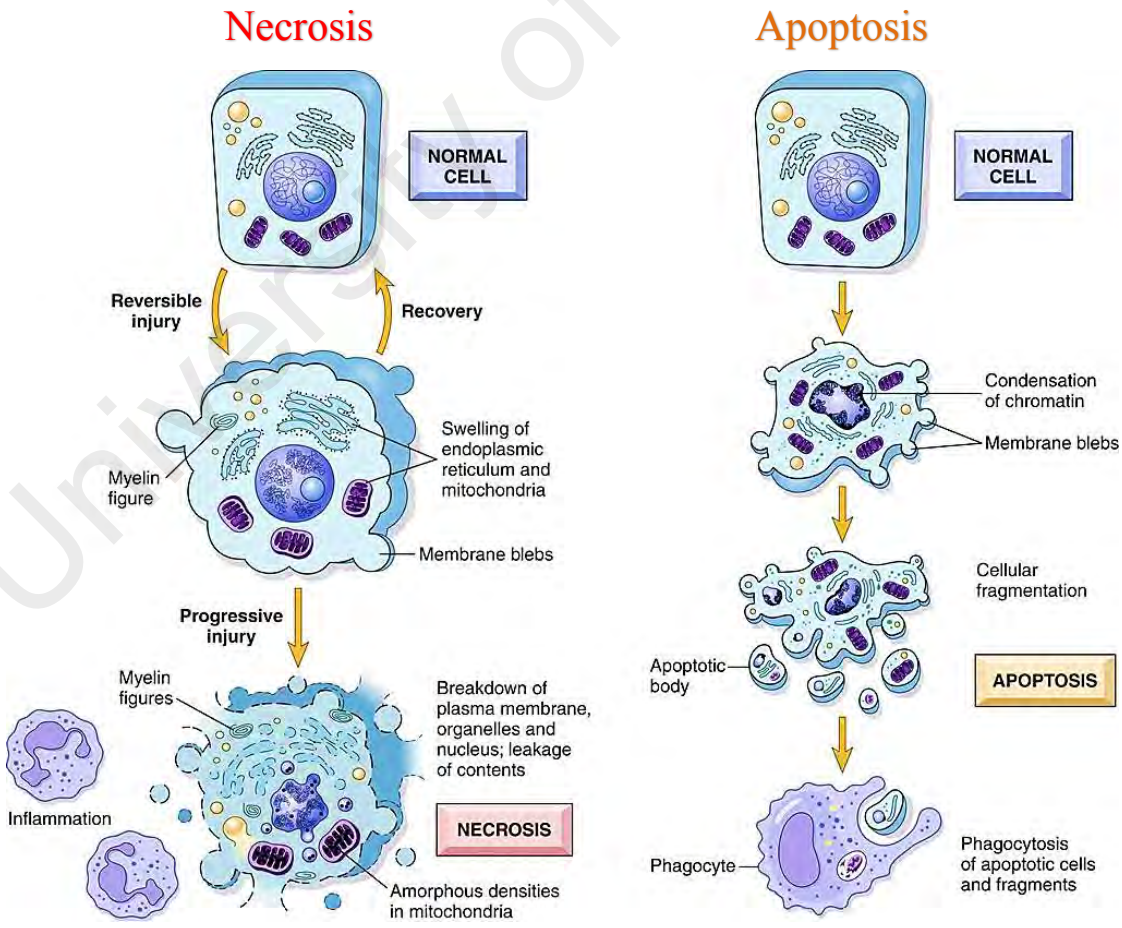

Figure 2. 3: Representation of cell death: apoptosis and necrosis (Kumar et al., 2014)

CHAPTER 3: ACUTE AND SUB-ACUTE ORAL TOXICITY OF *DRACAENA CINNABARI* EXTRACT IN RATS

3.1 Introduction

Toxicological studies subjected to make a decision about whether a new drug should be accepted for clinical use or not (Aneela et al., 2011). Toxicity study gives information on toxic doses and therapeutic indices of drugs and xenobiotic (Rahman et al., 2014). The outcome of the toxicity study in animals is vitally needed to determine the safety of medicinal plants if they are found to be suitable for development into pharmacological products. In addition, to determine the appropriate dose for long-term toxicity tests and to determine the affected organs at the end of the treatment, the toxicity test is achieved (Jothy et al., 2011). It is a scientific, ethical and regulatory requirement that before any potential new medicine administered to humans, its safety must be investigated in animals to define safe human doses (Robinson et al., 2009).

Medicinal plants are used worldwide to treat many diseases, and new drugs continue to be developed through research from these plants (Gupta et al., 2008). Medicinal plant preparations can be formulated into many forms including liquids, and they have been used worldwide for many years (Kamal et al., 2012). The use of a medicinal plant for the treatment of disease without the scientific foundation to adequately support conclusions of safety and efficacy can be potentially dangerous as well as useless. In addition, misuse of these medicinal plants may cause serious toxicity for humans (Mir et al., 2013). As chemical compositions of the medicinal plant are complex, some moderate to severe side effects may arise from the use of herbal medicines. Therefore, it is important to establish medicinal plant safety through the use of well-controlled and validated scientific toxicity studies or protocols (Bhushan et al., 2014).

Dracaena cinnabari (*DC*) is a perennial tree located on the Southern coast of Yemen native to the Socotra Island. This tree produces a deep red resin which called the Dragon's blood or the Twobrother's Blood (Al-Awthan et al., 2010). *DC* belongs to Agavaceae family, which is commonly known as Damm Alakhwain in Yemen (Yehia et al., 2013). The red "dragon's blood" resin of the *DC* tree exudes from fissures and wounds in the bark or branches (Gupta et al., 2008). Phytochemical studies of *DC* resin have led to the isolation of several active compounds belonging to the flavanoids, homoisoflavonoids, chalcones, sterols and terpenoids. Some homoisoflavonoids and chalcones isolated from the *DC* resin exhibited a strong antioxidant activity (Machala et al., 2001). *DC* resin is showing enormous potential interaction with antimicrobial and antioxidants activities (Gupta & Gupta, 2011; Mothana & Lindequist, 2005; Yehia et al., 2013) and considered as good source of food preservative due to its inhibitory effect on various foodborne pathogens (Gupta & Gupta, 2011).

Despite its wide uses, no study has been done to know about its oral toxicity which in turn will provide proper safety information regarding *DC* resin and to define safe human doses. Thus, the aim of the current study is to evaluate the safety of the *DC* resin methanol extract after single or 28 consecutive daily oral administrations.

3.2 Materials and Methods

3.2.1 Preparation of *Dracaena cinnabari* (*DC*) Resin Extract

The resin of *DC* collected from Socotra Island (Yemen) in May 2013. The plant samples were identified and authenticated by Environmental Protection Authority of Yemen. A voucher specimen of the resin (DC/2013-8/122) has been deposited at the herbarium of Department of Pharmacognosy, Faculty of Pharmacy, University of Sana'a, Yemen.

DC resin was ground into powder form using an electrical blender to ease the extraction process. 50 g of the powdered resin of *DC* placed into a 1L conical flask then macerated with methanol (500 ml methanol was added in, where 1g of dried and ground *DC* is to 10 ml methanol) in the ratio of (1:10). Methanol has high polarity and thus greater efficacy towards the extraction of polar phytochemicals such as phenolics and flavonoids (Anwar et al., 2010). The conical flask was left at room temperature for approximately 3 days on a shaker at 100 rpm. The resultant extract was filtered through a fine muslin cloth, and then a filter paper Whatman (Grade 1-Circles, 150 mm) was used to remove the crude part. For separating the methanol from the extract, rotary vacuum evaporator (EYELA) (Figure 3.1) used under reduced pressure at 40 C^o which produced a gummy red resin extract. After that, a freeze dryer was used (Figure 3.2) to produce 28.0 g of a dry powder extract (Figure 3.3).

The extract was then wrapped with aluminium foil to prevent a photo-oxidation that might be occurred and was stored at 4 C^o until used. This methanol extract was dissolved in (10 % DMSO) before use as mentioned elsewhere (Alabsi et al., 2016).

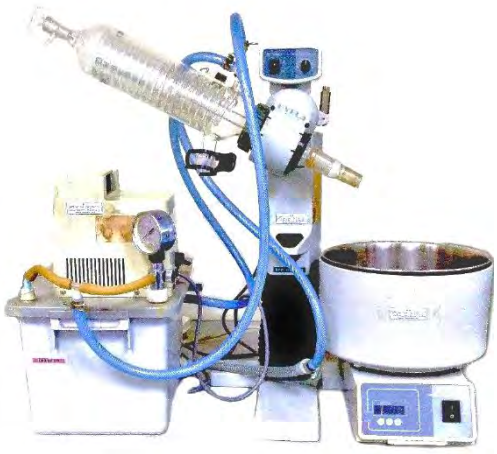

Figure 3. 1: EYELA rotary vacuum evaporator

Figure 3. 2: Freeze dryer

Figure 3. 3: *Dracaena cinnabari* resin methanol extract in a powder form.

3.2.2 Experimental Animals

Female and male Sprague Dawley (SD) rats were used for the acute and sub-acute oral toxicology tests. Nine rats (females) were used in an acute oral toxicity test, whereas 60 rats (30 males and 30 females) used in sub-acute oral toxicity test (the repeated dose 28 day). The toxicity tests were carried out according to Organization for Economic Cooperation and Development (OECD) test guideline OECD Guideline 423 for the acute oral test (OECD, 2001) and OECD Guideline 407 for the sub-acute oral test (OECD, 2008) with slight modifications. The rats were obtained from the Animal Experimental

Unit, Faculty of Medicine, University of Malaya, Kuala Lumpur. SD rats (8-10-week-old) weight 235±15 g were used. Prior to the start of the experiment, body weight of animals was recorded individually for calculating proper treatment dosage. The volume was adjusted depending on the body weight of the rat using 10 ml/kg as this is the normal volume to be used in the rat as mentioned elsewhere (Perret-Gentil, 2005; Turner et al., 2011). The female SD rats were nulliparous and non-pregnant. The SD rats were given standard rat pellets and reverse osmosis (RO) water *ad libitum*. They were acclimatised to laboratory conditions for 7 days before the experiments and housed in groups of three for acute oral toxicity and groups of five for sub-acute oral toxicity. The rats were maintained at a room temperature of 24^{o}C, with a 12 h light/dark cycle. Protocols approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Medicine, University of Malaya, Malaysia (Ethics No. 2014-02-14/OBBS/R/NAA). The endpoint of all rats considered when around 20% of body weight loss has been shown.

3.2.3 Acute Oral Toxicity

The *DC* extract was administered to the female rats under overnight fasting by using oral gavage in a volume of 10 ml/kg body weight.

Nine female SD rats were randomly divided into 3 groups of 3 rats each. The starting dose at 300 mg/kg body weight of *DC* extract that dissolved in 10 % DMSO administered to the group 1. The rats were observed for general behavioural changes; symptoms of toxicity and mortality after treatment for the first 4 h, then over a period of 48 h. Group 2 was administrated sequentially at 48-hour intervals with the next higher dose 2000 mg/kg body weight of *DC* extract that dissolved in 10 % DMSO when there were no signs of toxicity or mortality showed in group 1 after 48 h of treatment. In parallel, group 3 added and treated with vehicle (10% DMSO) to establish a comparative negative control group according to the OECD guideline (OECD, 2001). All animals observed at least once

during the first 30 minutes in the first 24 hours with great consideration given for the first 4 h following vehicle or *DC* extract administration and then once a day for 14 days. This observation was done to check the onset of clinical or toxicological symptoms according to the OECD guideline (OECD, 2001). All observations included changes in skin and fur, eyes and mucous membranes and behavioural pattern were systematically recorded and maintained with an individual record. In addition, consideration was given for observations of convulsions, tremors, diarrhoea, salivation, lethargy, sleep, coma and mortality. The food consumption and water intake recorded daily. The body weights of animals recorded shortly before the administration of the tested substance and at the end of each week. The percentage of body weight change calculated according to the following equation:

$$\frac{\text{Body weight at the end of each week} - \text{initial body weight}}{\text{Initial body weight}} \times 100$$

3.2.4 Sub-Acute Oral Toxicity (Repeated Dose 28-Day Oral Toxicity)

The SD rats were randomly divided into six groups of 10 rats each (n = 10/group, 5 males and 5 females). Four groups were administered daily with *DC* extract at different concentration dissolved in 10 % DMSO and two groups administered with the vehicle by using oral gavage. Group 1 received vehicle (10% DMSO) and served as control. Groups 2, 3 and 4 received doses of *DC* extract at 500, 1000 and 1500 mg/kg body weight, respectively. The 5^{th} and 6^{th} groups namely satellite groups added to determine the reversibility or recovery from toxic effects of the test material and given the vehicle (10% DMSO) and the top dose of *DC* extract 1500 mg/kg body weight, respectively. They were handled as the previous groups. The test material was administered orally (gavage) once daily for 28 consecutive days. The satellite groups were scheduled for follow-up observations for the next 14 days without a vehicle or *DC* extract administration.

Mortality, food consumption and water intake, as well as observation for general toxicity signs of the animals, were monitored and recorded daily during the study. The Initial body weight of all the groups has been recorded before the tested material is administered and at the end of each week. A summary of the groups of acute and sub-acute oral toxicity tests is illustrated in Figure. 3.4.

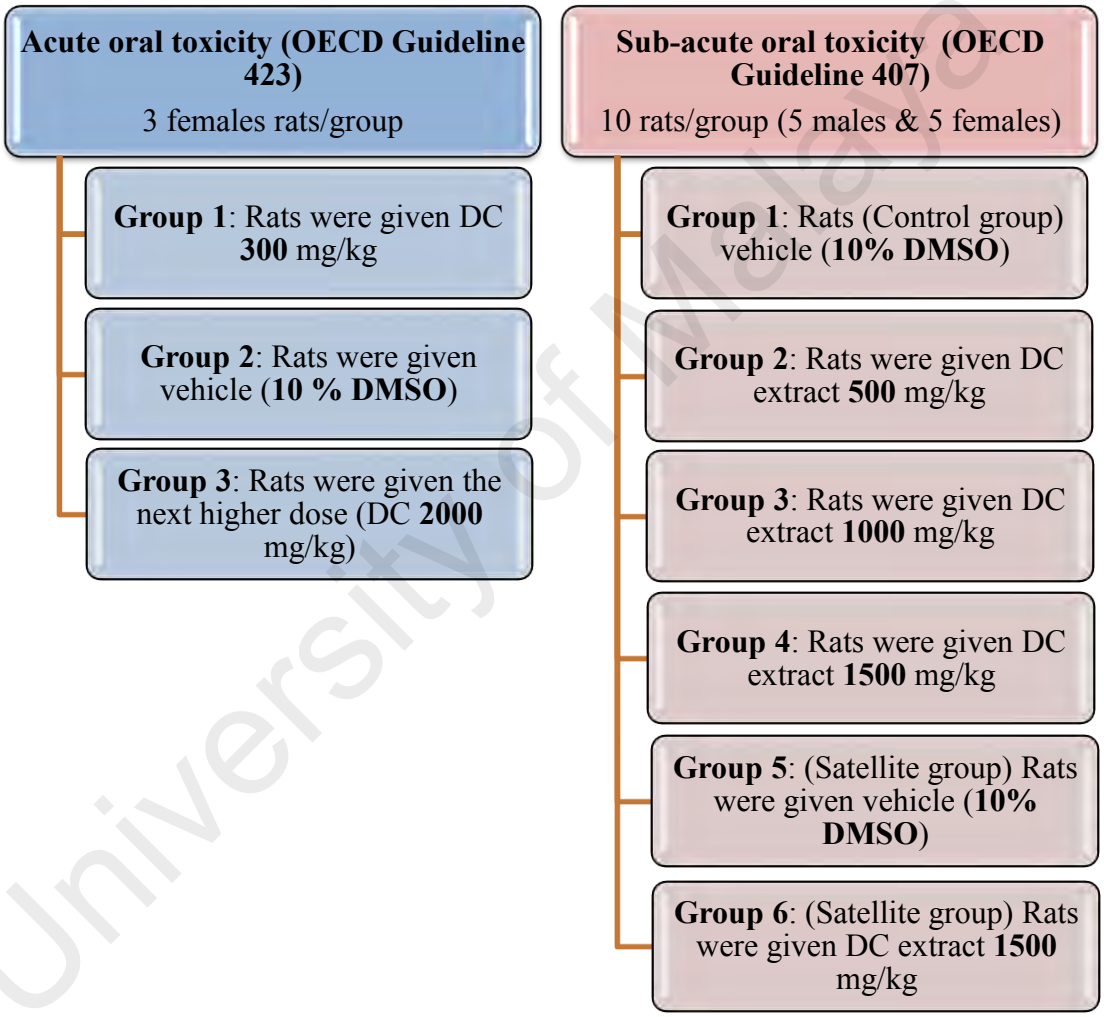

Figure 3. 4: Summary of acute and sub-acute oral toxicity tests groups

3.2.5 Haematology and Serum Biochemistry

At the end of each experiment (at 15th day for acute oral toxicity and at 29th day for sub-acute oral toxicity tests except the satellite groups which were at 43rd day), the rats generally anesthetized by intraperitoneal injection of 80 mg/kg of ketamine 100 mg/ml + 7 mg/kg of xylazine 100 mg/ml (Troy Laboratories PTY. Limited, Smithfield, Australia). Blood sample (5 ml) by cardiac puncture was collected using a disposable syringe. The blood kept in K2EDTA tube for analysing haematological parameters [haemoglobin (HGB), white blood cell (WBC), Neutrophil, Lymphocyte, Monocyte, Eosinophil and Basophil] and in Plain tubes for biochemical parameters [Renal Function - Urea and Creatinine, and liver function - Albumin, Globulin, Total bilirubin, Conjugate Bilirubin, Alkaline phosphatase (ALP), Alanine transaminase (ALT) and Aspartate aminotransferase (AST)]. The blood sample that placed in a plain tube left for 15 - 20 minutes at room temperature to promote blood coagulation. The blood sample was centrifuged at 5000 rpm for 20 min at 4°C; the serum obtained then analysed. Immediately, after blood collection, rats were sacrificed by cervical dislocation.

3.2.6 Relative Organ's Weight

Necropsy was done in acute and sub-acute oral toxicity tests groups of animals on day 15 and 29 respectively, and for the satellite groups, on day 43. After the blood collection, rats were sacrificed by cervical dislocation, and the vital organs (liver, kidney, heart, spleen and lung) removed through a midline incision in the Rat's abdomen. The organs were cleaned of fat and blotted with clean tissue paper, and then weighed on a balance. The relative organ's weight (ROW) was calculated and recorded in proportion to the body weight according to the following equation:

$$\mathrm{RWO} = \frac{\text{Absolute organ weight}}{\text{Body weight at sacrifice}} \times 100$$

3.2.7 Histopathological Observation

Samples from the vital organs (liver, kidney, heart, lung and spleen) of both acute and sub-acute oral toxicity tests were subjected to histopathological evaluation. They fixed in 10% buffered formalin, routinely processed and embedded in paraffin wax. Paraffin sections (5 μm) was cut on glass slides and stained with haematoxylin and eosin. An experienced pathologist who was unaware of the experimental groups to which each section belonged conducted the analysis. The slides were examined under a light microscope (Nikon E50i, Nikon Corporation, Japan) as mentioned elsewhere (Xu et al., 2014).

3.3 Statistical analysis

Results expressed as a mean ± standard deviation. Statistical analysis was performed using SPSS version 20. The differences between groups of acute and sub-acute toxicity tests determined by one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test, and Student *t* test for satellite groups comparisons. Differences were considered significant at $p < 0.05$.

3.4 Results

3.4.1 General Sign and Behavioural Analysis

Oral administration of *DC* extract showed no treatment-related mortality in both sexes of rats for both acute and sub-acute oral toxicity tests throughout the study. Physical observation of the *DC* treated rats for both acute and sub-acute oral toxicity tests throughout this study indicated that none of them showed signs of toxic effects such as changes in skin and fur, eyes and mucous membrane, behaviour pattern, tremors, salivation, diarrhoea and coma. No gross or microscopic pathological abnormalities in all groups (acute and sub-acute oral toxicity tests) observed in any animals. Concerning the Globally Harmonised Classification System, *DC* resin methanol extract can be classified as Category 5 and this provides direct relevance for protecting animal and human health up to the high dose level that used in this study.

3.4.2 Effect of *DC* Extract on Body Weight, Organ's Weight, Food Consumption and Water Intake in Acute and Sub-Acute Oral Toxicity Tests

The body weight of the control and *DC* treated rats were as shown in Table 3.1 (A and B). There was a gradual increase in body weight of the control and *DC* treated groups in both acute and sub-acute oral toxicity tests. The percentage changes in body weight of the *DC* treated groups were not significantly different compared to the control rats as $p > 0.05$ (Table 3.1 A and B). In addition, there is no significant difference has been shown in Satellite groups in both sexes with $p > 0.05$ (Table 3.2).

There was no statistically significant difference in ROW between control and *DC* treated groups of both tests as $p > 0.05$ (Table 3.3 A and B). No significant difference has been shown in Satellite group in both sexes as $p > 0.05$ (Table 3.4).

The food consumption of the *DC* treated groups in both tests was not significantly different compared to the control group ($p > 0.05$) measured throughout the study (Table 3.5 A and B) as well as in the satellite groups in both sexes as $p > 0.05$ (Table 3.6).

The water intake of the control and *DC* treated groups in acute oral toxicity test showed no significant difference with $p > 0.05$ (Table 3.7 A) while in the sub-acute oral toxicity test a significant difference has been demonstrated between the control and *DC* treated groups as $p < 0.05$ (Table 3.7 B). A post hoc Tukey test showed significant differences between the *DC* treated (male and female) and control groups as shown in Table 3.8 with $p < 0.05$. Similarly, a significant difference has been shown in Satellite groups between the *DC* treated group and the control group in both sexes as $p < 0.05$ (Table 3.9).

Table 3. 1 (A and B): Percentage of body weight gain of rats in acute and sub-acute oral toxicity tests.

A: Acute oral toxicity

	Sex	Control	*DC* 300 mg/kg	*DC* 2000 mg/kg	*P* value
0 day (g)	**F (n=3)**	235.33±5.03	250.00±8.54	249.67±9.61	
Week 1 (%)	**F (n=3)**	5.82±1.44	4.92±1.62	4.15±0.37	0.340
Week 2 (%)	**F (n=3)**	8.65±1.96	8.53±1.59	7.37±1.17	0.586

B: Sub-acute oral toxicity

	Sex	Control	*DC* 500 mg/kg	*DC* 1000 mg/kg	*DC* 1500 mg	*P* value
0 day (g)	**M (n=5)**	232.00±4.70	223.40±5.22	226.40±5.13	229.80±3.96	
	F (n=5)	247.20±6.14	233.40±2.70	228.60±8.02	232.20±2.56	
Week 1 (%)	**M (n=5)**	8.28±0.91	8.56±0.79	8.11±1.15	8.61±1.28	0.853
	F (n=5)	5.03±2.44	4.71±1.09	5.44±1.71	5.50±2.21	0.907
Week 2 (%)	**M (n=5)**	16.73±1.03	16.37±1.19	16.78±0.83	16.09±1.87	0.811
	F (n=5)	7.53±3.21	7.62±2.09	7.27±2.59	8.49±2.30	0.889
Week 3 (%)	**M (n=5)**	24.25±2.13	24.17±1.09	23.69±1.64	23.48±2.00	0.881
	F (n=5)	11.42±2.77	10.44±2.82	10.55±2.64	12.30±3.20	.0715
Week 4 (%)	**M (n=5)**	29.34±2.70	28.55±1.60	29.71±2.08	29.75±4.94	0.920
	F (n=5)	13.83±2.76	13.18±2.24	13.36±3.05	15.23±2.94	0.649

Values expressed as a mean ± standard deviation. Sex (male, M and female, F). *p-value less than 0.05, ($p < 0.05$) significant value.

Table 3. 2: Satellite group/ Percentage of body weight change of rats in sub-acute oral toxicity test

	Sex	Control	*DC* 1500 mg/kg	*P* value
0 day	M (n=5)	229.20±3.70	235.40±3.85	
	F (n=5)	222.20±5.12	221.00±6.67	
Week 1 (%)	M (n=5)	8.38±0.72	8.09±1.21	0.657
	F (n=5)	4.86±0.59	4.90±1.54	0.9601
Week 2 (%)	M (n=5)	15.36±1.57	15.13±0.77	0.782
	F (n=5)	7.57±0.66	7.56±1.67	0.994
Week 3 (%)	M (n=5)	19.90±1.34	19.89±1.62	0.988
	F (n=5)	10.09±1.12	9.80±1.68	0.756
Week 4 (%)	M (n=5)	24.45±1.99	24.30±0.83	0.883
	F (n=5)	12.96±0.96	12.77±1.35	0.807
Week 5 (%)	M (n=5)	28.81±2.02	28.48±1.68	0.788
	F (n=5)	15.22±0.71	14.78±1.81	0.631
Week 6 (%)	M (n=5)	34.03±2.50	33.16±2.69	0.612
	F (n=5)	17.28±0.75	16.59±1.65	0.415

Values expressed as a mean ± standard deviation. Sex (male, M and female, F). *p-value less than 0.05, ($p < 0.05$) significant value.

Table 3. 3 (A and B): Relative organs weight (g%) of rats in acute and sub-acute oral toxicity tests

A: Acute oral toxicity

	Sex	Control	*DC* 300 mg/kg	*DC* 2000 mg/kg	*P* value
Heart	F (n=3)	0.37±0.02	0.35±0.01	0.35±0.03	0.422
Liver	F (n=3)	2.58±0.14	2.60±0.12	2.50±0.13	0.601
Kidney	F (n=3)	0.70±0.02	0.67±0.03	0.68±0.02	0.484
Spleen	F (n=3)	0.18±0.02	0.18±0.02	0.18±0.01	0.952
Lung	F (n=3)	0.49±0.01	0.48±0.02	0.48±0.02	0.874

B: Sub-acute oral toxicity

	Sex	Control	*DC* 500 mg/kg	*DC* 1000 mg/kg	*DC* 1500 mg/kg	*P* value
Heart	M (n=5)	0.36±0.02	0.35±0.02	0.34±0.02	0.34±0.03	0.757
	F (n=5)	0.37±0.02	0.37±0.03	0.37±0.02	0.38±0.02	0.938
Liver	M (n=5)	2.92±0.15	2.81±0.10	2.87±0.17	2.92±0.14	0.593
	F (n=5)	2.45±0.09	2.38±0.07	2.35±0.05	2.43±0.07	0.114
Kidney	M (n=5)	0.75±0.02	0.75±0.03	0.76±0.02	0.76±0.03	0.930
	F (n=5)	0.69±0.03	0.70±0.02	0.70±0.05	0.72±0.04	0.639
Spleen	M (n=5)	0.19±0.02	0.19±0.02	0.18±0.03	0.19±0.02	0.937
	F (n=5)	0.18±0.02	0.18±0.02	0.18±0.02	0.19±0.02	0.835
Lung	M (n=5)	0.40±0.02	0.40±0.02	0.39±0.02	0.42±0.01	0.241
	F (n=5)	0.49±0.02	0.48±0.02	0.49±0.01	0.50±0.02	0.536

Values expressed as a mean ± standard deviation. Sex (male, M and female, F). *p-value less than 0.05, ($p < 0.05$) significant value.

Table 3. 4: Satellite group/ Relative organs weight (g%) of rats in the sub-acute oral toxicity test

	Sex	Control	*DC* 1500 mg/kg	*P* value
Heart	**M**	0.34±0.03	0.34±0.02	0.797
	F	0.38±0.01	0.38±0.01	0.493
Liver	**M**	2.73±0.09	2.76±0.08	0.508
	F	2.59±0.13	2.64±0.12	0.524
Kidney	**M**	0.76±0.02	0.75±0.02	0.655
	F	0.72±0.03	0.72±0.03	0.696
Spleen	**M**	0.19±0.01	0.19±0.01	0.370
	F	0.20±0.02	0.20±0.01	0.832
Lung	**M**	0.40±0.02	0.38±0.02	0.308
	F	0.50±0.02	0.50±0.01	0.722

Values expressed as a mean ± standard deviation. n = 5. Sex (male, M and female, F). *p-value less than 0.05, ($p < 0.05$) significant value.

Table 3. 5 (A and B): Food consumption (g) of rats in acute and sub-acute oral toxicity tests

A: Acute oral toxicity

	Sex	Control	*DC* 300 mg/kg	*DC* 2000 mg/kg	*P* value
Week 1	**F (n=3)**	64.68±2.80	63.43±2.87	59.14±14.67	0.089
Week 2	**F (n=3)**	66.16±2.89	67.05±5.14	63.74±2.15	0.236

B: Sub-acute oral toxicity

	Sex	Control	*DC* 500 mg/kg	*DC* 1000 mg/kg	*DC* 1500 mg/kg	*P* value
Week 1	**M (n=5)**	81.75±1.21	81.15±2.17	80.99±2.55	79.26±2.42	0.189
	F (n=5)	74.87±2.59	72.99±1.63	72.47±2.28	71.55±2.80	0.089
Week 2	**M (n=5)**	84.52±1.13	83.93±0.93	83.80±1.77	82.59±1.11	0.061
	F (n=5)	78.69±3.26	77.56±3.21	75.99±3.58	74.66±4.64	0.220
Week 3	**M (n=5)**	88.05±2.54	87.92±2.07	86.10±1.94	85.19±2.58	0.075
	F (n=5)	86.55±3.64	86.15±3.11	85.17±3.75	83.21±3.68	0.320
Week 4	**M (n=5)**	91.84±1.60	91.08±0.79	90.30±1.26	90.23±1.11	0.069
	F (n=5)	88.69±3.66	87.35±3.26	87.75±3.32	87.04±2.77	0.798

Values expressed as a mean ± standard deviation. Sex (male, M and female, F). *p-value less than 0.05, ($p < 0.05$) significant value.

Table 3. 6: Satellite group/ Food consumption (g) of rats in the sub-acute oral toxicity test

	Sex	Control	*DC* 1500 mg/kg	*P* value
Week 1	**M (n=5)**	81.92±1.44	79.30±3.81	0.113
	F (n=5)	73.05±3.11	71.26±1.96	0.221
Week 2	**M (n=5)**	84.60±2.38	82.68±1.56	0.099
	F (n=5)	73.68±2.27	72.77±1.93	0.435
Week 3	**M (n=5)**	89.75±1.96	87.72±1.69	0.059
	F (n=5)	81.89±3.76	78.62±4.60	0.170
Week 4	**M (n=5)**	91.96±0.66	91.07±0.93	0.061
	F (n=5)	83.55±2.00	82.43±1.59	0.265
Week 5	**M (n=5)**	92.96±1.63	91.87±1.80	0.256
	F (n=5)	85.81±1.98	83.92±3.91	0.179
Week 6	**M (n=5)**	101.90±3.70	100.25±2.85	0.366
	F (n=5)	86.55±3.82	83.07±4.73	0.155

Values expressed as a mean ± standard deviation. Sex (male, M and female, F). *p-value less than 0.05, ($p < 0.05$) significant value.

Table 3. 7 (A and B): Water intake (ml) of rats in the acute and sub-acute oral toxicity tests

A: Acute oral toxicity

	Sex	Control	*DC* 300 mg/kg	*DC* 2000 mg/kg	*P* value
Week 1	**F (n=3)**	102.56±1.27	104.03±1.78	104.34±2.12	0.157
Week 2	**F (n=3)**	102.54±0.97	104.00±1.77	103.93±2.01	0.199

B: Sub-acute oral toxicity

	Sex	Control	*DC* 500 mg/kg	*DC* 1000 mg/kg	*DC* 1500 mg/kg	*P* value
Week 1	**M (n=5)**	123.18±1.39	128.09±2.34	128.03±1.91	127.40±2.11	**0.000**
	F (n=5)	122.39±0.42	125.51±0.50	125.48±0.37	125.46±0.53	**0.000**
Week 2	**M (n=5)**	124.06±1.17	127.31±1.50	127.44±1.48	127.97±1.85	**0.000**
	F (n=5)	123.33±0.55	125.68±0.45	125.99±0.41	125.87±0.41	**0.000**
Week 3	**M (n=5)**	125.23±0.96	127.99±1.33	127.98±1.80	127.86±1.69	**0.004**
	F (n=5)	124.44±0.49	127.29±0.66	127.12±0.61	127.16±0.77	**0.000**
Week 4	**M (n=5)**	125.89±0.78	128.29±1.95	128.54±1.22	128.34±1.61	**0.007**
	F (n=5)	124.99±0.50	127.28±0.86	127.36±0.63	127.56±0.61	**0.000**

Values expressed as a mean ± standard deviation. Sex (male, M and female, F). *p-value less than 0.05, ($p < 0.05$) significant value.

Table 3. 8: Tukey test of water intake (ml) of male and female rats in sub-acute oral toxicity test

Dependent Variable	(I) water intake	(J) water intake	Mean Difference (I-J) in male rat	*P* value	Mean Difference (I-J) in female rat	*P* value
Week 1	Control	*DC* 500 mg/kg	-4.91*±1.05	**0.001**	-3.12*±0.24	**0.000**
		DC 1000 mg/kg	-4.86*±1.05	**0.001**	-3.09*±0.24	**0.000**
		DC 1500 mg/kg	-4.23*±1.05	**0.003**	-3.07*±0.24	**0.000**
Week 2	Control	*DC* 500 mg/kg	-3.25*±0.81	**0.003**	-2.35*±0.25	**0.000**
		DC 1000 mg/kg	-3.38*±0.81	**0.002**	-2.67*±0.25	**0.000**
		DC 1500 mg/kg	-3.91*±0.81	**0.000**	-2.54*±0.25	**0.000**
Week 3	Control	*DC* 500 mg/kg	-2.77*±0.79	**0.009**	-2.85*±0.34	**0.000**
		DC 1000 mg/kg	-2.75*±0.79	**0.010**	-2.68*±0.34	**0.000**
		DC 1500 mg/kg	-2.63*±0.79	**0.014**	-2.72*±0.34	**0.000**
Week 4	Control	*DC* 500 mg/kg	-2.40*±0.78	**0.025**	-2.29*±0.35	**0.000**
		DC 1000 mg/kg	-2.65*±0.78	**0.012**	-2.37*±0.35	**0.000**
		DC 1500 mg/kg	-2.45*±0.78	**0.021**	-2.57*±0.35	**0.000**

Values expressed as a mean ± Standard Error. n = 5. **p*-value less than 0.05, ($p < 0.05$) significant value.

Table 3. 9: Satellite group/ Water intake (ml) of rats in the sub-acute oral toxicity test

	Sex	Control	*DC* 1500 mg/kg	*P* value
Week 1	**M**	124.39±0.41	125.86±0.85	**0.001**
	F	121.24±1.79	123.59±1.96	**0.037**
Week 2	**M**	124.87±0.64	127.14±1.09	**0.000**
	F	123.33±1.85	125.03±0.76	**0.044**
Week 3	**M**	126.40±0.87	128.30±1.37	**0.009**
	F	123.80±0.95	125.80±1.00	**0.002**
Week 4	**M**	127.48±0.95	128.89±1.21	**0.032**
	F	124.53±1.10	125.94±1.01	**0.027**
Week 5	**M**	128.38±1.11	129.03±0.86	0.244
	F	125.68±0.99	125.97±0.68	0.538
Week 6	**M**	128.94±1.28	129.30±1.34	0.620
	F	126.48±0.90	126.58±0.68	0.825

Values expressed as a mean ± standard deviation. n = 5. Sex (male, M and female, F). **p*-value less than 0.05, ($p < 0.05$) significant value.

3.4.3 Effect of *DC* Extract on Haematological Parameters in Acute and Sub-Acute Oral Toxicity Tests

The haematological profile of control and *DC* treated groups summarised in Table 3.10 (A and B). The results concluded that all haematological parameters such as haemoglobin (HGB) and total white blood cell count are within the normal range in both the control and the *DC* treated groups. In ANOVA test, there is no significant association between the groups in both acute and sub-acute toxicity tests as $p > 0.05$. In the satellite groups, there was no significant difference showed in both sexes with $p > 0.05$ (Table 3.11).

Table 3. 10 (A and B): Haematological parameters of the rats in acute and sub-acute oral toxicity tests

A: Acute oral toxicity

Haematological Parameters	Sex	Control	*DC* 300 mg/kg	*DC*2000 mg/kg	*P* value
HGB (g/l)	F(n=3)	153.00±10.00	145.50±9.50	149.33±17.95	0.789
WBC (10^9/L)	F(n=3)	6.83±0.23	7.77±0.38	8.05±0.75	0.057
Neutrophil (10^9/L)	F(n=3)	0.55±0.13	0.56±0.07	0.58±0.14	0.961
Lymphocyte (10^9/L)	F(n=3)	6.39±0.87	6.72±1.15	7.29±0.52	0.498
Monocyte (10^9/L)	F(n=3)	0.14±0.03	0.15±0.02	0.18±0.3	0.228
Eosinophil (10^9/L)	F(n=3)	0.08±0.02	0.10±0.02	0.11±0.02	0.125
Basophil (10^9/L)	F(n=3)	0.02±0.01	0.03±0.02	0.03±0.02	0.531

B: Sub-acute oral toxicity

Haematological Parameters	Sex	Control	*DC* 500 mg/kg	*DC*1000 mg/kg	*DC*1500 mg/kg	*P* value
HGB (g/l)	M (n=5)	149.40±6.47	148.80±5.40	150.40±12.72	156.80±12.91	0.575
	F (n=5)	156.40±5.81	148.80±7.82	150.60±3.85	153.40±5.81	0.243
WBC (10^9/L)	M (n=5)	9.22±1.03	9.30±2.45	9.46±1.33	9.56±3.67	0.996
	F (n=5)	6.86±0.59	7.40±1.15	7.38±0.93	7.82±0.81	0.431
Neutrophil (10^9/L)	M (n=5)	0.98±0.12	1.01±0.16	1.03±0.18	1.14±0.30	0.626
	F (n=5)	0.81±0.21	0.77±0.11	0.87±0.17	0.89±0.14	0.646
Lymphocyte (10^9/L)	M (n=5)	9.66±2.12	9.64±1.67	10.39±1.99	10.77±2.08	0.753
	F (n=5)	8.22±1.07	8.44±1.22	8.67±1.00	9.20±1.14	0.564

Table 3.10 - B continued						
Monocyte (10^9/L)	**M (n=5)**	0.16±0.02	0.15±0.02	0.17±0.04	0.19±0.04	0.411
	F (n=5)	0.17±0.03	0.16±0.03	0.18±0.02	0.18±0.03	0.623
Eosinophil (10^9/L)	**M (n=5)**	0.08±0.02	0.09±0.02	0.10±0.02	0.10±0.03	0.388
	F (n=5)	0.08±0.01	0.09±0.02	0.07±0.03	0.10±0.02	0.215
Basophil (10^9/L)	**M (n=5)**	0.06±0.2	0.04±0.03	0.04±0.03	0.04±0.03	0.537
	F (n=5)	0.02±0.02	0.03±0.02	0.03±0.02	0.04±0.02	0.397

Values expressed as a mean ± standard deviation. Sex (male, M and female, F). *p-value less than 0.05, ($p < 0.05$) significant value.

Table 3. 11: Haematological parameters in sub-acute oral toxicity test in Satellite group

	Sex	**Control**	***DC* 1500 mg/kg**	***P* value**
HGB (g/l)	**M**	152.40±6.80	153.20±7.46	0.863
	F	155.00±6.82	154.80±5.97	0.961
WBC (10^9/L)	**M**	9.30±1.36	10.76±1.23	0.112
	F	6.70±0.72	6.74±0.60	0.926
Neutrophil (10^9/L)	**M**	1.08±0.13	1.31±0.23	0.084
	F	1.25±0.11	1.33±0.11	0.295
Lymphocyte (10^9/L)	**M**	9.77±1.07	11.25±1.41	0.098
	F	9.29±0.95	9.31±0.70	0.977
Monocyte (10^9/L)	**M**	0.21±0.06	0.23±0.07	0.649
	F	0.22±0.05	0.23±0.03	0.773
Eosinophil (10^9/L)	**M**	0.10±0.01	0.10±0.02	0.856
	F	0.09±0.02	0.10±0.03	0.596
Basophil (10^9/L)	**M**	0.03±0.02	0.04±0.03	0.636
	F	0.02±0.01	0.03±0.01	0.545

Values expressed as a mean ± standard deviation. n = 5. Sex (male, M and female, F). *p-value less than 0.05, ($p < 0.05$) significant value.

3.4.4 Effect of *DC* Extract on Biochemical Parameters in Acute and Sub-Acute Oral Toxicity Tests

The data on biochemical parameters (liver and renal functions) in control and *DC* treated groups of the acute and sub-acute oral toxicity tests presented in Table 3.12 and Table 3.13, respectively. There was no significant difference shown in the biochemical parameters between the groups of acute and sub-acute oral toxicity tests in both sexes (male and female rats) as well as in the satellite groups as $p > 0.05$ (Table 3.14).

Table 3. 12: Biochemical parameters of the rats in acute oral toxicity test

Biochemical parameters	Sex	Groups			*P* value
		Control	*DC* 300 mg/kg	*DC* 2000 mg/kg	
Urea (mmol/L)	**F**	3.80±0.53	4.00±0.62	4.13±0.29	0.727
Creatinine (umol/L)	**F**	32.67±1.53	32.00±4.58	31.67±1.53	0.920
Albumin (g/L)	**F**	40.67±4.04	37.67±3.51	36.67±1.53	0.350
Globulin (g/L)	**F**	24.00±1.00	22.33±2.08	20.00±2.65	0.128
Total bilirubin (umol/L)	**F**	2.00±0.00	1.00±0.00	1.00±0.00	-
Conjugate bilirubin (umol/L)	**F**	1.00±0.00	1.00±0.00	1.00±0.00	-
ALP (U/L)	**F**	166.33±4.73	164.67±6.03	177.33±7.02	0.081
ALT (U/L)	**F**	24.67±1.53	25.67±1.15	26.67±1.53	0.296
AST (U/L)	**F**	96.00±9.17	99.67±7.57	107.33±3.06	0.216

Values expressed as a mean ± standard deviation, n = 3. *p-value less than 0.05, ($p < 0.05$) significant value.

Table 3. 13: Biochemical parameters of the rats in sub-acute oral toxicity test

Biochemical parameters	Sex	Groups				*P* value
		Control	*DC* 500 mg/kg	*DC* 1000 mg/kg	*DC* 1500 mg/kg	
Urea (mmol/L)	**M**	3.76±0.59	3.92±0.34	4.14±0.38	4.18±0.51	0.466
	F	3.90±0.74	4.06±0.44	3.97±0.69	4.16±1.24	0.963
Creatinine (umol/L)	**M**	24.20±4.15	24.00±2.55	23.60±6.91	27.20±4.15	0.615
	F	28.60±3.05	28.40±2.70	27.40±2.70	28.80±1.92	0.839
Albumin (g/L)	**M**	35.00±2.24	34.60±1.14	36.20±5.26	34.60±1.67	0.817
	F	35.00±1.58	34.60±2.40	36.20±2.17	34.00±2.55	0.470
Globulin (g/L)	**M**	20.60±1.14	21.00±1.58	21.20±1.48	21.20±1.30	0.890
	F	22.40±1.14	21.80±1.10	22.80±1.30	21.20±1.48	0.245
Total bilirubin (umol/L)	**M**	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	-
	F	2.00±0.00	2.00±0.00	2.00±0.00	2.00±0.00	-
Conjugate bilirubin (umol/L)	**M**	1.40±1.14	1.00±0.00	0.40±0.55	1.00±1.22	0.379
	F	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	-
ALP (U/L)	**M**	216.20±16.99	218.40±12.70	222.40±18.51	236.40±12.74	0.203
	F	204.20±14.64	210.80±29.52	209.60±23.70	197.60±16.33	0.769
ALT (U/L)	**M**	29.60±3.29	29.40±1.52	29.60±2.30	32.00±2.83	0.356
	F	23.40±3.21	23.60±3.05	25.40±3.36	26.80±1.64	0.243
AST (U/L)	**M**	104.80±6.69	106.00±3.94	105.40±5.55	109.00±4.95	0.623
	F	99.60±7.40	103.00±2.30	103.60±3.44	104.60±2.41	0.320

Values expressed as a mean ± standard deviation, n = 5. Sex (male, M and female, F). *p-value less than 0.05, ($p < 0.05$) significant value.

Table 3. 14: Biochemical parameters of the rats in satellite group for subacute oral toxicity test

Biochemical parameters	Satellite group			*P* value
	Sex	Control	*DC* 1500 mg/kg	
Urea (mmol/L)	**M**	3.78±0.34	3.92±0.48	0.608
	F	3.96±0.70	4.06±0.17	0.765
Creatinine (umol/L)	**M**	25..00±3.87	26.40±3.78	0.579
	F	29.40±3.05	27.20±3.96	0.354
Albumin (g/L)	**M**	35.20±1.30	34.40±1.67	0.423
	F	34.20±2.77	32.40±3.36	0.382
Globulin (g/L)	**M**	20.20±0.84	19.40±1.34	0.290
	F	22.00±1.58	21.20±1.30	0.408
Total bilirubin (umol/L)	**M**	1.00±0.00	1.00±0.00	-
	F	2.00±0.00	2.00±0.00	-
Conjugate bilirubin (umol/L)	**M**	1.00±0.00	1.00±0.00	-
	F	1.00±0.00	1.00±0.00	-
ALP (U/L)	**M**	223.60±29.98	226.20±28.35	0.891
	F	184.40±29.11	190.40±29.21	0.753
ALT (U/L)	**M**	31.00±3.39	30.00±3.81	0.673
	F	25.20±1.79	25.60±2.07	0.752
AST (U/L)	**M**	107.00±6.04	111.40±5.94	0.279
	F	103.40±3.29	105.00±2.74	0.427

Values expressed as a mean ± standard deviation, n = 5. Sex (male, M and female, F). **p*-value less than 0.05, ($p < 0.05$) significant value.

3.4.5 Histopathological Observation

A histopathological study carried to confirm biochemical findings and to identify any structural changes. Light microscopic examination of the vital organs including liver, kidney, heart, lung and spleen of the rats in all the *DC* treated and control groups for acute oral toxicity (Figure 3.5), and sub-acute oral toxicity (Figure 3.6) did not reveal any gross pathological lesions.

The photomicrographs of the liver and kidney of the control and *DC* treated groups as well as of satellite group, both male and female, showed normal morphological architecture. Under microscopic examination, the liver of *DC* treated animals showed normal cellular architecture and binucleation and was without any distortions similar to the control groups. Furthermore, signs of injury, necrosis, congestion, fatty acid accumulation, or haemorrhagic regions around the central vein or sinusoids of the liver

not observed. The hepatocytes arranged in cords and clearly visible. The cross-section of the liver showed no lyses in the blood cells, or infiltration of neutrophil, lymphocyte, or macrophage in the acute oral toxicity group and the sub-acute oral toxicity. For the kidneys, histologically there was no morphological change for all *DC* treated groups. The appearance of the glomerular architecture showed normal similar to the control groups. The glomeruli, distal, and proximal tubules in the kidney appeared normal in both male and female rats. In addition, there was no interstitial and intraglomerular congestion or tubular atrophies. All the nephron cells showed normal and clearly visible nucleoli with no degeneration, bleeding, or necrosis infiltration in acute oral toxicity group as well as in the sub-acute oral toxicity. In both the control and *DC* treated female and male rats, the heart shows normal cardiac muscle fibers and lungs show a normal alveolar structure with no treatment-related inflammatory response in acute oral toxicity group as well as in the sub-acute oral toxicity. Similarly, normal structure and histology of the spleen also observed in all the rats of acute and sub-acute oral toxicity tests. There is mild congestion seen in the lung, liver, and kidney of the control and *DC* treated groups of both sexes which were incidental or spontaneous with no relation to *DC* resin methanol extract treatment.

Thus, the histopathological evaluations of the selected organs did not reveal any morphological abnormalities that could be attributed to the oral administration of *DC* resin methanol extract to the rats.

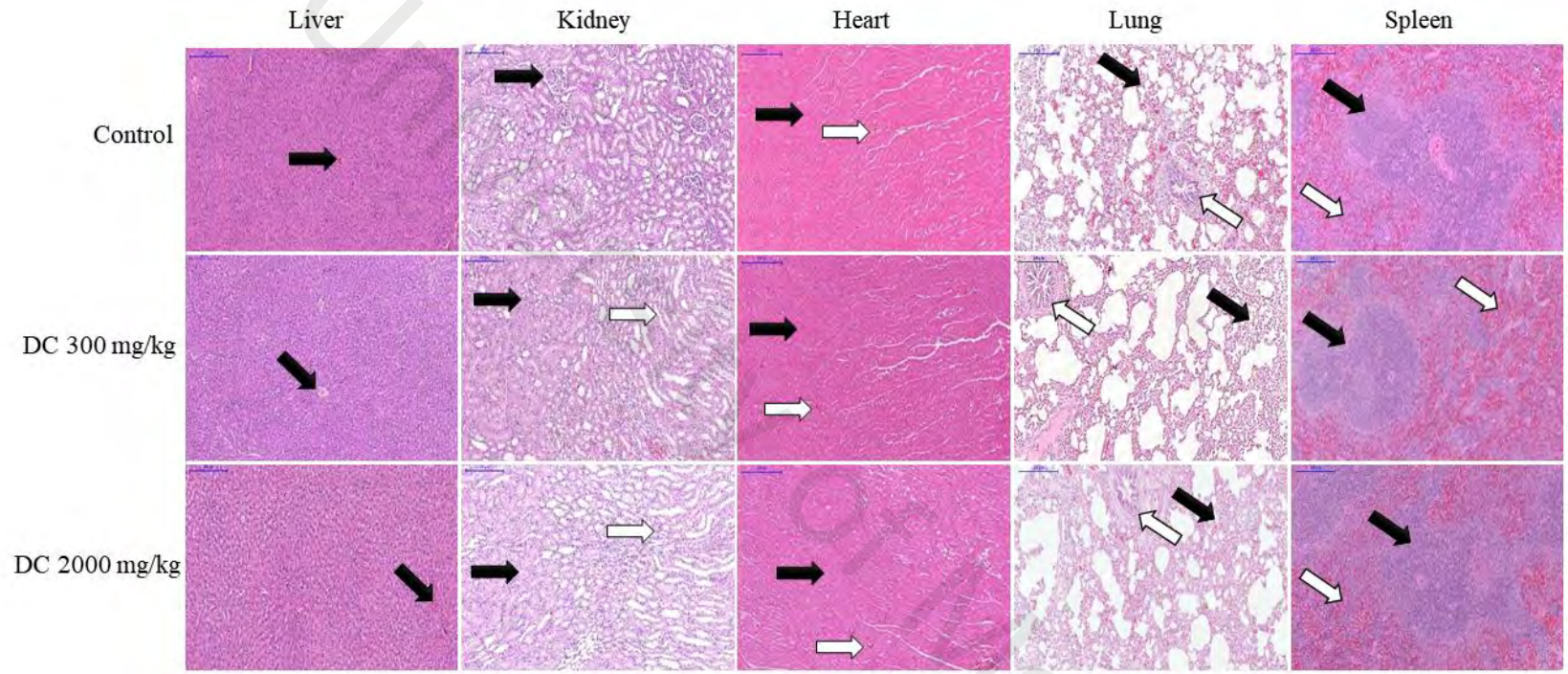

Figure 3. 5: Photomicrograph of vital organs in acute oral toxicity (H&E Stain, ×100). Liver: black arrow – portal vein; white arrow – portal triad. Kidney: black arrow – cortex; white arrow – medulla. Heart: black arrow – myocardium; white arrow – blood vessel. Lung: Black arrow – alveoli; white arrow – bronchiole. Spleen: Black arrow – white pulp, white arrow – red pulp

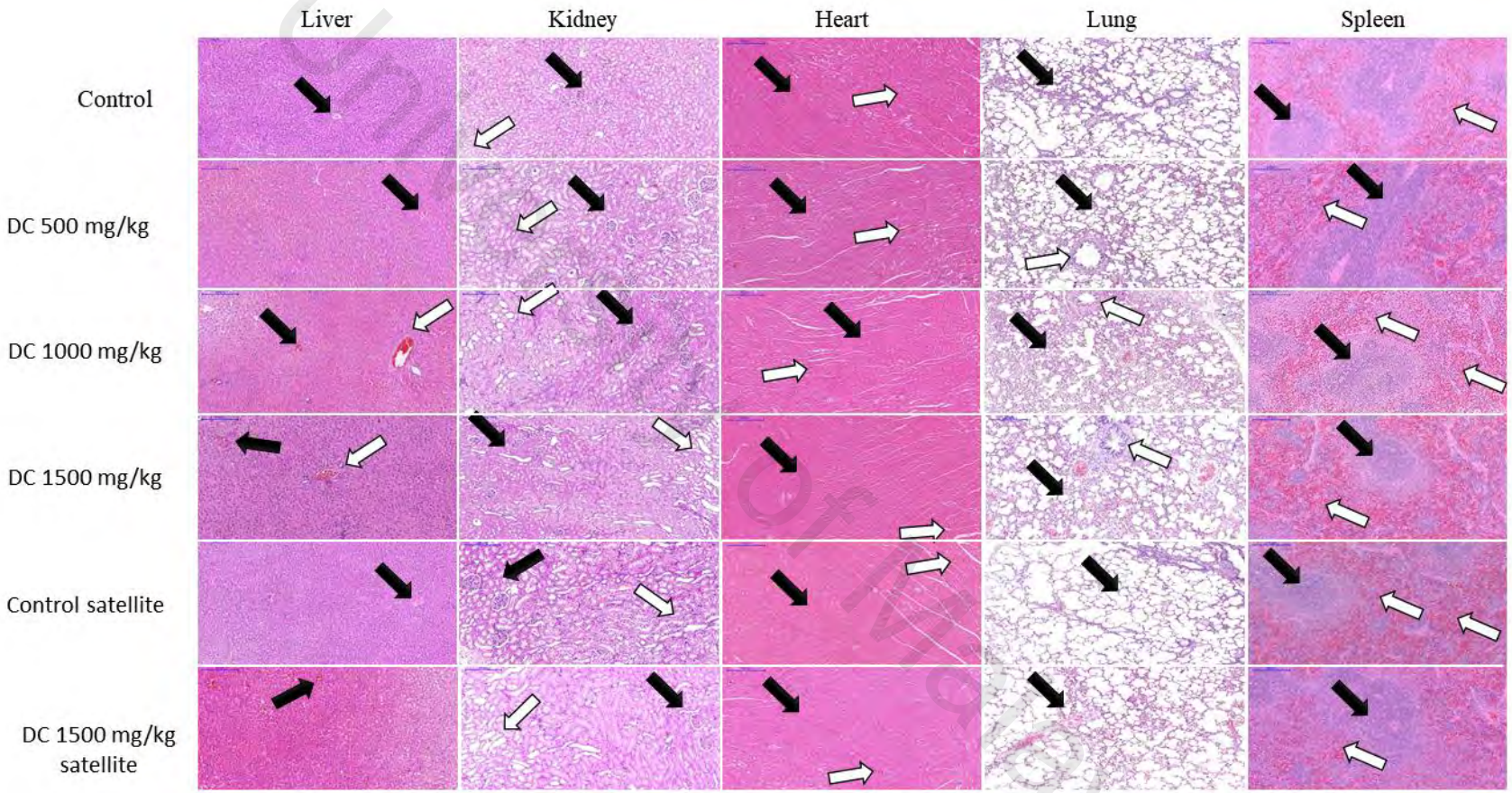

Figure 3. 6: Photomicrograph of vital organs in sub-acute oral toxicity (H&E Stain, ×100). Liver: black arrow – portal vein; white arrow – portal triad. Kidney: black arrow – cortex; white arrow – medulla. Heart: black arrow – myocardium; white arrow – blood vessel. Lung: Black arrow – alveoli; white arrow – bronchiole. Spleen: Black arrow – white pulp, white arrow – red pulp

3.5 Discussion

Herbal medicines have acquired greater importance as a substitute for conventional therapy (Ekor, 2014; Ramaswamy et al., 2012). As the use of medicinal plants increases, screening plant products to assess and evaluate the toxic characteristics of a natural product extract, fraction, or compound consider an initial step (Yuet Ping et al., 2013).

During the evaluation of the toxic characteristics of medicinal plants, an initial assessment of toxic manifestations is one of the initial screening experiments performed with all compounds. In addition, Data from the acute toxicity study may serve as the basis for classification and labelling of the test material (Ukwuani et al., 2012). Thus, the current study was assumed to evaluate and focus on the acute and sub-acute toxicity of *DC* resin methanol extract in an animal model.

The oral route administration is the most useful and normally used one while doing toxicity study. The absorption may be slow; however, this methodology expenses less and is painless to the animals. As the crude extracts administered orally, the animals need to fast before administering the material because food and other chemicals within the digestive system may affect the reaction(s) of the tested materials. All the procedures were performed based on the appropriate OECD guideline (Kumar & Lalitha, 2013).

Test method with a starting dose of 300 mg/kg body weight primarily used in situations where the investigator has no information indicating that the test material is likely to be toxic (OECD, 2001). In this study, the rats in control and *DC* treated groups administrated with the vehicle and crude extracts, respectively. From the experiment performed, the starting dose of 300 mg/kg body weight has revealed no mortality in the experimental animals. Thus, the next higher dose of 2000 mg/kg body weight selected as described in the OECD Guidelines 423. The rats monitored daily until the last day of the experiment (day 14^{th}) for any toxic signs and mortality. The clinical symptom is one amongst the

most important observations to indicate the toxicity effects on organs within the treated groups (Jothy et al., 2011). During the 14 days of acute toxicity assessment period, all rats orally administrated with *DC* extract at a single dose of 300 mg/kg and 2000 mg/kg showed no obvious signs of distress, and there were no noticeable symptoms of either toxicity or deaths. All of the rats showed no significant changes in wellness parameters. Physical appearance features such as skin, fur, eyes, mucous membrane, salivation, behavioural pattern, the sleep of the animals in control and *DC* treated groups (300 mg and 2000 mg) of *DC* extract were found to be normal. Lethargy, tremors, diarrhoea and coma did not occur in any of the animals. Moreover, the body weight of the rats showed an increase in both control and *DC* treated groups with no significant difference seen (Table 3.1 A); this indicates that the *DC* extract has no adverse effect on the growth of the animals.

This study estimated that *DC* extract does not cause acute toxicity effects and no rat has died. Based on OECD guidelines 423 (Annex 2), the results of this test allow the substance to be ranked and classified according to the Globally Harmonised System of Classification and Labelling of Chemicals. Thus, the *DC* extract can be classified as category 5 with low acute toxicity hazard, which was the lowest toxicity class (OECD, 2001). Therefore, it can be concluded that *DC* extract is tolerated up to 2000 mg/kg body weight when administered at a single dose. In a like manner, a study performed by Ramaswamy et al. (2012) using Nuna Kadugu (a Siddha medicine prepared from leaves and fruits of *Morinda Pubescens*) revealed that Nuna Kadugu can be classified under category-5 when administered at single dose 2000 mg/kg in accordance with Globally Harmonised System of Classification and Labelling of Chemicals, and this provides a direct relevance for protecting human and animal health.

Acute toxicity information is of limited clinical application because cumulative toxic effects do occur even at very low doses. Consequently, multiple dose studies are typically useful in evaluating the safety profile of phytomedicines. Therefore, sub-acute (Repeated dose 28-day oral toxicity) test has been used. Body weight changes are an indicator of adverse side effects (Chitra et al., 2015), and lose more than 20% of the animal body weight is regarded as critical and has been defined as one of the humane endpoints in several international guidelines (CCAC, 1998; OECD, 2000). In this study, all rats in the vehicle control and *DC* treated groups were gaining weight; however, there were no significant changes in body weight gain between the groups in a sub-acute oral test at each week (Table 1 B). Moreover, no significant change detected between the groups in both acute and sub-acute oral toxicity test regarding the ROW (Table 3 A and B). In satellite groups, nothing abnormal detected, and no significant difference was shown in body weight gain (Table 3.2) and ROW (Table 3.4).

Sub-acute oral toxicity test was conducted to evaluate the adverse effects of test medicinal plant *DC* extract and was carried out to provide information about the possible health threats that probable to arise from sub-acute exposure over a period of time, the possibilities of cumulative effects, and an estimate of the dose at which there is no observed adverse effect. Evaluation of safety margin between different dose level that produces the therapeutic effect and that which produces the adverse effects is necessary. Evaluation of safety is exactly to provide benefit to risk assessment. Animal experimental model is the only method that can assess this matter (Prajapati et al., 2006). Determination of food consumption is an important to study the safety of a product with therapeutic purpose as proper intake of nutrients is essential to the physiological status of the animal and give a good impression of the appropriate response to the treatment (Sathish et al., 2012).

For food consumption, no significant changes observed in all groups (vehicle and *DC* extract treated groups) in both acute and subacute oral toxicity tests and this reveals that it did not adversely affect the basic metabolic processes of the experimental animals. On the other hand, water intake showed more in the *DC* extract treated groups than the control, with a significant difference showed in sub-acute oral toxicity test (Table 3.8) and satellite group (Table 3.9) for both male and female rats during the administration period. This result could be referred to that *DC* resin extract can produce vasodilatation (hypotension) due to relaxation of smooth muscles of blood vessels (Al-Awthan et al., 2010) which in turn stimulate thirst and increase water intake (Thunhorst et al., 2010).

Haematological and biochemistry data play a major role in determining the toxicity induced by drugs (Petterino & Argentino-Storino, 2006). Blood parameters analysis is appropriate to risk evaluation as the haematological system has a higher prognostic value for toxicity (Olson et al., 2000). Blood serves as the main medium of transport for many drugs and xenobiotics in the body and for that reason components of the blood exposed to substantial concentrations of toxic compounds. Damage to and destruction of the blood cells are inimical to normal functioning of the body both in humans and animals (Abotsi et al., 2011). In the present study, the haematological parameters data indicated that *DC* extract did not affect blood cells production as there was no significant difference between the groups in acute and sub-acute oral toxicity tests (Table 3.10 A and B). In the satellite group, no significant difference showed between the two groups (Table 3.11). The change in haematological parameters was within the normal range as showed elsewhere (Abiola & Said, 2014; Petterino & Argentino-Storino, 2006).

Evaluation of Kidney and Liver function is important in the assessment toxicity of plant extracts as both of them are necessary for the survival of an organism (Olorunnisola et al., 2012). In animal model toxicity studies, the serum level of creatinine remains the

most widely used laboratory test to estimate renal function. It kept within a relatively stable range as daily production and renal excretion are continuous in healthy mammals (Yilmaz et al., 2007). In the present study, for kidney function test, two serum renal biochemical parameters, namely urea and creatinine were analysed as previously mentioned (P'ng et al., 2013). There were no significant changes observed in urea and creatinine levels between the control and all doses of *DC* extract groups in both acute and sub-acute oral toxicity tests.

The enzymatic activity of the liver such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) was studied to evaluate liver malfunctions. Liver enzymes levels are usually raised in acute hepatoxicity but tend to decrease with prolonged intoxication due to damage to the liver (Imafidon & Okunrobo, 2012). In the present study, there were no significant differences shown in the biochemical analysis in acute and sub-acute oral toxicity test. The level was within normal expected range for the rat species used in this study. Bilirubin formed from the breakdown of haemoglobin in the liver, spleen, and bone marrow. An increase in tissue or serum bilirubin level occurs through increased breakdown of RBC (haemolysis) or in the case of hepatitis or bile duct obstruction (liver damage) (Chitra et al., 2015). Reduction in serum albumin level may suggest infection or continuous loss of albumin (Yakubu et al., 2003). Thus, the insignificant change in serum concentration of albumin and globulin in control and *DC* treated groups at all doses used in this study proposed that *DC* extracts not do damage in hepatocellular or secretory functions of the liver which in turn indicated non-adverse effects of the tested material. For biochemical analysis, in the satellite group, there was no significant difference has been noted which concluded that the tested material (*DC* extract) would not produce the delayed onset of toxicity.

The assessment of histopathological alterations in organs considered as a basic test in the safety assessment of tested materials (Greaves, 2012). No abnormality observed on gross or histopathological evaluations of organs examined in this study. Histopathological findings of liver, kidney, heart, lung and spleen were normal in all animals that given different doses of *DC* resin methanol extract in both acute and sub-acute oral toxicity tests.

3.6 Conclusions

In conclusion, according to Globally Harmonised Classification System, *DC* resin methanol extract can be classified as Category 5. In addition, we may conclude that *DC* resin methanol extract is well tolerated up to the dose of 1500 mg/kg body weight administered daily for 28 days. *DC* resin methanol extract did not cause any lethality or produce any serum chemical alteration or important histopathological signs. The present investigation demonstrates, at least in part, the safety of *DC* resin methanol extract suggesting its promising potential for pharmaceutical uses.

CHAPTER 4: CHEMOPREVENTIVE AND CHEMOTHERAPEUTIC ACTIVITIES OF *DRACAENA CINNABARI* EXTRACT *IN VIVO*

4.1 Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common human cancers in the world. Almost 60–70% of oral-cavity carcinoma cases are diagnosed only after the tumours have become locally progressive (Tang et al., 2014). The World Health Organization (WHO) stated that the oral cavity cancer is having one of the highest mortality ratios of all malignancies because of extensive local invasion and distant metastasis even at initial diagnosis (Koh et al., 2011). Importantly, the mortality ratios do not take into account severe morbidity arising from the facial defacement and aerodigestive dysfunction that associated with surgery/radiotherapy (Wali et al., 2012). Unfortunately, unacceptable results always associated with the commonly used cancer chemotherapy, as the therapy is detrimental to patient health by making patients more vulnerable to other diseases and often cause death by debilitating the immune system of the patient body (Carelle et al., 2002). In addition, the drug-resistant cancer cells that remain alive after chemotherapy are responsible for the recurrence of tumours and the poor prognosis for patients. The occurrence of drug resistance leads to the failure of tumour treatment (Gottesman, 2002). Therefore, prevention of this malignancy represents a major healthcare imperative (Wali et al., 2012).

In a period of 2006 to 2012, United states collected data for patients with oral or pharyngeal cancer, and they demonstrate that a 5-year survival rate was 64% which is more than the rate reported in 1975-1977 as it was 52.5% (Howlader N et al., 2016). A relatively improvement in 5-year survival of patients with oral or pharyngeal cancer over more than four decades, propose that presence of primary prevention efforts to decrease exposure to major risk factors (e.g., tobacco, alcohol and HPV) and secondary prevention efforts to reduce mortality from this disease that involving cancer chemoprevention are

necessary (McCormick et al., 2015). Cancer chemoprevention was first well defined by Sporn (1976). This term refers to the use of natural, synthetic, or biologic chemical agents in an attempt to reverse, suppress, or prevent carcinogenic progression. A material of natural or synthetic origin considered as chemopreventive agents. The difference between these agents and other drugs (which do not prevent disease) is by reducing the incidence of diseases such as cancer before clinical symptoms have to take place (Tanaka et al., 2011). It can suppress the elevation of cancer and prevent the transformation of premalignant cells by modifying differentiation (Schwartz, 2000).

Dracaena cinnabari (*DC*) (Dragon's Blood Tree) is an endemic species, and the most iconic plant of the Socotra Island belongs to the Republic of Yemen (Hubálková et al., 2015). The resin of *DC* has been reported to possess a wide spectrum of therapeutic properties, including antimicrobial activity (Mothana & Lindequist, 2005) and antioxidant activity (Gupta et al., 2014). Recently, the methanol extract of the *DC* resin was studied by Alabsi et al. (2016), and the cytotoxic effects were observed in a panel of OSCC cell lines and were most pronounced in H400 with an IC_{50} value of 5.9 µg/ml. *DC* resin methanol extract inhibited H400 cells growth in a time-dependent manner, and the mode of cell death that induced was via apoptosis (Alabsi et al., 2016). Therefore, in the present study, the activity of *DC* resin methanol extract that daily administered orally in 4NQO induced oral cancer for a short interval to decrease the oral tumour burden (tumour size) and as inhibitors of the induction of OSCC was evaluated.

4.2 Materials and Methods

4.2.1 Animals Studies and Tumour Induction

Prior to the beginning of *in vivo* study, animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Medicine, University of Malaya, Malaysia (Ethics No. 2014-02-14/OBBS/R/NAA). Humane endpoint of all rats was considered following specific criteria in the present study as described elsewhere (McCormick et al., 2015) which included: loss of > 15% of body weight in one week; steady but constant decrease in body weight; sufficient signals that a study rat may not survive until the next scheduled observation; and protracted unhealthy appearance such as rough coat or hunched posture.

Male Sprague Dawley (SD) rats (6-8 weeks) weigh between (160-200 g) were housed in a climate controlled environment (24 C^o, with a 12 h light/dark cycle). They were acclimatised to laboratory conditions for 7 days prior to the experiments.

Rats were provided *ad libitum* rat chow and drinking water supplemented with 4-nitroquinoline 1-oxide (4NQO) (Sigma Aldrich) to a final concentration of 0.02 g/l (20 ppm) to induce hyperplastic and neoplastic lesions in the tongue of the rats as described by Tanaka and Ishigamori (2011). Freshly made 4NQO supplemented water was dispensed to rats in bottles wrapped with foil to preclude possible photodegradation of the carcinogen. Animals received drinking water containing 4NQO for 8 weeks that were changed at two- to three-day intervals throughout the administration period.

4.2.2 *Dracaena cinnabari* (*DC*) Resin Extract Preparation and Dose Selection

Preparation of *DC* resin methanol extract was done as mentioned previously in section (3.2.1. Preparation of *Dracaena cinnabari* (*DC*) Resin Extract).

The dose levels of *DC* extract used in chemopreventive and chemotherapeutic studies were selected based on the results of the acute and sub-acute oral toxicity studies that performed in our laboratory. The result indicated that *DC* resin methanol extract is well tolerated up to the dose of 1500 mg/kg body weight administered daily for 28 days and did not produce any toxic signs or symptoms. The aims of oral toxicity studies were to identify levels of *DC* resin methanol extract that induced no mortality, body weight suppression, or other evidence of limiting toxicity (McCormick et al., 2015). On this basis, in this study, doses of *DC* resin methanol extract lesser than 1500 mg/kg such as 100 mg/kg (low), 500 mg/kg (medium) and 1000 mg/kg (high) body weight were chosen for the present study and administered orally once per day for 10 consecutive weeks.

4.2.3 Cisplatin Preparation

Cisplatin 50 mg Freeze-dried yellowish crystalline powder, soluble in physiological saline solution, purchased from (Axon scientific sdn Bhd) was used. Preparation: 50 mg of the powder was diluted in 50 ml of saline solution 0.9 %, making up a final concentration of 1 mg/ml (Freitas et al., 2009). In clinical practice, administration of Cisplatin 100 mg/m^2 once every 3 weeks parallel with radiotherapy is a commonly recommended schedule for head and neck SCC treatment. Chemotherapy protocol schedule was achieved by administration of Cisplatin in week 1, 4 and 7 (Cisplatin 100 mg/m^2) over a period of 2 to 3 days every 3 weekly chemotherapy (Geeta et al., 2006; Tsan et al., 2012). In the present study, the dose of Cisplatin was decided based on the clinical doses converted into animal doses using surface area and weight factors. Therefore, Cisplatin 3 mg/kg body weight was administered in week 9, 12 and 15 over a period of 2 to 3 days every 3 weeks.

4.2.4 Chemoprevention Study

This study was designed to evaluate the effects of *DC* resin methanol extract during the initiation phase of 4NQO-induced oral carcinogenesis in male SD rats.

The SD rats were divided randomly into 6 groups of seven rats per each. Group I was provided with normal reverse osmosis (RO) water *ad libitum* and served as normal control group and kept in the same conditions. These rats were left untreated but weighed every week to compare their body weights and other parameters with those of the experimental rats in which 4NQO was administered. Group II was received only 4NQO supplemented water *ad libitum* and served as induced cancer or positive control. Group III was received 4NQO supplemented water *ad libitum* and given a vehicle (10% DMSO) orally. Groups IV, V and VI were orally given *DC* extract in different concentrations 100, 500 and 1000 mg/kg respectively, starting one week before exposure to the carcinogen (4NQO) until one week after the stop of the carcinogen exposure (total of 10 consecutive weeks) parallel with 4NQO supplemented water *ad libitum* which was administered for 8 consecutive weeks as previously described (Tanaka et al., 1998; Zhang et al., 2013). Then, animals from groups II-VI were switched to RO water and continued on this water until study termination after week 22 (Figure 4.1). Oral administration to the rats was done by gastric intubation using force-feeding needle size 18G (Harvard Apparatus, INC).

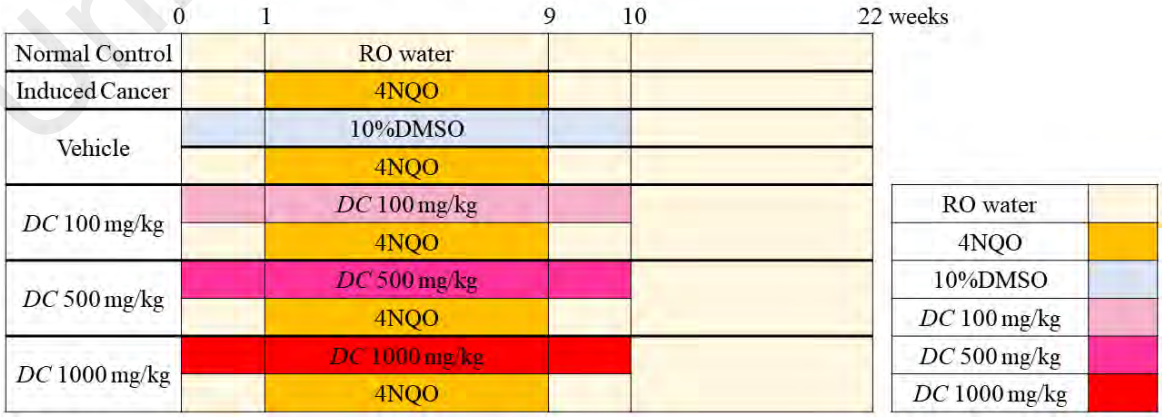

Figure 4. 1: Diagrammatic representation of experimental protocol in chemopreventive study

4.2.5 Chemotherapeutic Study

This study was designed to evaluate the effects of *DC* resin methanol extract during the post-initiation phase of 4NQO-induced oral carcinogenesis in male SD rats.

A total of 70 male SD rats were used in this study (10 groups of seven rats per each). Group 1 was provided with RO water *ad libitum* and served as normal control group. The rats in this group were left untreated but weighed every week to compare their body weights and other parameters with those of the experimental rats in which 4NQO was administered. The remaining 63 male SD rats were given 4NQO (20 ppm) for 8 weeks in their drinking water *ad libitum*; after 8 weeks, rats received drinking water without added 4NQO. Then, the rats were randomized into nine groups of 7 rats per each. From 9-22 weeks, Group 2 served as induced cancer group. Group 3 was given 10% DMSO (orally) and served as a vehicle. In group 4, Cisplatin 3 mg/kg (axon scientific sdn Bhd) (a widely used chemotherapeutic agent for oral cancer) was administered I.P to the rats. *DC* extract 100 mg/kg, *DC* extract 500 mg/kg and *DC* extract 1000 mg/kg were administered orally to group 5, 6 and 7 respectively. Cisplatin 3 mg/kg and *DC* extract at doses of 100, 500 and 1000 mg/kg were given as a combination to group 8, 9 and 10, respectively, (i.e. *DC* extract was administered orally 2 h before the Cisplatin 3 mg/kg I.P). The Cisplatin 3 mg/kg was administered once every three weeks, whereas the *DC* extract was given orally/day. Oral administration to the rats was done by gastric intubation using force-feeding needle size 18G (Harvard Apparatus, INC) starting 1 week after cessation of 4NQO exposure and maintained on this treatment for 10 consecutive weeks as described elsewhere (Tanaka et al., 1998; Zhang et al., 2013). The experiment was terminated after week 22 (Figure 4.2). All animals were sacrificed to assess the incidences of pre-neoplastic and neoplastic lesions in the tongue.

Figure 4. 2: Diagrammatic representation of experimental protocol in chemotherapeutic study

For both studies, cage-side observations were done at least twice daily to assess animal health and identify possible toxicities resulting from administration of carcinogen (4NQO). The body weights of the rats were recorded weekly throughout the study. Body weight is a key metric in oral cancer chemoprevention studies in the 4NQO model since body weight loss provides a useful indication of the clinical progression of OSCC (Tanaka & Ishigamori, 2011). Hand-held observations were performed weekly to monitor the presence of oral lesions.

The food consumption and water intake were recorded daily. The body weight gain was calculated by the difference between the body weight at the end of each week and the body weight at the beginning of the study (Study Day 0) (Barcessat et al., 2014).

The surviving rats were generally anaesthetized by intraperitoneal injection of 80 mg/kg of ketamine 100 mg/ml + 7 mg/kg of xylazine 100 mg/ml (Troy laboratories PTY. Limited, Smithfield, Australia). Blood sample (5 ml) by cardiac puncture was collected using a disposable syringe for analysing the haematological and biochemical parameters as described in section (3.2.5 Haematology and serum biochemistry), then the rats were euthanised by cervical dislocation. All rats (whether found dead, euthanised in extremis,

or euthanised at study termination) received a limited gross necropsy that focused on the tongue and oral cavity. The tongue from each rat was carefully excised, all gross lesions were recorded, and the tongue was bisected longitudinally. At necropsy, one-half of each tongue tissue was fixed in 10% buffered formalin, routinely processed, embedded in paraffin wax and subjected to histological processing and immunohistochemical analysis. The other half was stored in RNAlater solution (Qiagen) to ensure the stability of RNA and then transferred and frozen at -80°C for use in molecular analyses.

4.2.6 Estimation of Tumour Volume (Size)

The dissected tongues were inspected for the presence of overt tumours. The tumour volume (size) was measured according to the formula ($\pi/6 \times$ width $\times$ length $\times$ height) as mentioned elsewhere (Tomayko & Reynolds, 1989; Wali et al., 2012). Detected lesions were having different sizes ranging from small well-defined white papules to extensive exophytic formations. Histopathological evaluations for the presence of epithelial changes in the tongue tissue were performed after staining with haematoxylin and eosin.

4.2.7 Histopathological Evaluation (H&E) on the Incidence of Pre-Neoplasms and Neoplasms in the Tongue of the Rats

4.2.7.1 Tissue Preparation

(a) ***Fixation***

The tongue tissue specimen fixed in 10 % natural buffer formalin (NBF). The half of the tongue placed in a container containing 10% NBF and fixed for 24 hours. The ratio of tissue to fixative volume was 1:10.

(b) ***Processing for blocking***

The block processing was carried out using a Leica TP 1020 tissue processor. It is an automatic machine where the specimen underwent a long cycle schedule. The samples were treated through twelve stations with series of graded concentrations of ethanol (70 %, 95 %, 100 %, 100 % and 100 %) and xylene, and then Paraplast wax (56°C melting point). Finally, the samples were embedded in molten (56°C) paraffin wax. The blocks were stored in airtight container for future use.

(c) ***Sectioning***

A microtome (Leica, Germany), with disposable microtome blade (Leica 818 high profile disposable microtome blade) was used for sectioning to get a ribbon section. Four µm thin sections were floated on a water bath (45 °C) before being mounted on the glass slide, MUTO. Then, forceps was used to put a section on a glass slide and followed by a drop of 20% Alcohol.

(d) ***Staining***

Fixed sections were placed in fresh xylene bath and incubated at room temperature for 5 minutes. The previous step was repeated with fresh xylene for 4 minutes. Excess liquid was drained, and the slides were placed in a fresh absolute ethyl alcohol bath, 95 %, 70 % alcohol bath for 3 minutes and then running water bath for 3 minutes at room temperature. Sections were stained with Harris' haematoxylin working solution for 5 minutes. Then, it washed in slow running tap water until excess blue colour went off (3 minutes). Sections were differentiated by dipping in 0.5% acid alcohol (count from 1 to 10) and washed well in running tap water for 3 minutes. Sections were dipped in 2% sodium acetate (4 dips) and washed well in running tap water (3 minutes). The slides were placed in 80 % alcohol bath for 1 minute, and sections were stained with Eosin working

solution for 2-3 minutes. The slides were placed in 95 %, 95 % alcohol bath for four dips then 100% twice for 2 minutes. The sections were placed in xylene I, II and III bath for 3 minutes and finally, the slides mounted and coverslipped with mounting medium and checked under the microscope. H&E stain slides were evaluated for the incidence of pre-neoplastic and neoplastic lesions in the tongue.

The histopathological evaluation in this study was performed using a light microscope (Nikon E50i). An experienced pathologist who was unaware of the experimental groups to which each section belonged conducted the analysis. The tongue sections were analysed and graded as normal, hyperplasia, dysplasia and squamous cell carcinoma per animal as described by Ribeiro et al. (2007) and El-Rouby (2011). The dysplastic lesion was further graded as mild, moderate and severe according to the 2005 WHO Classification (Barnes et al., 2005).

4.2.8 Histopathological Evaluation of Oral Cancer Metastasis

To illustrate and examine the inhibitory effect of *DC* extract and/or in combination with Cisplatin on metastasis as well as Cisplatin group, we performed chemotherapy in a 4NQO-induce rat model and compared the inhibition of metastasis to the cervical lymph nodes histologically.

In the current study, cervical lymph nodes of all rats in the chemotherapeutic study were excised at the end of the study (after week 22). All samples were fixed in 10% NBF, embedded in paraffin and serial sections were cut and stained with haematoxylin and eosin as described previously in section (4.2.7 Histopathological Evaluation). The slides were stained and examined in detail to identify foci of metastasis.

4.2.9 Immunohistochemical (IHC) Analysis

The tongue tissue sections were subjected to IHC analysis to determine the effect of *DC* 1000 mg/kg on the expression of the following tumour marker; Cyclin D1, Ki-67, Bcl-2, p53, β-catenin and E-cadherin.

The effect of *DC* extracts was in a dose-dependant manner as presented in the histopathological part of this study. The highest dosage of *DC* extract showed the best inhibition effect on the 4NQO-induced oral cancer in rats for both the chemopreventive and chemotherapeutic studies. For this reason, a group of rats with the dose of *DC* 1000 mg /kg was selected to perform the IHC analysis and gene analysis evaluations.

Formalin-fixed paraffin-embedded (FFPE) blocks were sent to the Pathology Lab, Department of Pathology, Faculty of Medicine, University of Malaya. Sections at 4 μm were cut, mounted on slides, deparaffinized and rehydrated. Immunohistochemistry was performed with Ventana Benchmark XT autostainer (Ventana Medical Systems Inc., Tucson, Arizona) using the following antibodies; Bcl-2 (clone 124, Dako, Japan), p53 (clone DO-7, Dako, Japan), Ki-67 (clone MIB-1, Dako, Japan), E-cadherin (clone NCH-38, Dako Japan), beta-catenin (clone b-Catenin-1, Dako, Japan) and cyclin D1 (clone SP4, Thermo Fisher Scientific). Automated IHC staining was performed according to routine guidelines of Department of Pathology Faculty of Medicine, University of Malaya.

4.2.9.1 Digital Image Analysis of Immunohistochemical Stain Expression

The verification for the immunohistochemical reaction of Cyclin D1, Ki-67, Bcl-2, p53, β-catenin and E-cadherin in three samples (Dysplastic epithelia and SCC) from each group was done by light microscope. Then, immunostained sections were digitised using a digital slide scanner Panoramic Desk Scanner (3DHistech, Budapest, Hungary). Five fields at 400x magnification were randomly selected for each sample. Images were taken of these five fields. The immunoreactivity for each tumour marker was measured in the

form of area percent by using computer assisted image analysis (ImageJ, National Institute of Mental Health, Bethesda, MD, USA) with an IHC toolbox plugin (https://imagej.nih.gov/ij/plugins/ihc-toolbox/index.html) as mentioned in another study (Katsha et al., 2017). The percent area occupied by positive (brown) staining could be assessed by the functions of semi-automatic colour selection and automatic statistical colour detection model that presented in ImageJ's IHC Toolbox. Thresholds was set that successfully quantified staining in a positive staining specimen and this was then duplicated in every image for comparison purpose.

4.2.10 Gene Expression Analysis

The tongue tissue sections that stored in RNAlater solution (Qiagen) at -80°C were subjected to gene analysis study to determine the effect of *DC* 1000 mg/kg on the expression of selected apoptosis and proliferative gene related to oral cancer.

4.2.10.1 RNA Extraction

In both chemopreventive and chemotherapeutic studies, total cellular ribonucleic acid (RNA) was extracted from the tissue fragments dissected out of the posterior region of tongues of the induced cancer and *DC* 1000 mg/kg groups by using RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol.

Briefly, Fresh-frozen tissue sections were incubated in 350 µl Buffer RLT which contained 3.5 µl beta mercaptoethanol. The mixture was homogenised with 1 ml syringe. One volume of 70% ethanol was added to the mixture followed by vortexing for 30 seconds. The mixture was transferred into RNeasy MinElute spin column assembly with 2 ml collection tube and centrifuged for 15 seconds at 10,000 rpm. The flow through solution was discarded. The amount of 350 µl of buffer RW1 was added to the RNeasy MinElute spin column assembly with 2 ml collection tubes and centrifuged for 15 seconds at 10,000 rpm to wash the spin column. The flow through solution and collection tubes

was discarded. RNeasy MinElute spin column was placed in a new 2 ml collection tube and 500 µl Buffer RPE was added to the spin column continued by centrifuged for 15 seconds at 10,000 rpm to wash the spin column membrane. A total of 500 µl of 80% ethanol was added to the RNeasy MinElute spin column and centrifuged for 15 seconds at 10,000 rpm to wash the spin column membrane. The flow through solution and collection tube was discarded. The RNeasy MinElute spin column was placed in a new 2 ml collection tube. The lid of the spin column was opened during centrifugation at full speed for 5 minutes. The flow through solution and collection tube was discarded. The RNeasy MinElute spin column was placed in a new 1.5 ml collection tube. A total of 32 µl of RNase free water was directly added to the center of the spin column membrane and centrifuged for 3 minutes at full speed to elute the RNA.

The integrity of RNA was accessed using the Agilent Bioanalyzer-2100 (Agilent Technologies, CA, USA) to determine the quality of the extracted RNA from the samples. 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 being the most degraded total RNA and 10 being the most intact total RNA. RNA integrity number ≥ 7 were used for further analysis.

4.2.10.2 RNA Quantitation

RNA was assessed by measuring absorbance at 260 nm (A260) and 280 nm (A280) by using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A260/A280 ratio between 1.8 - 2.1 indicated high-quality RNA, while A260 determined the yield of total RNA.

4.2.10.3 First Strand cDNA Synthesis

Complementary DNA (cDNA) synthesis was carried out using the RT² First Strand Kit (Qiagen, USA) in a total of 1 µg of total RNA. Random hexamers and oligo-dT primed the reverse transcription reactions, and a reverse transcriptase synthesised the cDNA product.

The first step in cDNA synthesis was the genomic DNA elimination step. This step was done by addition of genomic DNA elimination mixture (Table 4.1) to total RNA isolated from tissues (1 µg of total RNA) followed by incubation at 42°C for 5 min. The mixture was then placed on ice immediately for at least one minute.

Table 4. 1: Genomic DNA Elimination Mixture

For each RNA sample, the following solutions were combined in a sterile PCR tube:

Total RNA	**25.0 ng to 5.0 µg**
GE (5X gDNA Elimination Buffer)	2.0 µl
RNase free H_2O to a final volume of	10 µl

The next step was reverse transcription using random hexamers and oligo-dT primers. This step was done by addition of RT cocktail (Table 4.2) to the above mixture, followed by incubation at 42°C for exactly 15 min in a PCR machine (Applied Biosystems Veriti Thermal Cycler). Then, the reaction was immediately stopped by heating at 95°C for 5 minutes. Sterile water (91 µL) was then added to each 20 µL of cDNA synthesis reaction and mixed well. The cDNA was then stored at -20 °C until further use.

Table 4. 2: RT Cocktail

RT Cocktail	**1 reaction**
BC3 (5X RT Buffer 3)	4 µl
P2 (Primer and External Control Mix)	1 µl
RE3 (RT Enzyme Mix 3)	2 µl
RNase free H_2O	3 µl
Final Volume	**10 µl**

4.2.10.4 Real-time qPCR

A total of 675 µl of master mix was prepared by mixing 25.5 µl of 1 µg of cDNA synthesised from each sample with 337.5 µl RT^2 SYBR Green/ROX qPCR Master Mix (SABiosciences) and water. Then, 25 µl of the aliquot mixture was loaded onto each well of a pre-designed 96-well RT^2 Profiler PCR Array (SABiosciences) customised for 4 samples. Each well from the Array plate was pre-hybridized with primers pair for a gene member that listed in Table 4.3. A housekeeping gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control.

Real-time PCR was performed by ABI 7500 HT FAST thermal cycler with recommended thermal cycler program: 95°C 10 mins; 40 cycles of 95°C for 15 secs and 60°C for 60 secs; Dissociation 95°C for 1 min, 60°C 2 mins and 95°C 2 mins. The reactions were performed in triplicate. The mRNA level of housekeeping gene GAPDH was used to normalize the gene expression data. Data were analysed using the GeneGlobe Data Analysis Centre on QIAGEN's website (https://www.qiagen.com/my/shop/genes-and-pathways/data-analysis-center-overview-page/).

Table 4. 3: List of the selected genes

Symbol	Description
Bax	-
Casp3	Caspase 3
Bcl-2	B-cell CLL/lymphoma 2
Tp53	Tumour protein p53
Ptgs2 (Cox-2)	Prostaglandin-endoperoxide e synthase 2
Ccnd1	Cyclin D1
EGFR	Epidermal growth factor receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

4.2.11 Reliability of Histopathological Evaluation (H&E) on the Incidence of Pre-Neoplasms and Neoplasms in the Tongue of the Rats

Intra-examiner reliability was assessed by re-evaluation of all samples twice after a seven-day interval and without the knowledge of the previous reading. Cohen's kappa (k) was run to determine if there was agreement between the two readings of the incidence of OSCC. There was a substantial agreement between the two readings, k = 0.774, p = 0.000 in chemopreventive study (Table 4.4), and k = 0.777, p = 0.000 in chemotherapeutic study (Table 4.5).

Table 4. 4: Reliability test (Kappa test) for chemopreventive study

Symmetric Measures					
		Value	**Asymp. Std. Error[a]**	**Approx. T[b]**	**Approx. Sig.**
Measure of Agreement	Kappa	0.774	0.092	6.354	0.000
N of Valid Cases		35			

Table 4. 5: Reliability test (Kappa test) for chemotherapeutic study

Symmetric Measures					
		Value	**Asymp. Std. Error[a]**	**Approx. T[b]**	**Approx. Sig.**
Measure of Agreement	Kappa	0.777	0.069	8.597	0.000
N of Valid Cases		63			

4.2.12 Statistical Analysis

The measured values were expressed as a mean ± standard deviation. Statistical analysis was performed using SPSS version 20.

Food consumption, water intake, body weights gain, haematological and biochemical parameters were compared by analysis of variance (ANOVA), with post-hoc comparisons made using Dunnett's test.

Comparison of the histopathological incidence (normal, hyperplasia, dysplasia and squamous cell carcinoma) was made using descriptive statistics and chi-square test.

For RT^2 Profiler PCR Array, Data were analysed using GeneGlobe Data Analysis Center on QIAGEN's website (https://www.qiagen.com/my/shop/genes-and-pathways/data-analysis-center-overview-page/).

For IHC, area percent of stained detection data was performed by independent *t* test and one-way ANOVA test for the chemopreventive and chemotherapeutic experiments, respectively.

Evidence of chemopreventive and chemotherapeutic activity was defined as a statistically significant ($p < 0.05$).

4.3 Results

4.3.1 Effect of *DC* Extract on Mean Food Consumption

The mean food consumption of all rats in each group was recorded daily throughout the study and the data were expressed in 1-week cumulative value/group as illustrated in Figures 4.3. and 4.4. For chemopreventive study, there were no significant differences presented in the first 11 weeks of the study, however, the significant differences were presented at 12 through 22 weeks between the groups (Appendix A). Whereas, in the chemotherapeutic study, no significant differences have been seen in the first 9 weeks and at week 11 ($p > 0.05$), however, the significant differences were presented at 10 and 12 through 22 weeks between the groups as $p < 0.05$ (Appendix B).

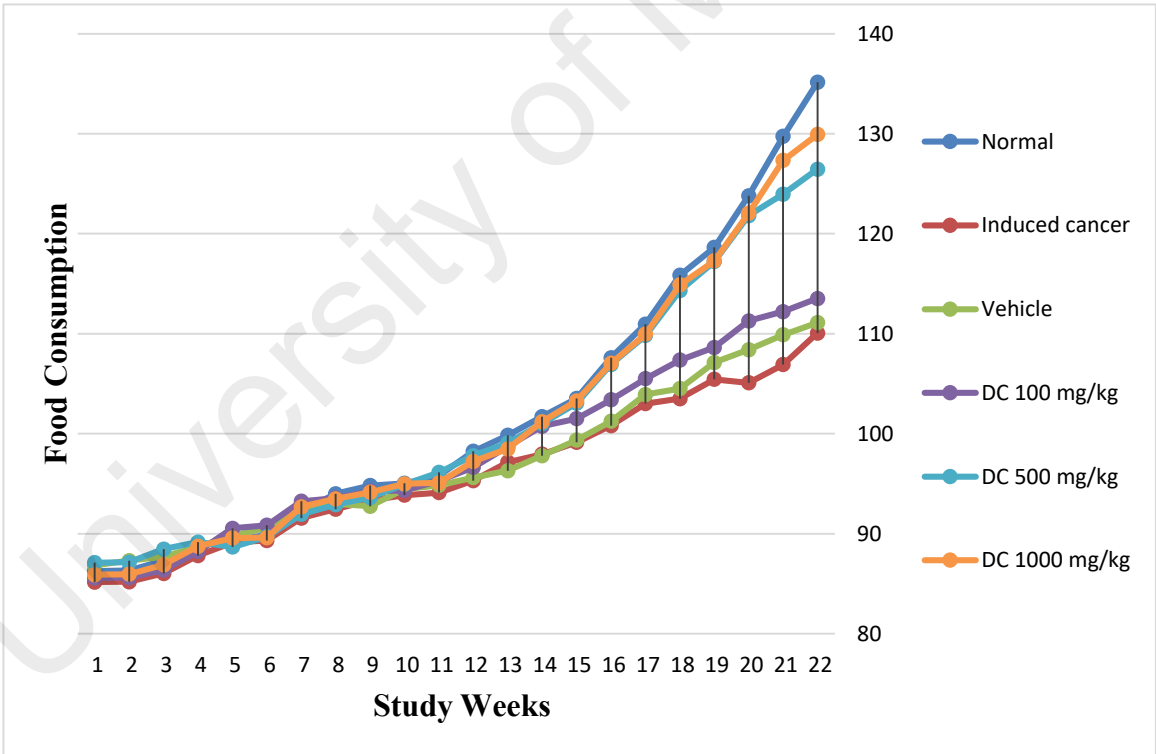

Figure 4. 3: Mean food consumption during 4NQO-induced oral carcinogenesis in the chemopreventive study

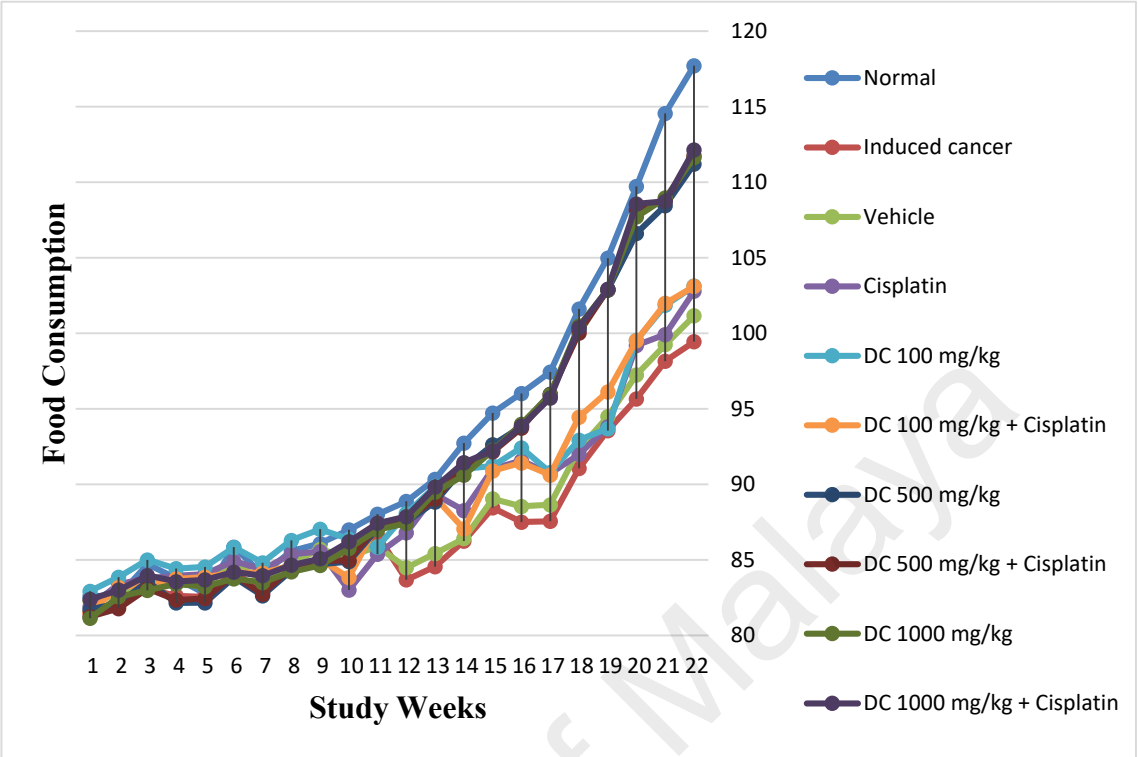

Figure 4. 4: Mean food consumption during 4NQO-induced oral carcinogenesis in the chemotherapeutic study

Additionally, in the chemopreventive study, a post hoc Dunnett's test showed that the food consumption decreased in induced cancer and vehicle groups with statistically significant differences showed at the following times: 12 through 22 weeks compared to the normal control group ($p < 0.05$). At 16 through 22 weeks, *DC* 100 mg/kg group showed a significantly decreased food consumption compared to the normal control group ($p < 0.05$). The food consumption of *DC* resin treated groups at doses 500 and 1000 mg/kg were comparable to the normal control group throughout the study except at the last two weeks (21 and 22 weeks) where a significant decreased of food consumption was showed in *DC* 500 mg/kg group and in the *DC* 1000 mg/kg group at week 22 compared to the normal control group as $p < 0.05$ (Table 4.6).

Table 4. 6: Mean Food consumption of rats during 4NQO-induced oral carcinogenesis in the chemopreventive study. A post hoc Dunnett's test.

Dependent Variable	(I) Food Consumption	(J) Food Consumption	Mean Difference (I-J)	Std. Error	*P* value
Week 12	Induced Cancer	Normal	-2.93*	0.98	0.022
	Vehicle	Normal	-2.62*	0.98	0.046
	DC 100 mg/kg	Normal	-1.60	0.98	0.359
	DC 500 mg/kg	Normal	-0.43	0.98	0.991
	DC 1000 mg/kg	Normal	-1.02	0.98	0.753
Week 13	Induced Cancer	Normal	-2.69*	0.80	0.008
	Vehicle	Normal	-3.53*	0.80	0.000
	DC 100 mg/kg	Normal	-1.13	0.80	0.492
	DC 500 mg/kg	Normal	-0.76	0.80	0.809
	DC 1000 mg/kg	Normal	-1.36	0.80	0.322
Week 14	Induced Cancer	Normal	-3.75*	0.73	0.000
	Vehicle	Normal	-3.90*	0.73	0.000
	DC 100 mg/kg	Normal	-0.95	0.73	0.567
	DC 500 mg/kg	Normal	-0.70	0.73	0.803
	DC 1000 mg/kg	Normal	-0.51	0.73	0.930
Week 15	Induced Cancer	Normal	-4.38*	0.77	0.000
	Vehicle	Normal	-4.16*	0.77	0.000
	DC 100 mg/kg	Normal	-2.02	0.77	0.050
	DC 500 mg/kg	Normal	-0.47	0.77	0.960
	DC 1000 mg/kg	Normal	-0.23	0.77	0.998
Week 16	Induced Cancer	Normal	-6.79*	0.84	0.000
	Vehicle	Normal	-6.35*	0.84	0.000
	DC 100 mg/kg	Normal	-4.21*	0.84	0.000
	DC 500 mg/kg	Normal	-0.68	0.84	0.883
	DC 1000 mg/kg	Normal	-0.57	0.84	0.938
Week 17	Induced Cancer	Normal	-7.96*	0.89	0.000
	Vehicle	Normal	-7.04*	0.89	0.000
	DC 100 mg/kg	Normal	-5.46*	0.89	0.000
	DC 500 mg/kg	Normal	-1.14	0.89	0.579
	DC 1000 mg/kg	Normal	-1.02	0.89	0.675
Week 18	Induced Cancer	Normal	-12.33*	1.10	0.000
	Vehicle	Normal	-11.32*	1.10	0.000
	DC 100 mg/kg	Normal	-8.49*	1.10	0.000
	DC 500 mg/kg	Normal	-1.54	1.10	0.497
	DC 1000 mg/kg	Normal	-0.97	1.10	0.845
Week 19	Induced Cancer	Normal	-13.20*	1.01	0.000
	Vehicle	Normal	-11.52*	1.01	0.000
	DC 100 mg/kg	Normal	-10.00*	1.01	0.000
	DC 500 mg/kg	Normal	-1.41	1.01	0.499
	DC 1000 mg/kg	Normal	-1.35	1.01	0.538

Table 4.6 continued					
Week 20	Induced Cancer	Normal	-18.71*	1.19	0.000
	Vehicle	Normal	-15.38*	1.19	0.000
	DC 100 mg/kg	Normal	-12.51*	1.19	0.000
	DC 500 mg/kg	Normal	-1.98	1.19	0.342
	DC 1000 mg/kg	Normal	-1.75	1.19	0.455
Week 21	Induced Cancer	Normal	-22.83*	1.07	0.000
	Vehicle	Normal	-19.84*	1.07	0.000
	DC 100 mg/kg	Normal	-17.53*	1.07	0.000
	DC 500 mg/kg	Normal	-5.78*	1.07	0.000
	DC 1000 mg/kg	Normal	-2.39	1.07	0.118
Week 22	Induced Cancer	Normal	-25.08*	0.62	0.000
	Vehicle	Normal	-24.04*	0.62	0.000
	DC 100 mg/kg	Normal	-21.63*	0.62	0.000
	DC 500 mg/kg	Normal	-8.70*	0.62	0.000
	DC 1000 mg/kg	Normal	-5.22*	0.62	0.000

*p value less than 0.05, ($p < 0.05$) significant value.

Similarly, in the chemotherapeutic study, a post hoc Dunnett's test showed a reduction in food consumption in induced cancer and vehicle groups with statistically significant differences showed at the following times: 12 through 22 weeks compared to the normal control group ($p < 0.05$). At 10 and 14 through 22 weeks, both a Cisplatin and *DC* 100 mg/kg + Cisplatin groups showed statistically significant decreased in food consumption compared to the normal control group ($p < 0.05$). The food consumption of *DC* 100 mg/kg group was decreased significantly at 15 through 22 weeks compared to the normal control group ($p < 0.05$). *DC* extract treated groups at doses of 500 and 1000 mg/kg with or without Cisplatin were practically comparable to the normal control group throughout the study except at the last three weeks (20, 21 and 22 weeks) as $p < 0.05$. However, *DC* 500 mg/kg showed a significant decrease in food consumption start from 20 through 22 weeks, and *DC* 500 mg/kg with Cisplatin, *DC* 1000 mg/kg with or without Cisplatin showed a significant decrease in food consumption only at 21 and 22 weeks compared to the normal control group with $p < 0.05$ (Table 4.7).

Table 4. 7: Mean food consumption of rats during 4NQO-induced oral carcinogenesis in the chemotherapeutic study. A post hoc Dunnett's test.

Dependent Variable	(I) Food consumption	(J) Food consumption	Mean Difference (I-J)	Standard Error	*P* value
Week 10	Induced cancer	Normal	-1.90	0.98	0.295
	Vehicle	Normal	-1.76	0.98	0.377
	Cisplatin	Normal	-3.98	0.98	0.001*
	DC 100 mg/kg	Normal	-0.61	0.98	0.995
	DC 100 mg/kg + Cisplatin	Normal	-3.16	0.98	0.015*
	DC 500 mg/kg	Normal	-2.10	0.98	0.200
	DC 500 mg/kg + Cisplatin	Normal	-2.00	0.98	0.244
	DC 1000 mg/kg	Normal	-1.23	0.98	0.757
	DC 1000 mg/kg + Cisplatin	Normal	-0.78	0.98	0.973
Week 12	Induced cancer	Normal	-5.20	1.07	0.000*
	Vehicle	Normal	-4.39	1.07	0.001*
	Cisplatin	Normal	-2.09	1.07	0.293
	DC 100 mg/kg	Normal	-0.80	1.07	0.981
	DC 100 mg/kg + Cisplatin	Normal	-1.04	1.07	0.920
	DC 500 mg/kg	Normal	-1.37	1.07	0.739
	DC 500 mg/kg + Cisplatin	Normal	-1.19	1.07	0.849
	DC 1000 mg/kg	Normal	-1.45	1.07	0.684
	DC 1000 mg/kg + Cisplatin	Normal	-1.01	1.07	0.929
Week 13	Induced cancer	Normal	-5.80	1.02	0.000*
	Vehicle	Normal	-4.93	1.02	0.000*
	Cisplatin	Normal	-1.02	1.02	0.905
	DC 100 mg/kg	Normal	-0.48	1.02	0.999
	DC 100 mg/kg + Cisplatin	Normal	-1.23	1.02	0.786
	DC 500 mg/kg	Normal	-1.50	1.02	0.596
	DC 500 mg/kg + Cisplatin	Normal	-1.33	1.02	0.723
	DC 1000 mg/kg	Normal	-0.86	1.02	0.961
	DC 1000 mg/kg + Cisplatin	Normal	-0.52	1.02	0.999
Week 14	Induced cancer	Normal	-6.48	0.98	0.000*
	Vehicle	Normal	-6.32	0.98	0.000*
	Cisplatin	Normal	-4.47	0.98	0.000*
	DC 100 mg/kg	Normal	-1.70	0.98	0.407
	DC 100 mg/kg + Cisplatin	Normal	-5.69	0.98	0.000*
	DC 500 mg/kg	Normal	-1.65	0.98	0.443
	DC 500 mg/kg + Cisplatin	Normal	-1.47	0.98	0.574
	DC 1000 mg/kg	Normal	-2.13	0.98	0.187
	DC 1000 mg/kg + Cisplatin	Normal	-1.29	0.98	0.707
Week 15	Induced cancer	Normal	-6.30	0.96	0.000*
	Vehicle	Normal	-5.70	0.96	0.000*
	Cisplatin	Normal	-3.72	0.96	0.002*
	DC 100 mg/kg	Normal	-3.51	0.96	0.004*
	DC 100 mg/kg + Cisplatin	Normal	-3.84	0.96	0.001*
	DC 500 mg/kg	Normal	-2.11	0.96	0.180
	DC 500 mg/kg + Cisplatin	Normal	-2.58	0.96	0.061
	DC 1000 mg/kg	Normal	-2.47	0.96	0.080
	DC 1000 mg/kg + Cisplatin	Normal	-2.51	0.96	0.072

Table 4.7 continued					
Week 16	Induced cancer	Normal	-8.53	0.84	0.000*
	Vehicle	Normal	-7.49	0.84	0.000*
	Cisplatin	Normal	-4.45	0.84	0.000*
	DC 100 mg/kg	Normal	-3.60	0.84	0.001*
	DC 100 mg/kg + Cisplatin	Normal	-4.60	0.84	0.000*
	DC 500 mg/kg	Normal	-2.19	0.84	0.076
	DC 500 mg/kg + Cisplatin	Normal	-2.30	0.84	0.056
	DC 1000 mg/kg	Normal	-2.04	0.84	0.114
	DC 1000 mg/kg + Cisplatin	Normal	-2.18	0.84	0.079
Week 17	Induced cancer	Normal	-9.88	0.65	0.000*
	Vehicle	Normal	-8.79	0.65	0.000*
	Cisplatin	Normal	-6.73	0.65	0.000*
	DC 100 mg/kg	Normal	-6.65	0.65	0.000*
	DC 100 mg/kg + Cisplatin	Normal	-6.81	0.65	0.000*
	DC 500 mg/kg	Normal	-1.45	0.65	0.170
	DC 500 mg/kg + Cisplatin	Normal	-1.50	0.65	0.144
	DC 1000 mg/kg	Normal	-1.50	0.65	0.141
	DC 1000 mg/kg + Cisplatin	Normal	-1.72	0.65	0.067
Week 18	Induced cancer	Normal	-10.55	0.60	0.000*
	Vehicle	Normal	-9.27	0.60	0.000*
	Cisplatin	Normal	-9.62	0.60	0.000*
	DC 100 mg/kg	Normal	-8.68	0.60	0.000*
	DC 100 mg/kg + Cisplatin	Normal	-7.15	0.60	0.000*
	DC 500 mg/kg	Normal	-1.36	0.60	0.158
	DC 500 mg/kg + Cisplatin	Normal	-1.58	0.60	0.069
	DC 1000 mg/kg	Normal	-1.13	0.60	0.326
	DC 1000 mg/kg + Cisplatin	Normal	-1.21	0.60	0.253
Week 19	Induced cancer	Normal	-11.42	0.80	0.000*
	Vehicle	Normal	-10.50	0.80	0.000*
	Cisplatin	Normal	-11.18	0.80	0.000*
	DC 100 mg/kg	Normal	-11.29	0.80	0.000*
	DC 100 mg/kg + Cisplatin	Normal	-8.85	0.80	0.000*
	DC 500 mg/kg	Normal	-2.12	0.80	0.069
	DC 500 mg/kg + Cisplatin	Normal	-2.09	0.80	0.075
	DC 1000 mg/kg	Normal	-2.08	0.80	0.077
	DC 1000 mg/kg + Cisplatin	Normal	-2.06	0.80	0.080
Week 20	Induced cancer	Normal	-14.06	0.83	0.000*
	Vehicle	Normal	-12.47	0.83	0.000*
	Cisplatin	Normal	-10.52	0.83	0.000*
	DC 100 mg/kg	Normal	-10.18	0.83	0.000*
	DC 100 mg/kg + Cisplatin	Normal	-10.20	0.83	0.000*
	DC 500 mg/kg	Normal	-3.11	0.83	0.003*
	DC 500 mg/kg + Cisplatin	Normal	-1.57	0.83	0.318
	DC 1000 mg/kg	Normal	-2.01	0.83	0.111
	DC 1000 mg/kg + Cisplatin	Normal	-1.16	0.83	0.644

Table 4.7 continued					
Week 21	Induced cancer	Normal	-16.40	0.73	0.000*
	Vehicle	Normal	-15.31	0.73	0.000*
	Cisplatin	Normal	-14.64	0.73	0.000*
	DC 100 mg/kg	Normal	-12.70	0.73	0.000*
	DC 100 mg/kg + Cisplatin	Normal	-12.57	0.73	0.000*
	DC 500 mg/kg	Normal	-6.10	0.73	0.000*
	DC 500 mg/kg + Cisplatin	Normal	-5.76	0.73	0.000*
	DC 1000 mg/kg	Normal	-5.60	0.73	0.000*
	DC 1000 mg/kg + Cisplatin	Normal	-5.83	0.73	0.000*
Week 22	Induced cancer	Normal	-18.28	0.80	0.000*
	Vehicle	Normal	-16.57	0.80	0.000*
	Cisplatin	Normal	-14.93	0.80	0.000*
	DC 100 mg/kg	Normal	-14.59	0.80	0.000*
	DC 100 mg/kg + Cisplatin	Normal	-14.61	0.80	0.000*
	DC 500 mg/kg	Normal	-6.52	0.80	0.000*
	DC 500 mg/kg + Cisplatin	Normal	-5.98	0.80	0.000*
	DC 1000 mg/kg	Normal	-6.10	0.80	0.000*
	DC 1000 mg/kg + Cisplatin	Normal	-5.57	0.80	0.000*

*p value less than 0.05, ($p < 0.05$) significant value.

4.3.2 Effect of *DC* Extract on Mean Water Intake

The mean water intake of all rats in each group was recorded daily throughout the study and the data were expressed in 1-week cumulative value/group as illustrated in Figures 4.5 and 4.6. In chemopreventive study, analysis of variance (one-way ANOVA) showed a significant difference at all weeks of the study ($p < 0.05$), except at the 1st, 10th,11th and 12th week between the groups (Appendix C). Whereas, in the chemotherapeutic study, no significant difference detected in the first 11 weeks of the study, however, a significant difference was showed at 12 through 22 weeks between the groups with $p < 0.05$ (Appendix D).

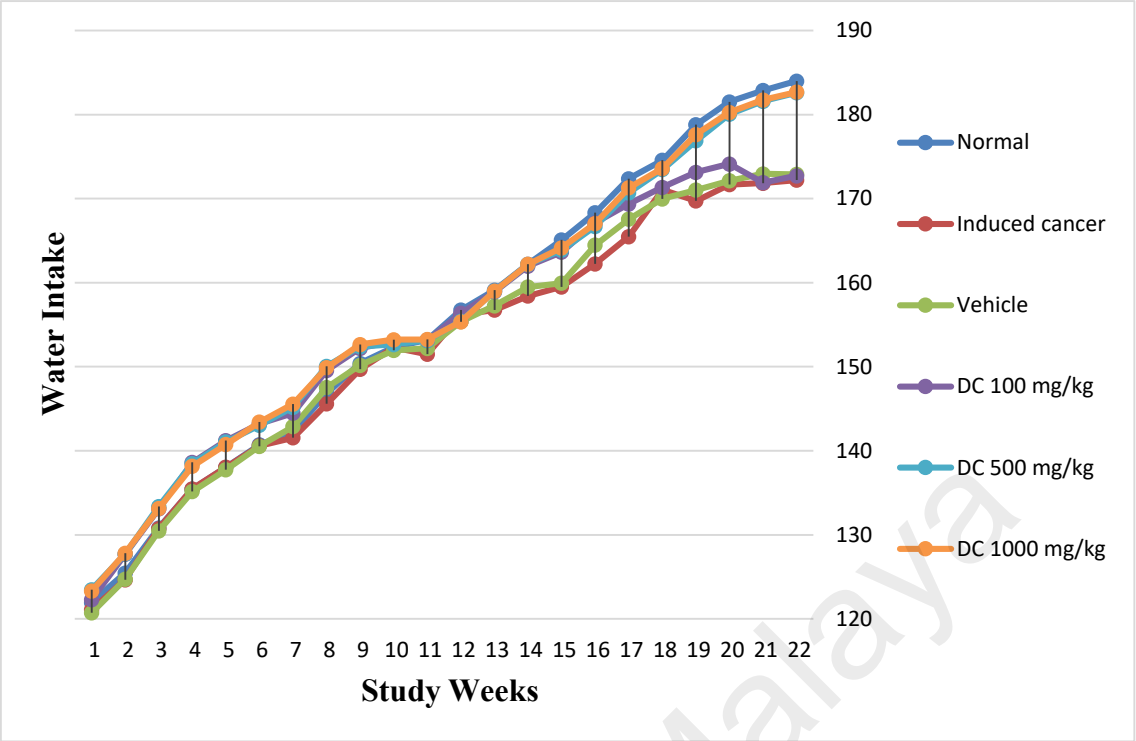

Figure 4. 5: Mean water intake during 4NQO-induced oral carcinogenesis in the chemopreventive study

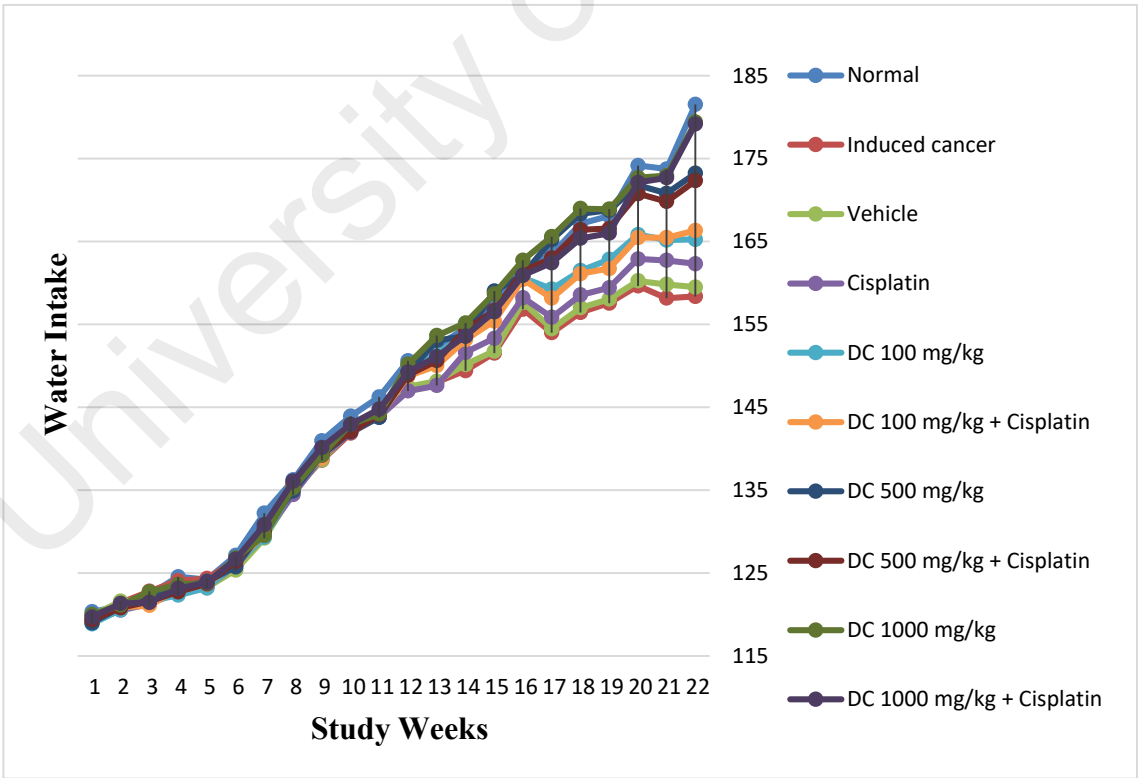

Figure 4. 6: Mean water intake during 4NQO-induced oral carcinogenesis in the chemotherapeutic study

Furthermore, in chemopreventive study, a post hoc Dunnett's test showed a significant increase in water intake in *DC* treated groups at doses 100, 500 and 1000 mg/kg in the following time: 2 through 9 weeks compared to the normal control group ($p < 0.05$). There was no significant difference between the normal control group and both induced cancer and vehicle groups at the first 12 weeks as $p > 0.05$ (Table 4.8). However, a significant decrease in water intake in both induced cancer and vehicle groups was detected at the following times: 13 through 22 weeks compared to normal control group ($p < 0.05$). At week 17 until the end of the study, *DC* 100 mg/kg group showed a significantly decreased in water intake compared to the normal control group ($p < 0.05$). The water intake in *DC* resin treated groups at doses of 500 and 1000 mg/kg from week 13 until the end of the study were comparable to the normal control group with $p > 0.05$ as shown in Table 4.8.

Table 4. 8: Water intake of rats during 4NQO-induced oral carcinogenesis in the chemopreventive study. A post hoc Dunnett's test.

Dependent Variable	(I) Water Intake	(J) Water Intake	Mean Difference (I-J)	Standard Error	*P* value
Week 2	Induced Cancer	Normal	-0.80	0.83	0.792
	Vehicle	Normal	-0.76	0.83	0.827
	DC 100 mg/kg	Normal	2.22	0.83	0.044*
	DC 500 mg/kg	Normal	2.26	0.83	0.039*
	DC 1000 mg/kg	Normal	2.36	0.83	0.029*
Week 3	Induced Cancer	Normal	-0.08	0.79	1.000
	Vehicle	Normal	-0.37	0.79	0.987
	DC 100 mg/kg	Normal	2.27	0.79	0.029*
	DC 500 mg/kg	Normal	2.56	0.79	0.011*
	DC 1000 mg/kg	Normal	2.30	0.79	0.026*
Week 4	Induced Cancer	Normal	0.03	0.92	1.000
	Vehicle	Normal	-0.28	0.92	0.998
	DC 100 mg/kg	Normal	3.19	0.92	0.006*
	DC 500 mg/kg	Normal	3.05	0.92	0.009*
	DC 1000 mg/kg	Normal	2.73	0.92	0.021*
Week 5	Induced Cancer	Normal	0.03	0.92	1.000
	Vehicle	Normal	-0.28	0.92	0.998
	DC 100 mg/kg	Normal	3.19	0.92	0.006*
	DC 500 mg/kg	Normal	3.05	0.92	0.009*
	DC 1000 mg/kg	Normal	2.73	0.92	0.021*
Week 6	Induced Cancer	Normal	-0.12	0.85	1.000
	Vehicle	Normal	-0.19	0.85	1.000
	DC 100 mg/kg	Normal	2.48	0.85	0.026*
	DC 500 mg/kg	Normal	2.30	0.85	0.043*

Table 4.8 continued					
	DC 1000 mg/kg	Normal	2.70	0.85	0.014*
Week 7	Induced Cancer	Normal	-0.75	0.82	0.823
	Vehicle	Normal	0.58	0.82	0.928
	DC 100 mg/kg	Normal	2.17	0.82	0.047*
	DC 500 mg/kg	Normal	2.89	0.82	0.005*
	DC 1000 mg/kg	Normal	3.25	0.82	0.001*
Week 8	Induced Cancer	Normal	-1.26	0.86	0.452
	Vehicle	Normal	0.68	0.86	0.890
	DC 100 mg/kg	Normal	2.72	0.86	0.013*
	DC 500 mg/kg	Normal	3.21	0.86	0.003*
	DC 1000 mg/kg	Normal	3.05	0.86	0.005*
Week 9	Induced Cancer	Normal	-0.70	0.68	0.750
	Vehicle	Normal	-0.24	0.68	0.996
	DC 100 mg/kg	Normal	1.79	0.68	0.049*
	DC 500 mg/kg	Normal	1.97	0.68	0.026*
	DC 1000 mg/kg	Normal	2.24	0.68	0.009*
Week 13	Induced Cancer	Normal	-2.37	0.58	0.001*
	Vehicle	Normal	-1.85	0.58	0.006*
	DC 100 mg/kg	Normal	-0.19	0.58	0.719
	DC 500 mg/kg	Normal	-0.12	0.58	0.766
	DC 1000 mg/kg	Normal	-0.10	0.58	0.778
Week 14	Induced Cancer	Normal	-3.78	0.52	0.000*
	Vehicle	Normal	-2.72	0.52	0.000*
	DC 100 mg/kg	Normal	-0.22	0.52	0.679
	DC 500 mg/kg	Normal	-0.03	0.52	0.817
	DC 1000 mg/kg	Normal	-0.01	0.52	0.827
Week 15	Induced Cancer	Normal	-4.71	0.53	0.000*
	Vehicle	Normal	-3.42	0.53	0.000*
	DC 100 mg/kg	Normal	-0.29	0.53	0.621
	DC 500 mg/kg	Normal	-0.24	0.53	0.663
	DC 1000 mg/kg	Normal	-0.22	0.53	0.683
Week 16	Induced Cancer	Normal	-6.08	0.78	0.000*
	Vehicle	Normal	-3.86	0.78	0.000*
	DC 100 mg/kg	Normal	-1.20	0.78	0.206
	DC 500 mg/kg	Normal	-0.63	0.78	0.505
	DC 1000 mg/kg	Normal	-0.28	0.78	0.705
Week 17	Induced Cancer	Normal	-6.89	0.69	0.000*
	Vehicle	Normal	-4.83	0.69	0.000*
	DC 100 mg/kg	Normal	-3.02	0.69	0.000*
	DC 500 mg/kg	Normal	-0.54	0.69	0.515
	DC 1000 mg/kg	Normal	-0.24	0.69	0.708
Week 18	Induced Cancer	Normal	-3.54	1.13	0.007*
	Vehicle	Normal	-4.58	1.13	0.001*
	DC 100 mg/kg	Normal	-3.22	1.13	0.015*
	DC 500 mg/kg	Normal	-0.38	1.13	0.713
	DC 1000 mg/kg	Normal	-0.10	1.13	0.804

Table 4.8 continued					
Week 19	Induced Cancer	Normal	-9.06	0.85	0.000*
	Vehicle	Normal	-7.76	0.85	0.000*
	DC 100 mg/kg	Normal	-5.64	0.85	0.000*
	DC 500 mg/kg	Normal	-0.90	0.85	0.394
	DC 1000 mg/kg	Normal	-0.43	0.85	0.643
Week 20	Induced Cancer	Normal	-9.87	0.72	0.000*
	Vehicle	Normal	-9.39	0.72	0.000*
	DC 100 mg/kg	Normal	-7.41	0.72	0.000*
	DC 500 mg/kg	Normal	-1.33	0.72	0.121
	DC 1000 mg/kg	Normal	-0.83	0.72	0.345
Week 21	Induced Cancer	Normal	-11.04	0.54	0.000*
	Vehicle	Normal	-9.94	0.54	0.000*
	DC 100 mg/kg	Normal	-10.99	0.54	0.000*
	DC 500 mg/kg	Normal	-1.00	0.54	0.122
	DC 1000 mg/kg	Normal	-0.84	0.54	0.202
Week 22	Induced Cancer	Normal	-11.78	0.66	0.000*
	Vehicle	Normal	-11.09	0.66	0.000*
	DC 100 mg/kg	Normal	-11.27	0.66	0.000*
	DC 500 mg/kg	Normal	-1.38	0.66	0.079
	DC 1000 mg/kg	Normal	-1.30	0.66	0.100

*p value less than 0.05, ($p < 0.05$) significant value.

Likewise, in the chemotherapeutic study, a post hoc Dunnett's test showed a significant decrease in water intake in groups of induced cancer, vehicle and Cisplatin compared to normal control group at the following times: 12 through 22 weeks ($p < 0.05$). At week 16 until the end of the study, *DC* 100 mg/kg with or without Cisplatin showed a significantly decrease in water intake compared to the normal control group ($p < 0.05$). The reduction of water intake in the combination of *DC* 500 mg/kg + Cisplatin group was detected at 20 through 22 weeks compared to normal control group. Whereas, *DC* 500 mg/kg group showed a significant decrease in water intake at the last two weeks 21 and 22 where $p < 0.05$. However, there was no significant difference detected in *DC* 1000 mg/kg groups with or without Cisplatin throughout the study compared to the normal control group as $p > 0.05$ (Table 4.9).

Table 4. 9: Mean water intake of rats during 4NQO-induced oral carcinogenesis in the chemotherapeutic study. A post hoc Dunnett's test.

Dependent Variable	(I) Water intake	(J) Water intake	Mean Difference (I-J)	Standard Error	*P* value
Week 12	Induced cancer	Normal	-2.82*	0.98	0.039
	Vehicle	Normal	-3.12*	0.98	0.017
	Cisplatin	Normal	-3.22*	0.98	0.013
	DC 100 mg/kg	Normal	0.22	0.98	1.000
	DC 100 mg/kg + Cisplatin	Normal	-0.70	0.98	0.987
	DC 500 mg/kg	Normal	0.13	0.98	1.000
	DC 500 mg/kg + Cisplatin	Normal	-1.31	0.98	0.702
	DC 1000 mg/kg	Normal	1.29	0.98	0.713
	DC 1000 mg/kg + Cisplatin	Normal	-1.06	0.98	0.868
Week 13	Induced cancer	Normal	-4.29*	0.92	0.000
	Vehicle	Normal	-4.16*	0.92	0.000
	Cisplatin	Normal	-4.69*	0.92	0.000
	DC 100 mg/kg	Normal	-0.62	0.92	0.990
	DC 100 mg/kg + Cisplatin	Normal	-2.31	0.92	0.092
	DC 500 mg/kg	Normal	0.76	0.92	0.966
	DC 500 mg/kg + Cisplatin	Normal	-1.68	0.92	0.355
	DC 1000 mg/kg	Normal	1.33	0.92	0.614
	DC 1000 mg/kg + Cisplatin	Normal	-1.19	0.92	0.725
Week 14	Induced cancer	Normal	-5.15*	0.70	0.000
	Vehicle	Normal	-4.41*	0.70	0.000
	Cisplatin	Normal	-2.95*	0.70	0.001
	DC 100 mg/kg	Normal	-0.68	0.70	0.922
	DC 100 mg/kg + Cisplatin	Normal	-1.43	0.70	0.250
	DC 500 mg/kg	Normal	0.01	0.70	1.000
	DC 500 mg/kg + Cisplatin	Normal	-0.22	0.70	1.000
	DC 1000 mg/kg	Normal	0.60	0.70	0.960
	DC 1000 mg/kg + Cisplatin	Normal	-0.55	0.70	0.974
Week 15	Induced cancer	Normal	-5.83*	0.83	0.000
	Vehicle	Normal	-5.61*	0.83	0.000
	Cisplatin	Normal	-4.93*	0.83	0.000
	DC 100 mg/kg	Normal	-1.83	0.83	0.180
	DC 100 mg/kg + Cisplatin	Normal	-1.97	0.83	0.125
	DC 500 mg/kg	Normal	1.63	0.83	0.284
	DC 500 mg/kg + Cisplatin	Normal	-0.72	0.83	0.953
	DC 1000 mg/kg	Normal	1.30	0.83	0.530
	DC 1000 mg/kg + Cisplatin	Normal	-0.57	0.83	0.989
Week 16	Induced cancer	Normal	-5.70*	0.69	0.000
	Vehicle	Normal	-5.32*	0.69	0.000
	Cisplatin	Normal	-4.73*	0.69	0.000
	DC 100 mg/kg	Normal	-2.10*	0.69	0.024
	DC 100 mg/kg + Cisplatin	Normal	-2.35*	0.69	0.009
	DC 500 mg/kg	Normal	-0.86	0.69	0.761
	DC 500 mg/kg + Cisplatin	Normal	-0.39	0.69	0.997
	DC 1000 mg/kg	Normal	0.93	0.69	0.686
	DC 1000 mg/kg + Cisplatin	Normal	-0.88	0.69	0.737

Table 4.9 continued					
Week 17	Induced cancer	Normal	-9.49*	0.84	0.000
	Vehicle	Normal	-9.00*	0.84	0.000
	Cisplatin	Normal	-7.64*	0.84	0.000
	DC 100 mg/kg	Normal	-4.22*	0.84	0.000
	DC 100 mg/kg + Cisplatin	Normal	-5.32*	0.84	0.000
	DC 500 mg/kg	Normal	1.72	0.84	0.239
	DC 500 mg/kg + Cisplatin	Normal	-0.50	0.84	0.996
	DC 1000 mg/kg	Normal	2.11	0.84	0.089
	DC 1000 mg/kg + Cisplatin	Normal	-1.06	0.84	0.745
Week 18	Induced cancer	Normal	-10.72*	0.80	0.000
	Vehicle	Normal	-10.16*	0.80	0.000
	Cisplatin	Normal	-8.61*	0.80	0.000
	DC 100 mg/kg	Normal	-5.68*	0.80	0.000
	DC 100 mg/kg + Cisplatin	Normal	-6.04*	0.80	0.000
	DC 500 mg/kg	Normal	1.18	0.80	0.600
	DC 500 mg/kg + Cisplatin	Normal	-0.74	0.80	0.937
	DC 1000 mg/kg	Normal	1.81	0.80	0.164
	DC 1000 mg/kg + Cisplatin	Normal	-1.78	0.80	0.176
Week 19	Induced cancer	Normal	-10.51*	0.81	0.000
	Vehicle	Normal	-10.02*	0.81	0.000
	Cisplatin	Normal	-8.66*	0.81	0.000
	DC 100 mg/kg	Normal	-5.24*	0.81	0.000
	DC 100 mg/kg + Cisplatin	Normal	-6.34*	0.81	0.000
	DC 500 mg/kg	Normal	0.70	0.81	0.958
	DC 500 mg/kg + Cisplatin	Normal	-1.52	0.81	0.331
	DC 1000 mg/kg	Normal	0.82	0.81	0.900
	DC 1000 mg/kg + Cisplatin	Normal	-2.08	0.81	0.082
Week 20	Induced cancer	Normal	-14.51*	0.87	0.000
	Vehicle	Normal	-13.92*	0.87	0.000
	Cisplatin	Normal	-11.27*	0.87	0.000
	DC 100 mg/kg	Normal	-8.34*	0.87	0.000
	DC 100 mg/kg + Cisplatin	Normal	-8.70*	0.87	0.000
	DC 500 mg/kg	Normal	-2.38	0.87	0.055
	DC 500 mg/kg + Cisplatin	Normal	-3.40*	0.87	0.002
	DC 1000 mg/kg	Normal	-1.48	0.87	0.438
	DC 1000 mg/kg + Cisplatin	Normal	-2.05	0.87	0.131
Week 21	Induced cancer	Normal	-15.58*	0.99	0.000
	Vehicle	Normal	-13.93*	0.99	0.000
	Cisplatin	Normal	-11.02*	0.99	0.000
	DC 100 mg/kg	Normal	-8.57*	0.99	0.000
	DC 100 mg/kg + Cisplatin	Normal	-8.28*	0.99	0.000
	DC 500 mg/kg	Normal	-2.93*	0.99	0.031
	DC 500 mg/kg + Cisplatin	Normal	-3.91*	0.99	0.002
	DC 1000 mg/kg	Normal	-0.82	0.99	0.965
	DC 1000 mg/kg + Cisplatin	Normal	-1.06	0.99	0.868

Table 4.9 continued					
Week 22	Induced cancer	Normal	-23.16*	0.88	0.000
	Vehicle	Normal	-22.05*	0.88	0.000
	Cisplatin	Normal	-19.22*	0.88	0.000
	DC 100 mg/kg	Normal	-16.29*	0.88	0.000
	DC 100 mg/kg + Cisplatin	Normal	-15.20*	0.88	0.000
	DC 500 mg/kg	Normal	-8.32*	0.88	0.000
	DC 500 mg/kg + Cisplatin	Normal	-9.20*	0.88	0.000
	DC 1000 mg/kg	Normal	-2.15	0.88	0.108
	DC 1000 mg/kg + Cisplatin	Normal	-2.35	0.88	0.064

*p value less than 0.05, ($p < 0.05$) significant value.

4.3.3 Effect of *DC* Extract on Mean Body Weight Gain

Mean body weights gain for all groups/week is illustrated in Figures 4.7 and 4.8. For chemopreventive study, there were no significant differences showed in the first 11 weeks of the study with $p > 0.05$, however, started from week 12 until the end of the study a significant difference was detected between the groups as $p < 0.05$ (Appendix E). Whereas, in the chemotherapeutic study, no statistically significant differences were presented in the first 9 weeks ($p > 0.05$), however, at 10 throughout 22 weeks significant difference were detected between the groups ($p < 0.05$) as shown in Appendix F.

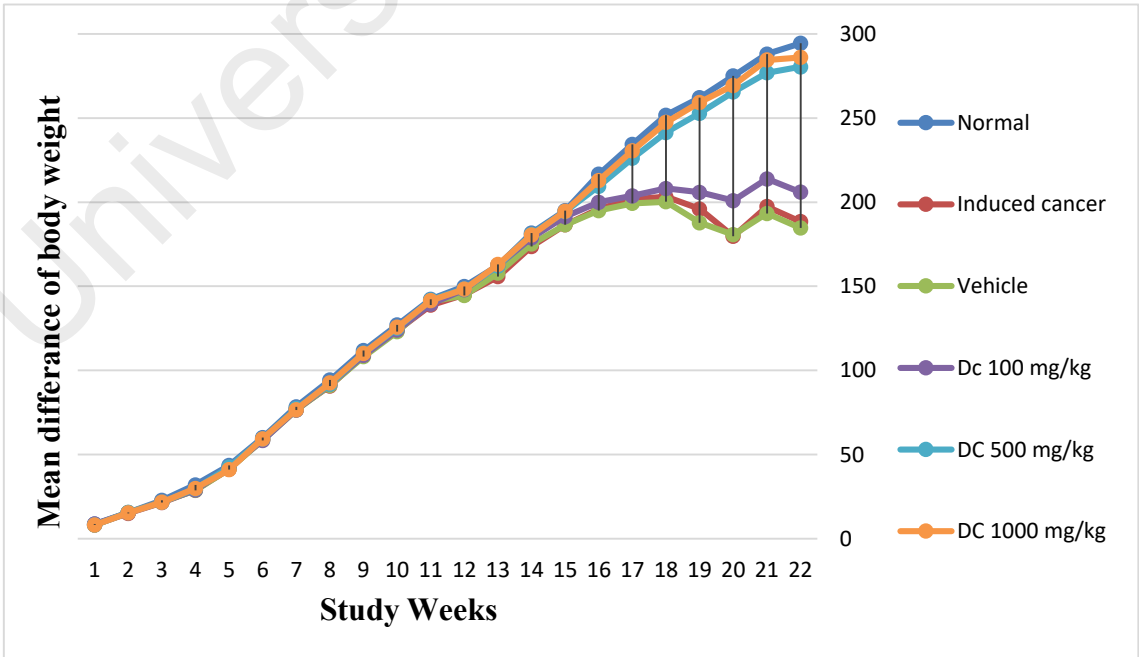

Figure 4. 7: Mean body weights gain during 4NQO-induced oral carcinogenesis in the chemopreventive study

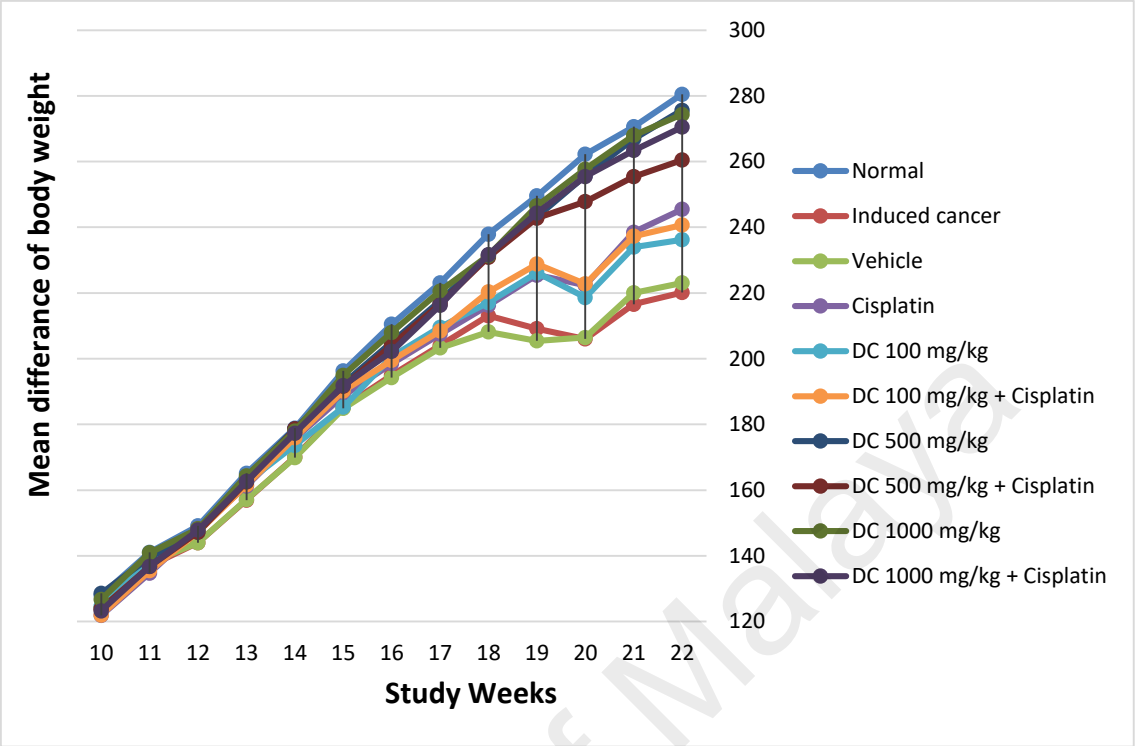

Figure 4. 8: Mean body weights gain during 4NQO-induced oral carcinogenesis in the chemotherapeutic study

Moreover, in the chemopreventive study, a post hoc Dunnett's test showed that the mean body weight gain of the induced cancer and vehicle groups was decreased with a significant difference at the following times: 12 through 22 weeks compared to normal control group ($p < 0.05$). For the *DC* 100 mg/kg group, a statistically significant difference was shown at 16 through 22 weeks compared to normal control group ($p < 0.05$). Whereas, *DC* 500 mg/kg group showed a significant decrease to the normal control group at the last two weeks (21 and 22 weeks) as $p < 0.05$. Mean body weights gain in the *DC* 1000 mg/kg group was comparable to the normal control group throughout the study and no significant difference was presented with $p > 0.05$ (Table 4.10).

Table 4. 10: Mean Body weights gain of rats during 4NQO-induced oral carcinogenesis in the chemopreventive study. A post hoc Dunnett's test.

Dependent Variable	(I) Body weights gain	(J) Body weights gain	Mean Difference (I-J)	Std. Error	*P* value
Week 12	Induced Cancer	Normal	-4.89	1.23	0.001*
	Vehicle	Normal	-5.24	1.23	0.001*
	DC 100 mg/kg	Normal	-1.85	1.23	0.431
	DC 500 mg/kg	Normal	-1.49	1.23	0.629
	DC 1000 mg/kg	Normal	-1.57	1.23	0.583
Week 13	Induced Cancer	Normal	-6.68	1.56	0.001*
	Vehicle	Normal	-4.58	1.56	0.024*
	DC 100 mg/kg	Normal	-0.02	1.56	1.000
	DC 500 mg/kg	Normal	-0.32	1.56	1.000
	DC 1000 mg/kg	Normal	0.52	1.56	0.997
Week 14	Induced Cancer	Normal	-7.99	1.82	0.000*
	Vehicle	Normal	-6.58	1.82	0.004*
	DC 100 mg/kg	Normal	-2.91	1.82	0.375
	DC 500 mg/kg	Normal	-0.80	1.82	0.990
	DC 1000 mg/kg	Normal	-1.25	1.82	0.937
Week 15	Induced Cancer	Normal	-8.43	2.21	0.002*
	Vehicle	Normal	-8.41	2.21	0.002*
	DC 100 mg/kg	Normal	-3.55	2.21	0.369
	DC 500 mg/kg	Normal	-0.60	2.21	0.999
	DC 1000 mg/kg	Normal	-0.33	2.21	1.000
Week 16	Induced Cancer	Normal	-20.58	3.20	0.000*
	Vehicle	Normal	-21.57	3.20	0.000*
	DC 100 mg/kg	Normal	-16.57	3.20	0.000*
	DC 500 mg/kg	Normal	-7.10	3.20	0.123
	DC 1000 mg/kg	Normal	-3.65	3.20	0.679
Week 17	Induced Cancer	Normal	-32.61	3.32	0.000*
	Vehicle	Normal	-35.06	3.32	0.000*
	DC 100 mg/kg	Normal	-30.63	3.32	0.000*
	DC 500 mg/kg	Normal	-8.25	3.32	0.070
	DC 1000 mg/kg	Normal	-3.80	3.32	0.678
Week 18	Induced Cancer	Normal	-48.45	3.96	0.000*
	Vehicle	Normal	-51.45	3.96	0.000*
	DC 100 mg/kg	Normal	-43.52	3.96	0.000*
	DC 500 mg/kg	Normal	-10.22	3.96	0.056
	DC 1000 mg/kg	Normal	-4.22	3.96	0.729
Week 19	Induced Cancer	Normal	-66.13	5.73	0.000*
	Vehicle	Normal	-74.31	5.73	0.000*
	DC 100 mg/kg	Normal	-56.22	5.73	0.000*
	DC 500 mg/kg	Normal	-9.22	5.73	0.368
	DC 1000 mg/kg	Normal	-2.80	5.73	0.984

Table 4.10 continued					
Week 20	Induced Cancer	Normal	-95.27	9.20	0.000*
	Vehicle	Normal	-94.26	9.58	0.000*
	DC 100 mg/kg	Normal	-74.05	9.20	0.000*
	DC 500 mg/kg	Normal	-9.50	9.20	0.755
	DC 1000 mg/kg	Normal	-5.52	9.20	0.963
Week 21	Induced Cancer	Normal	-90.55	3.54	0.000*
	Vehicle	Normal	-94.73	3.54	0.000*
	DC 100 mg/kg	Normal	-74.11	3.36	0.000*
	DC 500 mg/kg	Normal	-11.11	3.23	0.008*
	DC 1000 mg/kg	Normal	-3.44	3.23	0.744
Week 22	Induced Cancer	Normal	-106.02	3.47	0.000*
	Vehicle	Normal	-109.80	3.47	0.000*
	DC 100 mg/kg	Normal	-88.52	3.30	0.000*
	DC 500 mg/kg	Normal	-14.06	3.17	0.001*
	DC 1000 mg/kg	Normal	-8.44	3.17	0.050

*p value less than 0.05, ($p < 0.05$) significant value.

Accordingly, in the chemotherapeutic study, a post hoc Dunnett's test showed that the mean body weight gain in the induced cancer and vehicle groups was significantly decrease at the following times: 12 through 22 weeks compared to the normal control group ($p < 0.05$). For both groups, Cisplatin and combination of *DC* 100 mg/kg + Cisplatin, a statistically significant decrease showed at 10, 11 and 15 through 22 weeks. The *DC* 100 mg/kg group showed a statistically significant decrease at the following weeks: 15 through 22 compared to the normal control group ($p < 0.05$). However, at the last two weeks (21 and 22) a significant decrease has been shown in the combination of *DC* 500 mg/kg + Cisplatin group compared to normal control group with $p < 0.05$. No significant differences were shown between the normal control group and both groups of *DC* extract at 500 and 1000 mg/kg. Mean body weights gain in normal control and *DC* extract treated groups at 500 and 1000 mg/kg were comparable throughout the study as $p > 0.05$ (Table 4.11).

Table 4. 11: Mean body weights gain of rats during 4NQO-induced oral carcinogenesis in the chemotherapeutic study. A post hoc Dunnett's test.

Dependent Variable	(I) Body weights gain	(J) Body weights gain	Mean Difference (I-J)	Standard Error	*P* value
Week 10	Induced cancer	Normal	-4.40	1.71	0.081
	Vehicle	Normal	-3.85	1.71	0.163
	Cisplatin	Normal	-6.13*	1.71	0.005
	DC 100 mg/kg	Normal	-3.33	1.71	0.292
	DC 100 mg/kg + Cisplatin	Normal	-6.01*	1.71	0.007
	DC 500 mg/kg	Normal	0.59	1.71	1.000
	DC 500 mg/kg + Cisplatin	Normal	-4.01	1.71	0.134
	DC 1000 mg/kg	Normal	-1.26	1.71	0.983
	DC 1000 mg/kg + Cisplatin	Normal	-4.69	1.71	0.053
Week 11	Induced cancer	Normal	-4.13	1.77	0.137
	Vehicle	Normal	-3.27	1.77	0.345
	Cisplatin	Normal	-6.33*	1.77	0.005
	DC 100 mg/kg	Normal	-2.85	1.77	0.498
	DC 100 mg/kg + Cisplatin	Normal	-5.61*	1.77	0.017
	DC 500 mg/kg	Normal	-1.54	1.77	0.953
	DC 500 mg/kg + Cisplatin	Normal	-4.30	1.77	0.111
	DC 1000 mg/kg	Normal	-0.16	1.77	1.000
	DC 1000 mg/kg + Cisplatin	Normal	-4.30	1.77	0.111
Week 12	Induced cancer	Normal	-5.12*	1.55	0.012
	Vehicle	Normal	-5.09*	1.55	0.013
	Cisplatin	Normal	-0.77	1.55	0.999
	DC 100 mg/kg	Normal	-1.85	1.55	0.797
	DC 100 mg/kg + Cisplatin	Normal	-1.85	1.55	0.797
	DC 500 mg/kg	Normal	-1.89	1.55	0.779
	DC 500 mg/kg + Cisplatin	Normal	-1.88	1.55	0.783
	DC 1000 mg/kg	Normal	-0.86	1.55	0.998
	DC 1000 mg/kg + Cisplatin	Normal	-1.28	1.55	0.965
Week 13	Induced cancer	Normal	-8.17*	1.74	0.000
	Vehicle	Normal	-7.95*	1.74	0.000
	Cisplatin	Normal	-3.87	1.74	0.170
	DC 100 mg/kg	Normal	-2.74	1.74	0.520
	DC 100 mg/kg + Cisplatin	Normal	-3.53	1.74	0.247
	DC 500 mg/kg	Normal	-1.16	1.74	0.991
	DC 500 mg/kg + Cisplatin	Normal	-2.68	1.74	0.546
	DC 1000 mg/kg	Normal	-0.76	1.74	1.000
	DC 1000 mg/kg + Cisplatin	Normal	-2.48	1.74	0.631
Week 14	Induced cancer	Normal	-8.79*	1.96	0.000
	Vehicle	Normal	-8.97*	1.96	0.000
	Cisplatin	Normal	-3.15	1.96	0.497
	DC 100 mg/kg	Normal	-5.20	1.96	0.066
	DC 100 mg/kg + Cisplatin	Normal	-2.91	1.96	0.586
	DC 500 mg/kg	Normal	-0.23	1.96	1.000
	DC 500 mg/kg + Cisplatin	Normal	-0.23	1.96	1.000
	DC 1000 mg/kg	Normal	-0.82	1.96	1.000
	DC 1000 mg/kg + Cisplatin	Normal	-1.53	1.96	0.975

Table 4.11 continued					
Week 15	Induced cancer	Normal	-11.05*	2.13	0.000
	Vehicle	Normal	-11.40*	2.13	0.000
	Cisplatin	Normal	-7.53*	2.13	0.006
	DC 100 mg/kg	Normal	-11.00*	2.13	0.000
	DC 100 mg/kg + Cisplatin	Normal	-6.10*	2.13	0.039
	DC 500 mg/kg	Normal	-3.86	2.13	0.365
	DC 500 mg/kg + Cisplatin	Normal	-4.69	2.13	0.178
	DC 1000 mg/kg	Normal	-1.31	2.13	0.995
	DC 1000 mg/kg + Cisplatin	Normal	-4.45	2.13	0.223
Week 16	Induced cancer	Normal	-15.46*	2.48	0.000
	Vehicle	Normal	-16.20*	2.48	0.000
	Cisplatin	Normal	-11.98*	2.48	0.000
	DC 100 mg/kg	Normal	-9.87*	2.48	0.002
	DC 100 mg/kg + Cisplatin	Normal	-11.01*	2.48	0.000
	DC 500 mg/kg	Normal	-5.47	2.48	0.179
	DC 500 mg/kg + Cisplatin	Normal	-6.36	2.48	0.082
	DC 1000 mg/kg	Normal	-2.45	2.48	0.910
	DC 1000 mg/kg + Cisplatin	Normal	-8.24*	2.48	0.011
Week 17	Induced cancer	Normal	-19.05*	2.48	0.000
	Vehicle	Normal	-19.79*	2.48	0.000
	Cisplatin	Normal	-15.57*	2.48	0.000
	DC 100 mg/kg	Normal	-13.46*	2.48	0.000
	DC 100 mg/kg + Cisplatin	Normal	-14.60*	2.48	0.000
	DC 500 mg/kg	Normal	-5.47	2.48	0.179
	DC 500 mg/kg + Cisplatin	Normal	-6.36	2.48	0.082
	DC 1000 mg/kg	Normal	-2.45	2.48	0.911
	DC 1000 mg/kg + Cisplatin	Normal	-6.81	2.48	0.053
Week 18	Induced cancer	Normal	-24.85*	2.99	0.000
	Vehicle	Normal	-29.76*	2.99	0.000
	Cisplatin	Normal	-21.65*	2.99	0.000
	DC 100 mg/kg	Normal	-20.86*	2.99	0.000
	DC 100 mg/kg + Cisplatin	Normal	-17.57*	2.99	0.000
	DC 500 mg/kg	Normal	-6.39	2.99	0.203
	DC 500 mg/kg + Cisplatin	Normal	-7.00	2.99	0.133
	DC 1000 mg/kg	Normal	-6.55	2.99	0.183
	DC 1000 mg/kg + Cisplatin	Normal	-6.21	2.99	0.228
Week 19	Induced cancer	Normal	-40.44*	5.20	0.000
	Vehicle	Normal	-44.14*	5.20	0.000
	Cisplatin	Normal	-24.11*	5.20	0.000
	DC 100 mg/kg	Normal	-23.44*	5.20	0.000
	DC 100 mg/kg + Cisplatin	Normal	-20.79*	5.20	0.001
	DC 500 mg/kg	Normal	-6.40	5.20	0.772
	DC 500 mg/kg + Cisplatin	Normal	-6.81	5.20	0.717
	DC 1000 mg/kg	Normal	-3.04	5.20	0.996
	DC 1000 mg/kg + Cisplatin	Normal	-5.21	5.20	0.905

Table 4.11 continued					
Week 20	Induced cancer	Normal	-56.21*	9.91	0.000
	Vehicle	Normal	-55.78*	9.91	0.000
	Cisplatin	Normal	-39.86*	9.52	0.001
	DC 100 mg/kg	Normal	-43.63*	9.52	0.000
	DC 100 mg/kg + Cisplatin	Normal	-39.49*	9.52	0.001
	DC 500 mg/kg	Normal	-6.52	9.52	0.990
	DC 500 mg/kg + Cisplatin	Normal	-14.42	9.52	0.571
	DC 1000 mg/kg	Normal	-4.54	9.52	0.999
	DC 1000 mg/kg + Cisplatin	Normal	-6.80	9.52	0.986
Week 21	Induced cancer	Normal	-53.99*	2.85	0.000
	Vehicle	Normal	-50.58*	2.85	0.000
	Cisplatin	Normal	-32.11*	2.71	0.000
	DC 100 mg/kg	Normal	-36.60*	2.71	0.000
	DC 100 mg/kg + Cisplatin	Normal	-33.29*	2.71	0.000
	DC 500 mg/kg	Normal	-3.70	2.60	0.650
	DC 500 mg/kg + Cisplatin	Normal	-15.16*	2.60	0.000
	DC 1000 mg/kg	Normal	-2.64	2.60	0.908
	DC 1000 mg/kg + Cisplatin	Normal	-7.18	2.60	0.055
Week 22	Induced cancer	Normal	-60.35*	3.12	0.000
	Vehicle	Normal	-57.42*	3.12	0.000
	Cisplatin	Normal	-35.02*	2.96	0.000
	DC 100 mg/kg	Normal	-44.27*	2.96	0.000
	DC 100 mg/kg + Cisplatin	Normal	-39.79*	2.96	0.000
	DC 500 mg/kg	Normal	-4.85	2.84	0.448
	DC 500 mg/kg + Cisplatin	Normal	-20.03*	2.84	0.000
	DC 1000 mg/kg	Normal	-6.10	2.84	0.210
	DC 1000 mg/kg + Cisplatin	Normal	-9.95*	2.84	0.007

**p* value less than 0.05, ($p < 0.05$) significant value.

4.3.4 Effect of *DC* extract on haematological and biochemical parameters

4.3.4.1 Haematological parameters

The mean values ± SD of haematological and biochemical parameters for the all groups are presented in Appendix G and H. In the chemopreventive study, no significant differences have been shown with respect to the values of HGB, lymphocyte, eosinophil and basophil ($p > 0.05$), however, significant differences were detected in the values of WBC, neutrophil and monocyte between the groups with $p < 0.05$ (Appendix G). Whereas, in the chemotherapeutic study, no significant differences have been shown with respect to the values of lymphocyte and basophil ($p > 0.05$), however, significant

differences were detected in the values of HGB, WBC, neutrophil, monocyte and eosinophil between the groups as $p < 0.05$ (Appendix H).

Particularly, in the chemopreventive study, a post hoc Dunnett's test showed a significantly lower total white blood cell (WBC) and monocyte counts in the induced cancer and vehicle groups than those observed in the normal control group ($p < 0.05$). In addition, elevated neutrophil value showed with significant difference in the induced cancer and vehicle groups compared to the normal control group ($p < 0.05$). The administration of *DC* extract at various concentrations (100, 500 and 1000 mg/kg) returned the values of WBC, neutrophils and monocytes to near normal value compared to the normal control group as $p > 0.05$ (Table 4.12).

Table 4. 12: Effect of *DC* extract on haematological parameters in the chemopreventive study A post hoc Dunnett's test.

Dependent Variable	(I) Blood Analysis	(J) Blood Analysis	Mean Difference (I-J)	Standard Error	*P* value
WBC	Induced Cancer	Normal	-3.59*	1.08	0.011
	Vehicle	Normal	-2.95*	1.08	0.044
	DC 100 mg/kg	Normal	0.19	1.03	1.000
	DC 500 mg/kg	Normal	0.04	0.99	1.000
	DC 1000 mg/kg	Normal	-0.14	0.99	1.000
Neutrophil	Induced Cancer	Normal	0.53*	0.15	0.006
	Vehicle	Normal	0.51*	0.15	0.009
	DC 100 mg/kg	Normal	0.09	0.14	0.947
	DC 500 mg/kg	Normal	0.10	0.14	0.925
	DC 1000 mg/kg	Normal	0.06	0.14	0.993
Monocyte	Induced Cancer	Normal	-0.17*	0.03	0.000
	Vehicle	Normal	-0.17*	0.03	0.000
	DC 100 mg/kg	Normal	-0.04	0.03	0.622
	DC 500 mg/kg	Normal	-0.03	0.03	0.835
	DC 1000 mg/kg	Normal	-0.02	0.03	0.904

*p value less than 0.05, ($p < 0.05$) significant value.

As well as, in the chemotherapeutic study**,** a post hoc Dunnett's test showed a significant reduction in the values of haemoglobin (HGB) and eosinophil in Cisplatin-treated rats ($p < 0.05$), which returned to near normal level in rats that treated with *DC* extract. 4NQO administration to the rats does not affect the levels of HGB and eosinophil as the induced cancer and vehicle groups showed no significant difference compared to the normal control group ($p > 0.05$). The level of WBC was decreased in the groups of induced cancer, vehicle and Cisplatin compared to the normal control group ($p > 0.05$). However, a significant difference showed in the induced cancer group ($p < 0.05$). The value of monocyte was significantly decreased in the induced cancer and vehicle group. However, *DC* extract returned the value of monocyte to near normal value compared to the normal control group with $p > 0.05$ (Table 4.13).

Table 4. 13: Effect of *DC* extract on haematological parameters in the chemotherapeutic study. A post hoc Dunnett's test.

Dependent Variable	(I) Blood Analysis	(J) Blood Analysis	Mean Difference (I-J)	Standard Error	*P* value
HGB	Induced cancer	Normal	-0.37	4.05	1.000
	Vehicle	Normal	-2.57	4.05	0.994
	Cisplatin	Normal	-24.57*	3.85	0.000
	DC 100 mg/kg	Normal	-1.07	3.78	1.000
	DC 100 mg/kg + Cisplatin	Normal	-9.57	3.85	0.103
	DC 500 mg/kg	Normal	-1.00	3.70	1.000
	DC 500 mg/kg + Cisplatin	Normal	-4.57	3.70	0.782
	DC 1000 mg/kg	Normal	-1.57	3.70	1.000
	DC 1000 mg/kg + Cisplatin	Normal	-4.43	3.70	0.807
WBC	Induced cancer	Normal	-3.30*	1.17	.046
	Vehicle	Normal	-2.15	1.09	.292
	Cisplatin	Normal	-1.73	1.00	.427
	DC 100 mg/kg	Normal	1.00	1.04	.927
	DC 100 mg/kg + Cisplatin	Normal	1.70	1.04	.492
	DC 500 mg/kg	Normal	0.47	1.00	.999
	DC 500 mg/kg + Cisplatin	Normal	0.71	1.00	.988
	DC 1000 mg/kg	Normal	1.89	1.00	.334
	DC 1000 mg/kg + Cisplatin	Normal	0.74	1.00	.984

Table 4.13 continued					
Neutrophils	Induced cancer	Normal	0.54*	0.04	0.000
	Vehicle	Normal	0.53*	0.04	0.000
	Cisplatin	Normal	0.41*	0.04	0.000
	DC 100 mg/kg	Normal	0.13*	0.04	0.020
	DC 100 mg/kg + Cisplatin	Normal	0.30*	0.04	0.000
	DC 500 mg/kg	Normal	0.09	0.04	0.177
	DC 500 mg/kg + Cisplatin	Normal	0.10	0.04	0.073
	DC 1000 mg/kg	Normal	0.08	0.04	0.236
	DC 1000 mg/kg + Cisplatin	Normal	0.11	0.04	0.062
Monocyte	Induced cancer	Normal	-0.12*	0.04	0.038
	Vehicle	Normal	-0.14*	0.04	0.007
	Cisplatin	Normal	0.02	0.04	0.997
	DC 100 mg/kg	Normal	0.01	0.04	1.000
	DC 100 mg/kg + Cisplatin	Normal	-0.02	0.04	0.999
	DC 500 mg/kg	Normal	0.00	0.04	1.000
	DC 500 mg/kg + Cisplatin	Normal	-0.02	0.04	0.999
	DC 1000 mg/kg	Normal	-0.01	0.04	1.000
	DC 1000 mg/kg + Cisplatin	Normal	-0.03	0.04	0.989
Eosinophil	Induced cancer	Normal	0.02	0.02	0.942
	Vehicle	Normal	0.02	0.02	0.903
	Cisplatin	Normal	-0.07*	0.02	0.010
	DC 100 mg/kg	Normal	0.01	0.02	0.999
	DC 100 mg/kg + Cisplatin	Normal	-0.02	0.02	0.887
	DC 500 mg/kg	Normal	0.01	0.02	0.998
	DC 500 mg/kg + Cisplatin	Normal	-0.01	0.02	0.990
	DC 1000 mg/kg	Normal	0.01	0.02	1.000
	DC 1000 mg/kg + Cisplatin	Normal	-0.01	0.02	0.980

*p value less than 0.05, ($p < 0.05$) significant value.

4.3.4.2 Biochemical parameters

The mean values ±SD of biochemical parameters for all groups are presented in Appendix I and J. In the chemopreventive study, no significant differences have been shown with respect to the values of albumin and total bilirubin ($p > 0.05$), however, the value of albumin was lower in the induced cancer and vehicle groups than other groups ($p < 0.05$). A significant difference was detected in the values of urea, creatinine, ALP, ALT and AST between the groups (Appendix I). Whereas, in the chemotherapeutic study, statistically significant changes in all biochemical parameters were presented between the groups as $p < 0.05$ (Appendix J).

Furthermore, in the chemopreventive study as shown in Table 4.14, both induced cancer and vehicle groups exhibited significantly higher values for urea, creatinine, ALP, ALT, and AST compared to normal control group ($p < 0.05$). When rats were orally administered with *DC* extract, the values of biochemical parameters almost returned to the normal especially at doses of *DC* 500 and 1000 mg/kg. The *DC* extract showed values comparable to the normal control group with $p > 0.05$.

Table 4. 14: Effect of *DC* extract on biochemical parameters in the chemopreventive study A post hoc Dunnett's test.

Dependent Variable	(I) Blood Analysis	(J) Blood Analysis	Mean Difference (I-J)	Standard Error	*P* value
Urea (mmol/L)	Induced Cancer	Normal	3.90	0.80	0.000*
	Vehicle	Normal	2.74	0.80	0.008*
	DC 100 mg/kg	Normal	1.26	0.76	0.360
	DC 500 mg/kg	Normal	1.07	0.73	0.475
	DC 1000 mg/kg	Normal	0.64	0.73	0.859
Creatinine (umol/L)	Induced Cancer	Normal	31.00	2.95	0.000*
	Vehicle	Normal	31.20	2.95	0.000*
	DC 100 mg/kg	Normal	7.33	2.80	0.055
	DC 500 mg/kg	Normal	3.29	2.69	0.638
	DC 1000 mg/kg	Normal	3.00	2.69	0.711
ALP (U/L)	Induced Cancer	Normal	46.20	15.06	0.019*
	Vehicle	Normal	55.20	15.06	0.004*
	DC 100 mg/kg	Normal	8.33	14.31	0.969
	DC 500 mg/kg	Normal	11.71	13.74	0.871
	DC 1000 mg/kg	Normal	7.57	13.74	0.976
ALT (U/L)	Induced Cancer	Normal	16.91	3.35	0.000*
	Vehicle	Normal	18.31	3.35	0.000*
	DC 100 mg/kg	Normal	2.21	3.18	0.938
	DC 500 mg/kg	Normal	2.43	3.06	0.899
	DC 1000 mg/kg	Normal	3.00	3.06	0.799
AST (U/L)	Induced Cancer	Normal	67.94	13.20	0.000*
	Vehicle	Normal	73.14	13.20	0.000*
	DC 100 mg/kg	Normal	5.98	12.54	0.987
	DC 500 mg/kg	Normal	2.57	12.05	1.000
	DC 1000 mg/kg	Normal	4.00	12.05	0.997

*p value less than 0.05, ($p < 0.05$) significant value

Moreover, in the chemotherapeutic study as shown in Table 4.15, a higher value of urea was showed in both groups of Cisplatin and combination of *DC* 100 mg/kg + Cisplatin with a significant difference presented in the Cisplatin group compared to the normal control group ($p < 0.05$). Serum creatinine was significantly higher in the induced cancer, vehicle and Cisplatin groups compared to the normal control group ($p < 0.05$). However, the highest level was showed in Cisplatin group. The values of ALP, ALT and AST were significantly higher in the induced cancer, vehicle and Cisplatin groups compared to the normal control group. In addition, a significantly lower serum albumin value was seen in Cisplatin treated group ($p < 0.05$). In contrast, the administration of *DC* extract (especially at doses of *DC* 500 and *DC* 1000 mg/kg) with or without Cisplatin showed a considerable improvement of the tested parameters, which looks to be comparable to the normal control group ($p > 0.05$).

Table 4. 15: Effect of *DC* extract on biochemical parameters in chemotherapeutic study A post hoc Dunnett's test.

Dependent Variable	(I) Blood Analysis	(J) Blood Analysis	Mean Difference (I-J)	Std. Error	*P* value
Urea	Induced cancer	Normal	2.19	1.28	0.443
	Vehicle	Normal	1.81	1.28	0.655
	Cisplatin	Normal	7.21*	1.22	0.000
	DC 100 mg/kg	Normal	2.18	1.22	0.392
	DC 100 mg/kg + Cisplatin	Normal	3.40	1.22	0.050
	DC 500 mg/kg	Normal	0.67	1.17	0.997
	DC 500 mg/kg + Cisplatin	Normal	2.61	1.17	0.175
	DC 1000 mg/kg	Normal	0.67	1.17	0.997
	DC 1000 mg/kg + Cisplatin	Normal	2.06	1.17	0.413
Creatinine	Induced cancer	Normal	21.63*	4.16	0.000
	Vehicle	Normal	22.83*	4.16	0.000
	Cisplatin	Normal	44.93*	3.95	0.000
	DC 100 mg/kg	Normal	2.76	3.95	0.989
	DC 100 mg/kg + Cisplatin	Normal	17.26*	3.95	0.000
	DC 500 mg/kg	Normal	2.43	3.80	0.994
	DC 500 mg/kg + Cisplatin	Normal	8.43	3.80	0.181
	DC 1000 mg/kg	Normal	3.00	3.80	0.977
	DC 1000 mg/kg + Cisplatin	Normal	9.14	3.80	0.122

Table 4.15 continued					
albumin	Induced cancer	Normal	-3.31	2.27	0.620
	Vehicle	Normal	-4.31	2.27	0.325
	Cisplatin	Normal	-8.05*	2.15	0.004
	DC 100 mg/kg	Normal	-2.38	2.15	0.862
	DC 100 mg/kg + Cisplatin	Normal	-4.71	2.15	0.192
	DC 500 mg/kg	Normal	-2.14	2.07	0.897
	DC 500 mg/kg + Cisplatin	Normal	-3.00	2.07	0.630
	DC 1000 mg/kg	Normal	-0.43	2.07	1.000
	DC 1000 mg/kg + Cisplatin	Normal	-2.71	2.07	0.730
ALP	Induced cancer	Normal	59.89*	12.08	0.000
	Vehicle	Normal	56.09*	12.08	0.000
	Cisplatin	Normal	50.12*	11.48	0.000
	DC 100 mg/kg	Normal	14.12	11.48	0.786
	DC 100 mg/kg + Cisplatin	Normal	19.12	11.48	0.475
	DC 500 mg/kg	Normal	10.86	11.03	0.920
	DC 500 mg/kg + Cisplatin	Normal	17.86	11.03	0.507
	DC 1000 mg/kg	Normal	6.57	11.03	0.996
	DC 1000 mg/kg + Cisplatin	Normal	15.29	11.03	0.677
ALT	Induced cancer	Normal	24.54*	2.60	0.000
	Vehicle	Normal	25.54*	2.60	0.000
	Cisplatin	Normal	21.48*	2.47	0.000
	DC 100 mg/kg	Normal	5.98	2.47	0.120
	DC 100 mg/kg + Cisplatin	Normal	6.31	2.47	0.089
	DC 500 mg/kg	Normal	5.71	2.38	0.123
	DC 500 mg/kg + Cisplatin	Normal	5.29	2.38	0.179
	DC 1000 mg/kg	Normal	5.61	2.38	0.135
	DC 1000 mg/kg + Cisplatin	Normal	5.14	2.38	0.202
AST	Induced cancer	Normal	74.43*	8.56	0.000
	Vehicle	Normal	75.23*	8.56	0.000
	Cisplatin	Normal	55.93*	8.14	0.000
	DC 100 mg/kg	Normal	12.60	8.14	0.558
	DC 100 mg/kg + Cisplatin	Normal	21.76	8.14	0.067
	DC 500 mg/kg	Normal	12.14	7.82	0.555
	DC 500 mg/kg + Cisplatin	Normal	18.00	7.82	0.153
	DC 1000 mg/kg	Normal	7.86	7.82	0.912
	DC 1000 mg/kg + Cisplatin	Normal	11.71	7.82	0.595

*p value less than 0.05, ($p < 0.05$) significant value.

4.3.5 Effect of *DC* Extract on Rat's Survival Rate and Tongue Tumour size

In the chemopreventive study, the administration of *DC* extract started one week before the administration of 4NQO and continued one week more after the secession of 4NQO. The administration of *DC* extract for 10 consecutive weeks increase the survival rate to 100% in both *DC* 500 and 1000 mg/kg and significantly decrease the tongue

tumour size induced by 4NQO as shown in Table 4.16 and Figure 4.9. However, the Chi square test that used to compare the survival rate showed no significant difference (Chi.Sq = 4.667; $p = 0.323$) between the groups.

The induced cancer and vehicle groups developed large tumours in the oral cavity, mostly seen in the posterior region of the tongue. As presented in Table 4.16, the administration of *DC* extracts to the rats at doses of 100, 500 and 1000 mg/kg developed smaller tumours (tumour volume) than those seen in the induced cancer and vehicle groups.

Table 4. 16: Effect of *DC* extract on survival rate and tongue tumour size in 4NQO-induced oral carcinogenesis in the chemopreventive study.

Group	Survival[a] (%)	*P* value - Chi square test	Tongue Tumour Size (Mean± SD)	*P* value one-way ANOVA test
Induced cancer	5/7 (71.4)	0.323	110.16±21.26	0.000*
Vehicle	5/7 (71.4)		113.75±45.79	
***DC* 100 mg/kg**	6/7 (85.7)		74.50±29.52	
***DC* 500 mg/kg**	7/7 (100)		56.99±31.75	
***DC* 1000 mg/kg**	7/7 (100)		31.85±31.75	

[a] all early mortality was the result of oral neoplasia. 30/35 rats were survived until the end of the study; 5 rats were found dead prior to the terminal necropsy.
*p value less than 0.05, ($p < 0.05$) significant value.

A post hoc Dunnett's test showed significant differences between the *DC* groups (*DC* 500 and 1000 mg/kg) compared to the induced cancer group ($p < 0.05$) as shown in Table 4.17. These results indicated that the administration of *DC* extract, especially at the doses of 500 and 1000 mg/kg, during the 4NQO administration could effectively stood the development of tumours.

Table 4. 17: Effect of *DC* extract on tumour size in the chemopreventive study. A post hoc Dunnett's test.

(I) Group	(J) Group	Mean Difference (I-J)	Standard Error	*P* value
Vehicle	Induced cancer	3.59	18.67	0.999
DC 100 mg/kg	Induced cancer	-35.66	18.67	0.195
DC 500 mg/kg	Induced cancer	-53.17	18.67	0.027*
DC 1000 mg/kg	Induced cancer	-78.31	18.67	0.001*

**p* value less than 0.05, ($p < 0.05$) significant value.

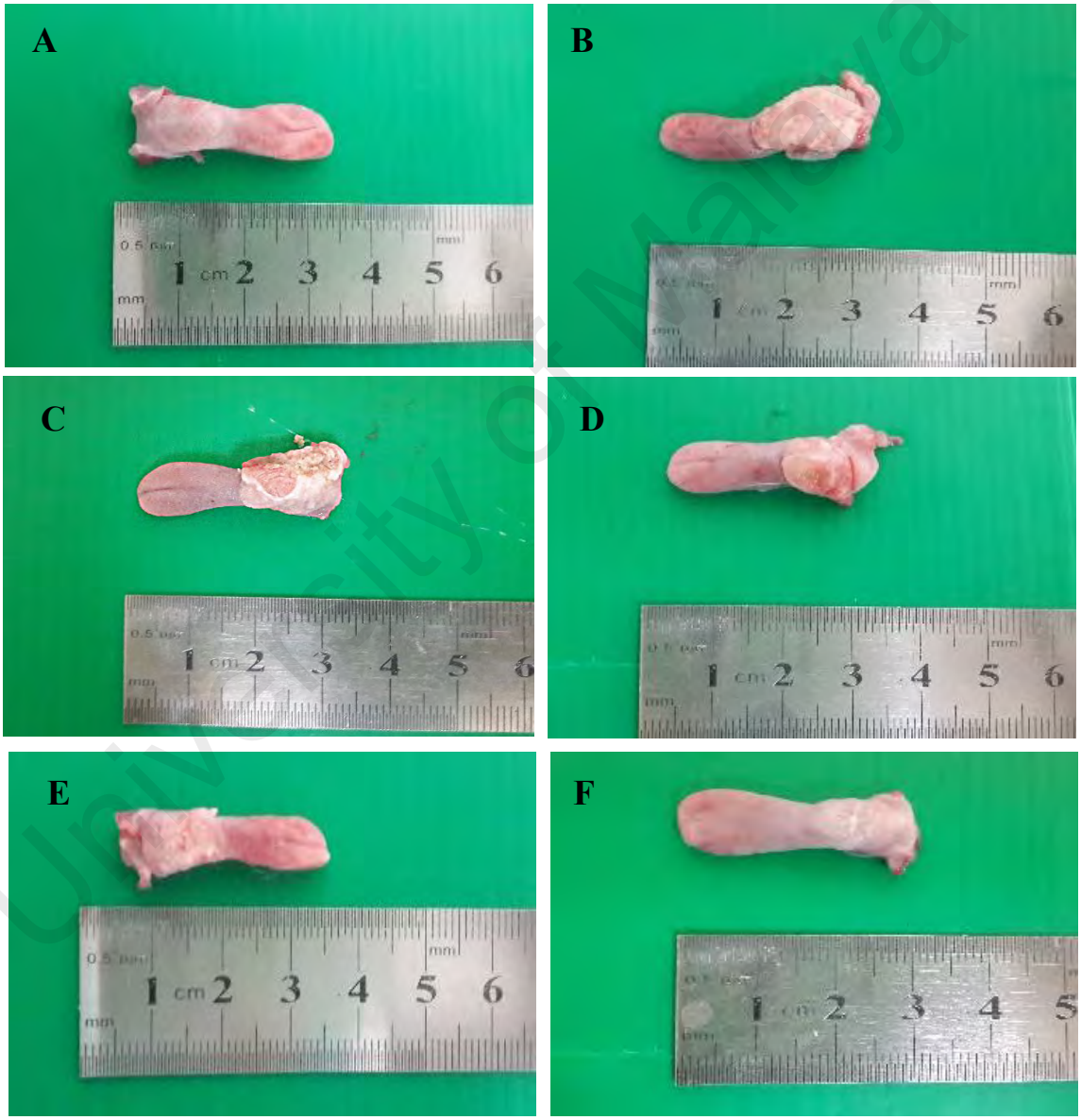

Figure 4. 9: Tongue tumour in different groups (A) normal control group. Following oral carcinogenesis induced by 4NQO and treated with *DC* extract. (B) Induced cancer group. (C) Vehicle group. (D) *DC* 100 mg/kg. (E) *DC* 500 mg/kg. (F) *DC* 1000 mg/kg

Nevertheless, in the chemotherapeutic study, the administration of *DC* extract is beginning after 1 week from the secession of 4NQO administration (after week 9) and continued for 10 consecutive weeks. Chemotherapeutic activity was showed as an increase in animal survival rate which was 100% in the groups that treated with *DC* extract at doses of 500 and 1000 mg/kg, and significantly decrease the tongue tumour size induced by 4NQO as shown in Table 4.18 and Figure 4.10. However, the Chi square test that used to compare the survival rate showed no significant difference (Chi.Sq = 8.877; p = 0.353) between groups.

The induced cancer and vehicle groups developed large tumours in the oral cavity, mostly seen in the posterior region of the tongue. As presented in Table 4.18, the administration of *DC* extract to the rats at doses of 100, 500 and 1000 mg/kg with or without Cisplatin, as well as Cisplatin alone, developed smaller tumours (tumour volume) than those seen in the induced cancer and vehicle groups.

Table 4. 18: Effect of *DC* extract on survival rate and tongue tumour size in 4NQO-induced oral carcinogenesis in the chemotherapeutic study.

Group	Survival[a] (%)	*P* value Chi-square test	Tongue Tumour Size (Mean± SD)	*P* value one-way ANOVA test
Induced cancer	5/7 (71.4)	0.353	106.11±19.52	0.000*
Vehicle	5/7 (71.4)		111.85±33.17	
Cisplatin	5/7 (71.4)		30.37±36.02	
***DC* 100 mg/kg**	6/7 (85.7)		75.41±31.05	
***DC* 100 mg/kg + Cisplatin**	6/7 (85.7)		34.60±37.69	
***DC* 500 mg/kg**	7/7 (100)		45.67±35.50	
***DC* 500 mg/kg + Cisplatin**	7/7 (100)		28.36±30.21	
***DC* 1000 mg/kg**	7/7 (100)		34.86±36.45	
***DC* 1000 mg/kg + Cisplatin**	7/7 (100)		24.25±33.38	

[a] all early mortality was the result of oral neoplasia. 55/63 rats were survived until the end of the study; 7 rats were found dead prior to the terminal necropsy.
**p* value less than 0.05, ($p < 0.05$) significant value.

A post hoc Dunnett's test showed significant decrease in the tongue tumour size between all the *DC* extract groups with or without Cisplatin (except *DC* 100 mg/kg) as well as Cisplatin group compared to the induced cancer group with $p < 0.05$ (Table 4.19). These results indicated that the administration of *DC* extract, especially at doses of 500 and 1000 mg/kg, after the secession of 4NQO administration conferred significant protection and can effectively stood the development of tumours in this animal model.

Table 4. 19: Effect of *DC* extract on tumour size in the chemotherapeutic study. A post hoc Dunnett's test.

(I) Group	(J) Group	Mean Difference (I-J)	Standard Error	*P* value
Vehicle	Induced cancer	5.73	17.62	1.000
Cisplatin	Induced cancer	-75.75	17.62	0.001*
DC 100 mg/kg	Induced cancer	-30.71	17.62	0.384
DC 100 mg/kg + Cisplatin	Induced cancer	-71.51	17.62	0.001*
DC 500 mg/kg	Induced cancer	-60.44	17.62	0.008*
DC 500 mg/kg + Cisplatin	Induced cancer	-77.75	17.62	0.000*
DC 1000 mg/kg	Induced cancer	-71.26	17.62	0.001*
DC 1000 mg/kg + Cisplatin	Induced cancer	-81.87	17.62	0.000*

**p* value less than 0.05, ($p < 0.05$) significant value.

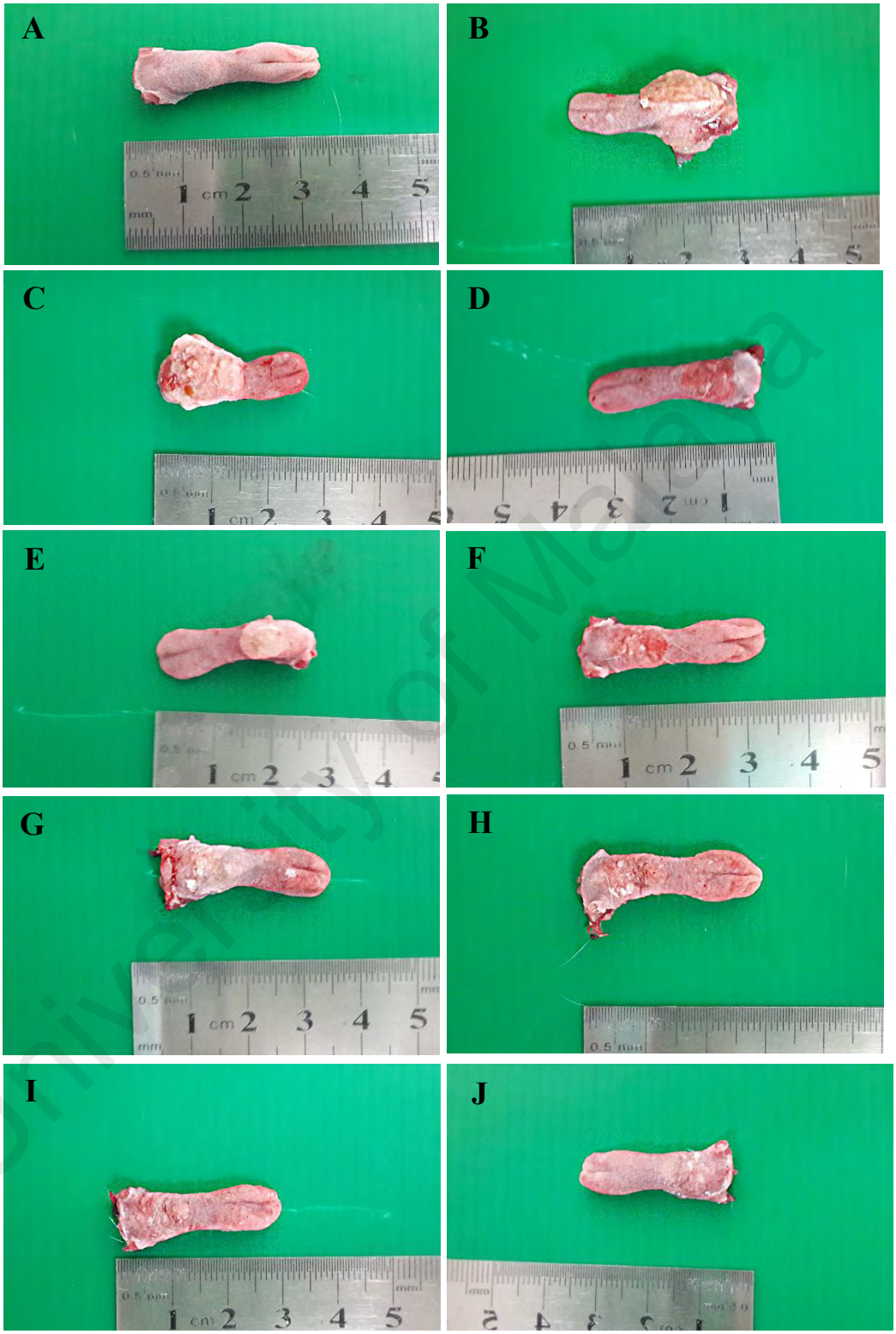

Figure 4. 10: Tongue tumour size in different groups (A) normal control group. Following oral carcinogenesis induced by 4NQO and treated with *DC* extract with/without Cisplatin or Cisplatin. (B) Induced cancer. (C) Vehicle. (D) Cisplatin. (E) *DC* 100 mg/kg. (F) *DC* 100 mg/kg + Cisplatin. (G) *DC* 500 mg/kg. (H) *DC* 500 mg/kg + Cisplatin. (I) *DC* 1000 mg/kg. (J) *DC* 1000 mg/kg + Cisplatin

4.3.6 Effect of *DC* Extract on the Incidence of Pre-Neoplasms and Neoplasms

The descriptive analysis indicated that most of the gross lesions associated with our model of 4NQO-induced rat oral cancer were seen in the posterior region of the tongue (on the dorsal surface of the tongue).

On microscopic examination, tongues of the normal control group showed normal oral mucosa of the tongue with surface keratinized stratified squamous epithelium with normal architecture, normal sizes and orientation of the papillae with underlying connective tissue and skeletal muscle bundle in both chemopreventive (Figure 4.11. A) and chemotherapeutic (Figure 4.12 A) studies.

Microscopic examination revealed that different histopathological changes such as hyperplasia, dysplasia and SCC appeared in all groups that given 20 ppm of 4NQO in their drinking water. The incidence of SCC was detected by the presence of discontinuation of the basement membrane and submucosal invasion of epithelial tumour cells as islands, nests and sheets. The epithelial tumour cells show nuclear and cellular pleomorphism, hyperchromatic nuclei, and altered nucleus: cytoplasmic ratio and keratin pearls in connective tissue (Figures. 4.13 A and B). These were observed in induced cancer (Figures. 4.11 B) and vehicle (Figures. 4.11 C) groups of chemopreventive and induced cancer (Figures. 4.12 B) and vehicle (Figures. 4.12 C) groups of chemotherapeutic studies.

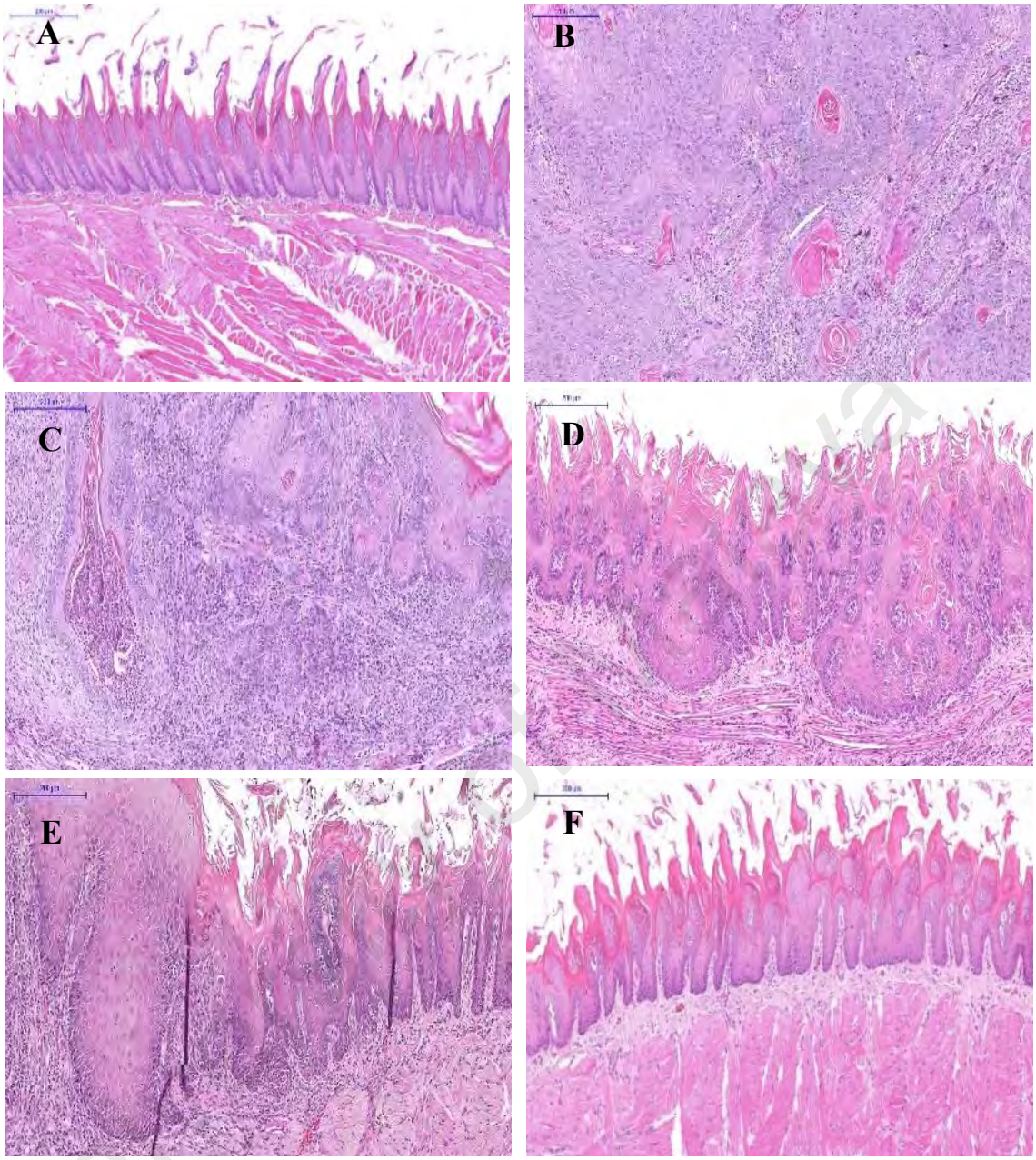

Figure 4. 11: Photomicrographs (A) normal control group showing normal oral mucosa with papillae having surface keratinized epithelium, underlying connective tissue and skeletal muscle bundles. Following oral carcinogenesis induced by 4NQO and treated with *DC* extract. (B) Induced cancer group & (C) vehicle group showing SCC invading the underlying connective tissue. (D) *DC* 100 mg/kg - Dysplasia. (E) *DC* 500 mg/kg - Dysplasia. (F) *DC* 1000 mg/kg - Hyperplasia. H&E stain, 100× magnification

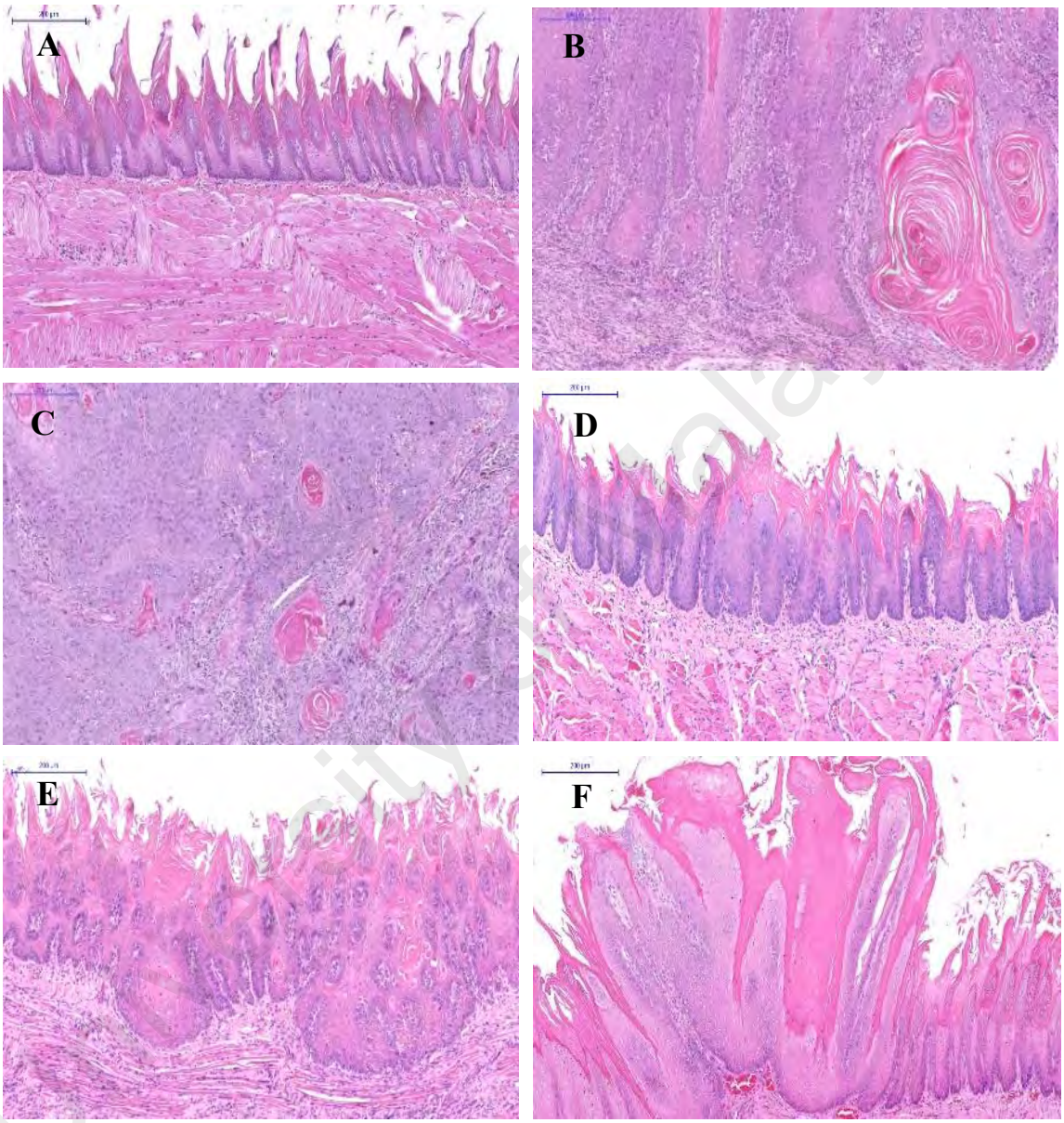
A
B
C
D
E
F

Figure 4.12, continued

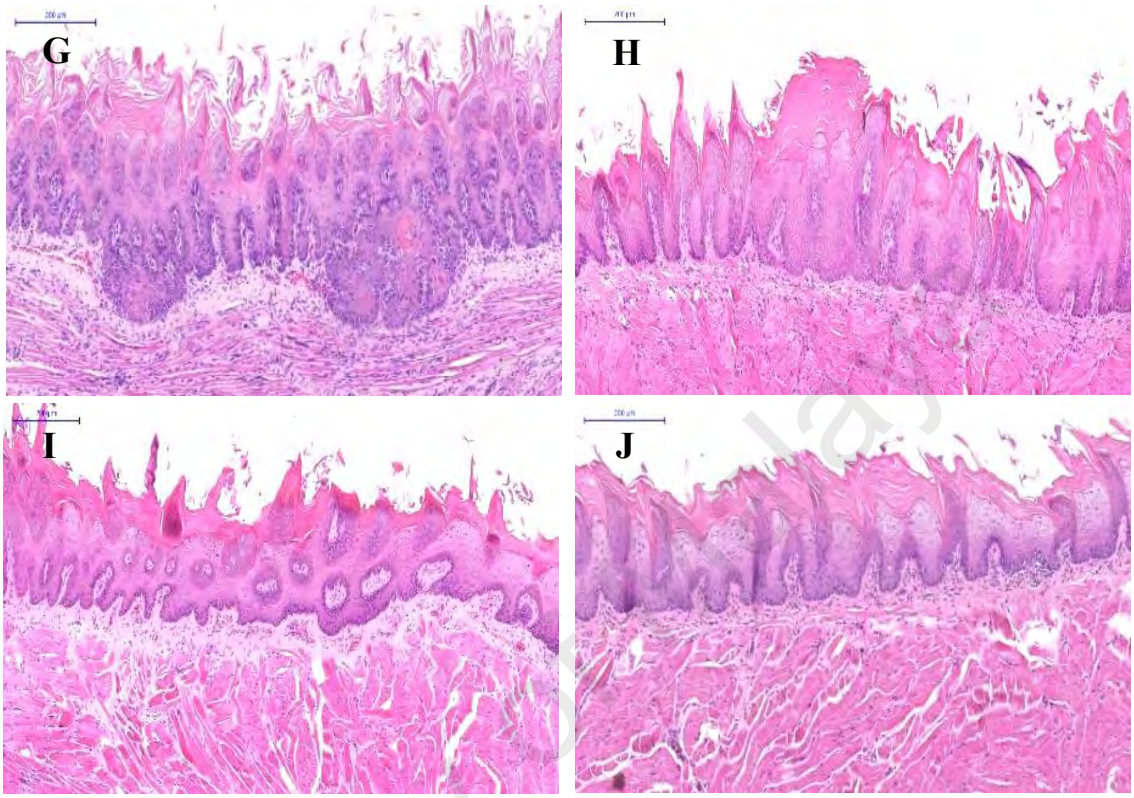

Figure 4. 12: Photomicrographs (A) normal control group showing normal oral mucosa with papillae having surface keratinized epithelium, underlying connective tissue and skeletal muscle bundles. Following oral carcinogenesis induced by 4NQO and treated with *DC* extract with/without Cisplatin or Cisplatin. (B) Induced cancer - SCC. (C) Vehicle - SCC. (D) Cisplatin - Dysplasia. (E) *DC* 100 mg/kg - Dysplasia. (F) *DC* 100 mg/kg + Cisplatin - Hyperplasia. (G) *DC* 500 mg/kg - Dysplasia. (H) *DC* 500 mg/kg + Cisplatin – Hyperplasia. (I) *DC* 1000 mg/kg- Hyperplasia. (J) *DC* 1000 mg/kg + Cisplatin- Hyperplasia. H&E stain, 100× magnification

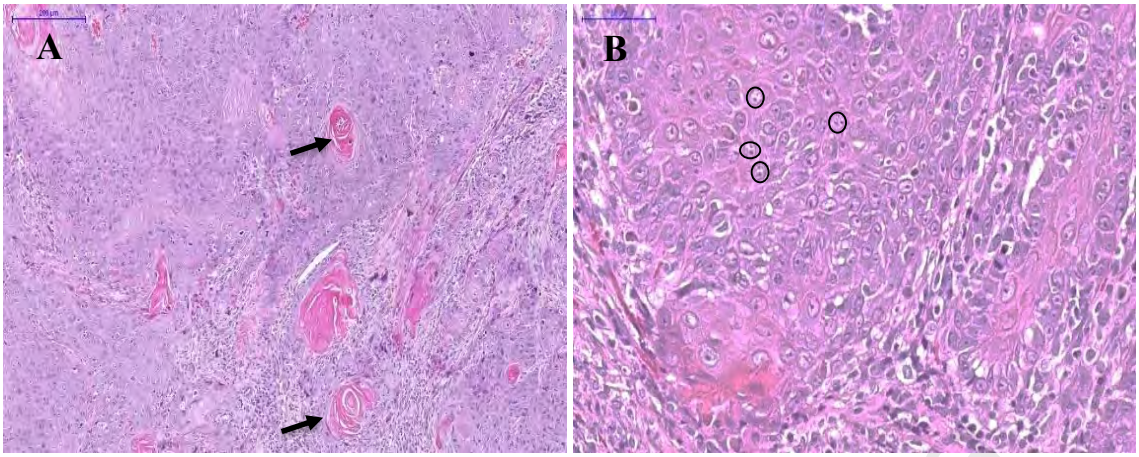

Figure 4. 13: Photomicrographs following oral carcinogenesis induced by 4NQO showed (A) the presence of keratin pearls in connective tissue (Black arrow) (100×) and (B) pleomorphic tumour cells (Black circle) (400×). H&E stain

In the chemopreventive study, administration of *DC* extracts conferred significant protection against the induction of OSCC by 4NQO. In the induced cancer and vehicle groups, the incidence of OSCC induced by 4NQO was 85.7% while in rats treated with *DC* extract at 100, 500 and 1000 mg/kg was 42.9%, 28.6 % and 14.3%, respectively, which was accompanied by an obvious decrease in the incidence of SCC, especially in the group received *DC* extract at 1000 mg/kg. Chemopreventive activity was confirmed as a statistically significant decrease in the incidence of OSCC (Table 4.20). The Chi square test that used to compare the incidence of development of hyperplasia, dysplasia and squamous cell carcinoma showed a statistical significant difference (Chi.Sq = 19.646; p = 0.012) between groups.

Moreover, in chemoprevention study, the hyperplasia with clearly defined basement membrane was seen in some samples in groups treated with *DC* 500 mg/kg (28.6%) and 1000 mg/kg (57.1%).

Table 4. 20: Effect of *DC* extract on the incidence of pre-neoplasms and neoplasms in 4NQO-induced oral carcinogenesis in the chemopreventive study.

	Incidence pre-neoplasms and neoplasms (%) within Treatment			*P* value
	Hyperplasia	**Dysplasia**	**SCC**	
Induced cancer	0/7 (0.0)	1/7 (14.3)	6/7 (85.7)	0.012*
Vehicle	0/7 (0.0)	1/7 (14.3)	6/7 (85.7)	
***DC* 100 mg/kg**	0/7 (0.0)	4/7 (57.1)	3/7 (42.9)	
***DC* 500 mg/kg**	2/7 (28.6)	3/7 (42.9)	2/7 (28.6)	
***DC* 1000 mg/kg**	4/7 (57.1)	2/7 (28.6)	1/7 (14.3)	

*p value less than 0.05, ($p < 0.05$) significant value.

The incidence of dysplasia in chemopreventive study was detected and graded into three grades of epithelial dysplasia (mild, moderate and severe), which are premalignant lesions, where mostly presented in the tongue of the rats that treated with *DC* extract at 100 mg/kg, 500 mg/kg and 1000 mg/kg during 4NQO administration (Table 4.21). The tongues in *DC* 1000 mg/kg group did not show severe dysplastic changes; two samples of mild dysplastic changes were detected in this group. Mild histological changes including basal cell hyperplasia, few mitosis, hyperchromatic nuclei and the level of atypia in the lower 1/3 with normal epithelial stratification were evident in the tongue epithelia of *DC* 1000 mg/kg group. These incidences were less than those seen in the other groups. While, only one rat in *DC* 500 mg/kg group, had mild dysplasia and two rats presented with moderate dysplasia. The moderate histological changes including hyperplasia of stratum spinosum, basal cell hyperplasia, superficial mitosis, increased number and size of nucleoli and level of atypia in the middle 1/3 with irregular epithelial stratification were evident in the tongue base epithelia of *DC* 500 mg/kg group. Whereas in *DC* 100 mg/kg group, two samples with moderate and two samples with severe dysplastic changes were seen. The severe histological changes including irregular rete ridges with keratin pearls within rete ridges, abnormally superficial mitotic figures,

premature keratinization in single cells (dyskeratosis), atypical mitotic figures in the basal layer, increased number and size of nucleoli and level of atypia in the upper 1/3 with irregular epithelial stratification were evident in the tongue base epithelia of *DC* 100 mg/kg group. One sample from each group (induced cancer and vehicle) demonstrated a sever dysplastic change.

Table 4. 21: Effect of *DC* extract on the severity of dysplastic lesions in the chemopreventive study.

	Incidence of Tongue Dysplasia			
	Total	**Mild**	**Moderate**	**Severe**
Induced cancer	1/7	0	0	1
Vehicle	1/7	0	0	1
***DC* 100 mg/kg**	4/7	0	2	2
***DC* 500 mg/kg**	3/7	1	2	0
***DC* 1000 mg/kg**	2/7	2	0	0

To illustrate, in the chemotherapeutic study, administration of *DC* extracts conferred significant protection against the induction of OSCC by 4NQO. In the induced cancer and vehicle groups, the incidence of OSCC that induced by 4NQO was 85.7% per each, while in rats treated with *DC* extracts at 100, 500 and 1000 mg/kg was 57.1%, 28.6% and 14.3%, respectively, which was accompanied by an obvious decrease in the incidence of OSCC, especially in the group received *DC* extract at 1000 mg/kg. Whereas, the incidence of OSCC in the group of Cisplatin and combination groups at 100 and 500 mg/kg with Cisplatin was 14.3% per each. Finally, in the combination group of *DC* 1000 mg/kg + Cisplatin, no incidence of OSCC was detected as the incidence was 0%, offering the best protection to the rats exposed to 4NQO. The chemotherapeutic activity of *DC* extract was confirmed as a statistical significant decrease in OSCC incidence (Table 4.22). The Chi square test that used to compare the incidence of development of hyperplasia, dysplasia

and SCC showed a statistical significant difference (Chi.Sq = 29.442; p = 0.021) between groups.

Furthermore, in the chemotherapeutic study, the hyperplasia was seen in some samples treated with Cisplatin (42.9%), *DC*100 mg/kg + Cisplatin (42.9%), *DC*500 mg/kg (28.6%), *DC*500 mg/kg + Cisplatin (42.9%), *DC*1000 mg/kg (42.9%) and *DC*1000 mg/kg + Cisplatin (71.4%).

Table 4. 22: Effect of *DC* extract on the incidence of pre-neoplasms and neoplasms in 4NQO-induced oral carcinogenesis in the chemotherapeutic study.

Group	Incidence pre-neoplasms and neoplasms (%) within Treatment			*P* value
	Hyperplasia	Dysplasia	SCC	
Induced cancer	0/7 (0)	1/7 (14.3)	6/7 (85.7)	0.021*
Vehicle	0/7 (0)	1/7 (14.3)	6/7 (85.7)	
Cisplatin	2/7 (28.6)	4/7 (57.1)	1/7 (14.3)	
***DC*100 mg/kg**	0/7 (0)	3/7 (42.9)	4/7 (57.1)	
***DC*100 mg/kg + Cisplatin**	2/7 (28.6)	4/7 (57.1)	1/7 (14.3)	
***DC*500 mg/kg**	2/7 (28.6)	3/7 (42.9)	2/7 (28.6)	
***DC*500 mg/kg + Cisplatin**	3/7 (42.9)	3/7 (42.9)	1/7 (14.3)	
***DC*1000 mg/kg**	3/7 (42.9)	3/7 (42.9)	1/7 (14.3)	
***DC*1000 mg/kg + Cisplatin**	3/7 (42.9)	4/7 (57.1)	0/7 (0)	

*p value less than 0.05, ($p < 0.05$) significant value.

The incidence of dysplasia in chemotherapeutic study was detected with three grades of epithelial dysplasia (mild, moderate and severe), were most presented in the tongues of the rats that given Cisplatin, *DC* extract at 100, 500 and 1000 mg/kg with or without Cisplatin after cessation of 4NQO administration (Table 4.23). The tongues with mild dysplastic changes were detected in one sample of Cisplatin, combination of *DC* 100 mg/kg + Cisplatin, combination of *DC* 500 mg/kg + Cisplatin and *DC* 1000 mg/kg groups, whereas two samples with mild dysplastic changes detected in the combination of *DC*

1000 mg/kg + Cisplatin group. Moderate dysplasia was seen in one sample of the combination of *DC* 500 mg/kg + Cisplatin, whereas two samples were detected in Cisplatin, combination of DC 100 mg/kg + Cisplatin, *DC* 500 and 1000 mg/kg groups. Severe dysplastic changes were presented in three samples of the *DC* 100 mg/kg group and one sample of *DC* 500 mg/kg, induced cancer and vehicle groups. The mild, moderate and severe histological changes are same as described earlier in the chemopreventive study.

Table 4. 23: Effect of *DC* extract on the severity of dysplastic lesions in the chemotherapeutic study.

Group	Incidence of Tongue Dysplasia			
	Total	Mild	Moderate	Severe
Induced cancer	**1/7**	0	0	1
Vehicle	**1/7**	0	0	1
Cisplatin	**3/7**	1	2	0
***DC* 100 mg/kg**	**3/7**	0	0	3
***DC* 100 mg/kg + Cisplatin**	**3/7**	1	2	0
***DC* 500 mg/kg**	**3/7**	0	2	1
***DC* 500 mg/kg + Cisplatin**	**2/7**	1	1	0
***DC* 1000 mg/kg**	**3/7**	1	2	0
***DC* 1000 mg/kg + Cisplatin**	**2/7**	2	0	0

In both chemopreventive and chemotherapeutic studies, groups that subjected to the effect of *DC* extract, Cisplatin and combination of both of them against 4NQO-induced oral cancer presented with different degrees of epithelial dysplasia as illustrated in Figure 4.14. Dysplastic lesions demonstrated both cytological and architectural changes extending to various thickness of the epithelium; severe dysplasia (Figure 4.14. A1), moderate dysplasia (Figure 4.14. B1) and mild dysplasia (Figure 4.14. C1).

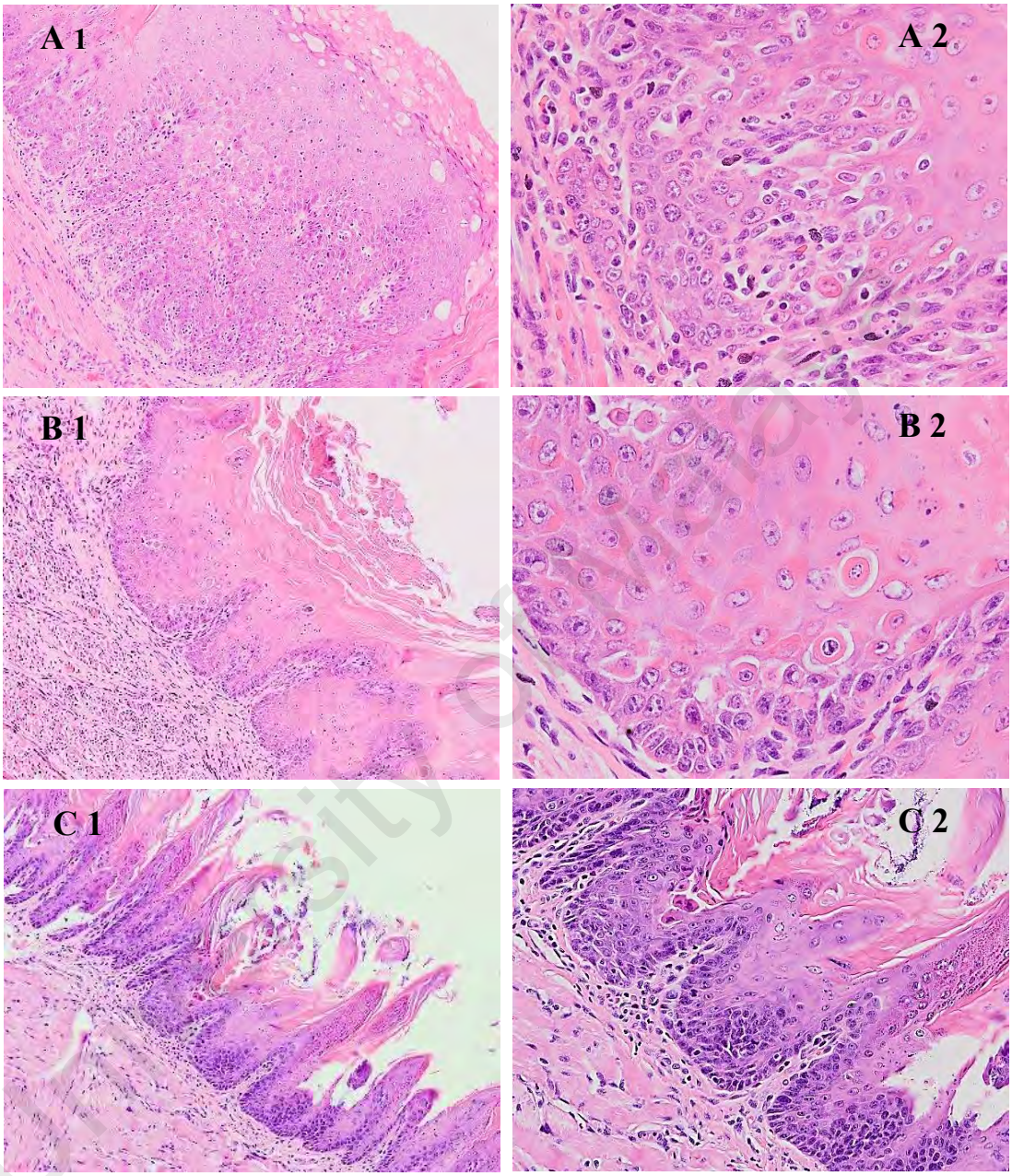

Figure 4. 14: Photomicrographs following oral carcinogenesis induced by 4NQO and treated with *DC* extract. (A1 and A2) shows features of severe dysplasia (*DC* 100 mg/kg), (B1 and B2) Moderate dysplasia with few mitosis (*DC* 500 mg/kg), and (C1 and C2) Mild dysplasia (*DC* 1000 mg/kg). (H&E stain, A1, B1 and C1 100× magnification and A2, B2 and C2 400× magnification)

4.3.7 Histological Findings of Oral Cancer Metastasis

In chemotherapeutic study, after 22 weeks, metastatic tumour in the cervical lymph node was presented and localised in the marginal sinus of the lymph node from one rat in each group of the induce cancer and vehicle (Figure 4.15), and thus, the metastasis rate was 14.3% per each. In general, normally functioning lymph nodes contain densely packed lymphocytes which usually exhibit strong nucleic acid signals, while metastatic cancer contains nucleic acid with reducing density in the sample volume, as epithelial cells have a higher relative volume of cytoplasm (Lloyd et al., 2013). Among the *DC* treated groups with or without Cisplatin as well as Cisplatin group, the cervical lymph node metastasis rate was 0%, which represented the effect of chemotherapy on a 4NQO-induced rat model. Lymph node metastasis was inhibited in the *DC* treated groups with or without Cisplatin as well as in the Cisplatin group compared to the induced cancer and vehicle groups.

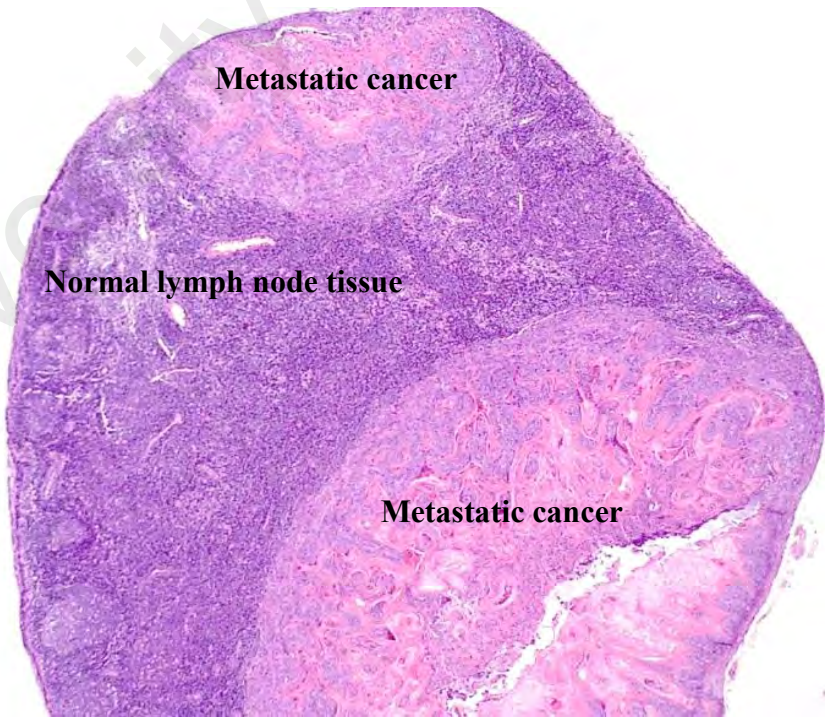

Figure 4. 15: Photomicrograph shows lymph node tissue and metastatic invasion of squamous cell carcinoma in a cervical lymph node of induced cancer group. (H&E stain, 20× magnification)

4.3.8 Immunohistochemical (IHC) Analysis

Immunohistochemical expression analysis of the selected tumour marker in the chemopreventive and the chemotherapeutic studies was performed to assess the localization and compare the positive (brown) stained tumour cell percentage area between the induced cancer group that given 4NQO alone and *DC* treated group that given *DC* 1000 mg/kg during (as in the chemopreventive study) or after secession (as in the chemotherapeutic study) of 4NQO administration. In addition, groups of Cisplatin and Combination of Cisplatin + *DC* 1000 mg/kg were added to analyse the IHC expression in the chemotherapeutic study.

Generally, in the chemopreventive study, photomicrographs of haematoxylin and eosin (H&E) stained tissue sections of the oral administered *DC* 1000 mg/kg group (Figure 4.16 b) showed decreased proliferation indices in 4NQO-treated rats when compared to the induced cancer (4NQO alone) group (Figure 4.16 a). Moreover, in the chemotherapeutic study, photomicrographs of H&E stained tissue sections of *DC* 1000 mg/kg (Figure 4.17 b), Cisplatin (Figure 4.17 c), and the combination of *DC* 1000 mg/kg + Cisplatin groups (Figure 4.17 d) decreased proliferation indices in 4NQO-treated rats when compared to induced cancer (4NQO alone) group (Figure 4.17 a).

4.3.8.1 Immunohistochemical Analysis of Cyclin D1 and Ki-67 (Cell-cycle proteins)

In the induced cancer (4NQO alone) group, there was an increase in the proliferation as represented with high Cyclin D1 and Ki-67-labelled epithelial cells. The Cyclin D1 and Ki-67 proliferative area found in the basal and parabasal compartment of the keratinized stratified squamous epithelium of induced cancer rats and extended into the epithelial tumour cells that have invaded the underlying connective tissue as shown in chemopreventive (Figure 4.16 c and e) and in chemotherapeutic (Figure 4.17 a1 and a2) studies. Inversely, administration of *DC* 1000 mg/kg (Figure 4.16 d) in chemopreventive

study, as well as *DC* 1000 mg/kg (Figure 4.17 b1), Cisplatin (Figure 4.17 c1) and the combination of *DC* 1000 mg/kg + Cisplatin (Figure 4.17 d1) in chemotheraputic study, does not show SCC and the surface epithelium show intense staining of Cyclin D1 at the basal and parabasal region of the surface parakeratinized stratified squamous epithelium (proliferative area). The immunostaining was almost negative for Ki-67 in *DC* 1000 mg/kg (Figure 4.16 f) of chemopreventive study, and *DC* 1000 mg/kg (Figure 4.17 b2), Cisplatin (Figure 4.17 c2) and the combination of *DC* 1000 mg/kg + Cisplatin (Figure 4.17 d2) in chemotheraputic study, indicating strong antiproliferative effects.

4.3.8.2 Immunohistochemical Analysis of Bcl-2 and p53 (Apoptotic proteins)

Bcl-2 and p53 proteins expression showed a characteristic cellular localisation. In induced cancer group, high positive Bcl-2 and p53 stained cells found in basal and parabasal layers and extended into the epithelial tumour cells that have invaded into the connective tissue as shown in the chemopreventive study (Figures 4.16 g and i) and chemotherapeutic study (Figures 4.17 a3 and a4). However, few positive Bcl-2 and p53 stained cells were detected in the *DC* 1000 mg/kg group (Figures 4.16 h and j) of the chemopreventive study, and in *DC* 1000 mg/kg (Figures 4.17 b3 and b4), Cisplatin (Figures 4.17 c3 and c4) and combination of *DC* 1000 mg/kg + Cisplatin (Figures 4.17 d3 and d4) groups of the chemotherapeutic study.

4.3.8.3 Immunohistochemical Analysis of β-catenin and E-cadherin (Cell adhesion proteins).

A membranous pattern of β-catenin and E-cadherin protein expression with strong positive expression in the parabasal layer and weak expression in the most superficial layers was showed in the group *DC* 1000 mg/kg of chemopreventive study (Figures 4.16 l for β-catenin and n for E-cadherin) and in the groups *DC* 1000 mg/kg, Cisplatin and *DC* 1000 mg/kg + Cisplatin of chemotherapeutic study (Figures 4.17 b5, c5 and d5 for β-

catenin) and (Figures 4.17 b6, c6 and d6 for E-cadherin). The immunostaining was principally limited to the basal and spinous layers, as opposite to the induced cancer group where β-catenin and E-cadherin protein expression exhibited a partially absent membranous staining with an altered distribution in the cytoplasm and was combined in the keratin pearl nest of the tumour as illustrated in chemopreventive study (Figures 4.16 k and m) and chemotherapeutic study (Figures 4.17 a5 and a6).

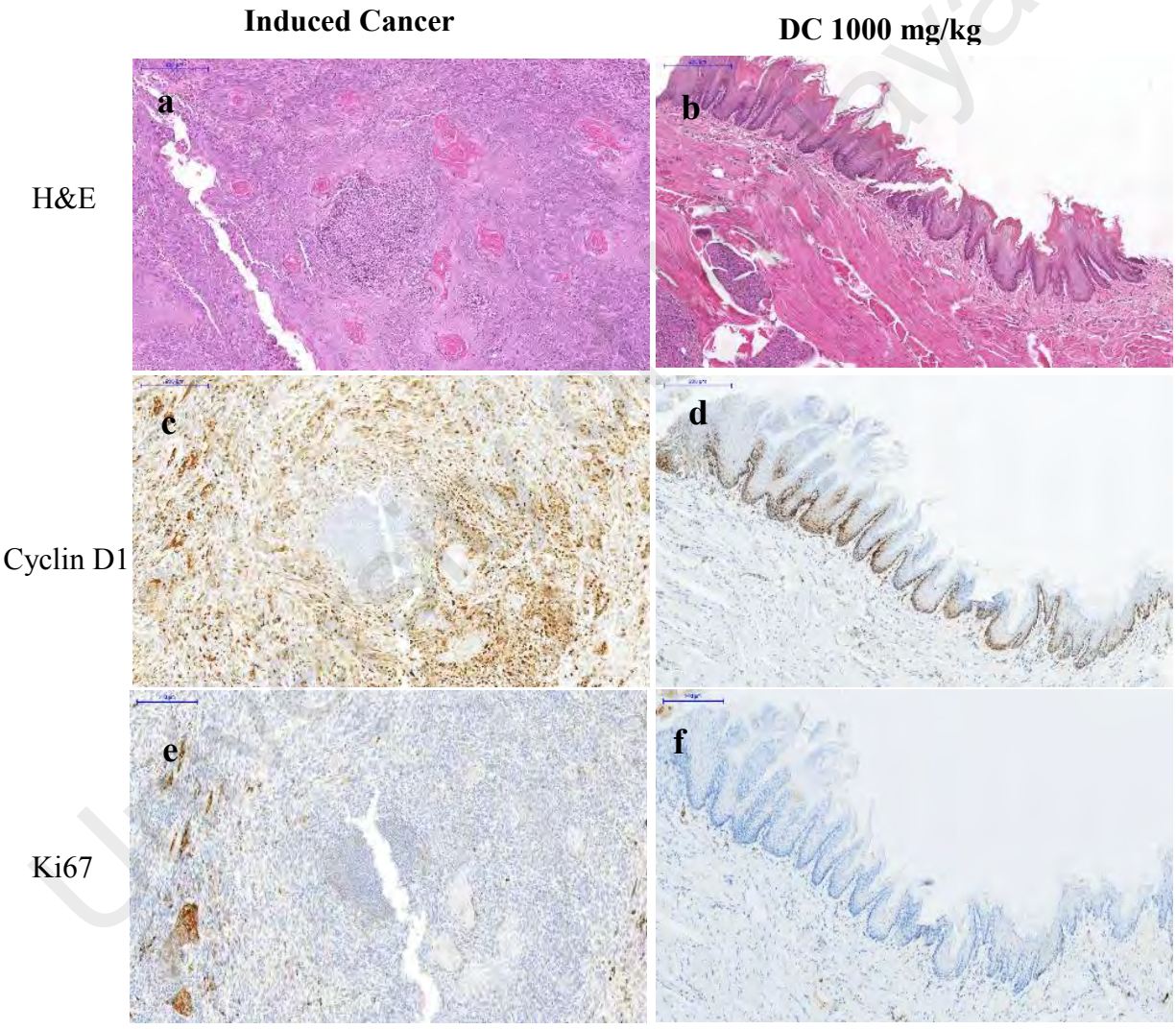

Figure 4.16, continued

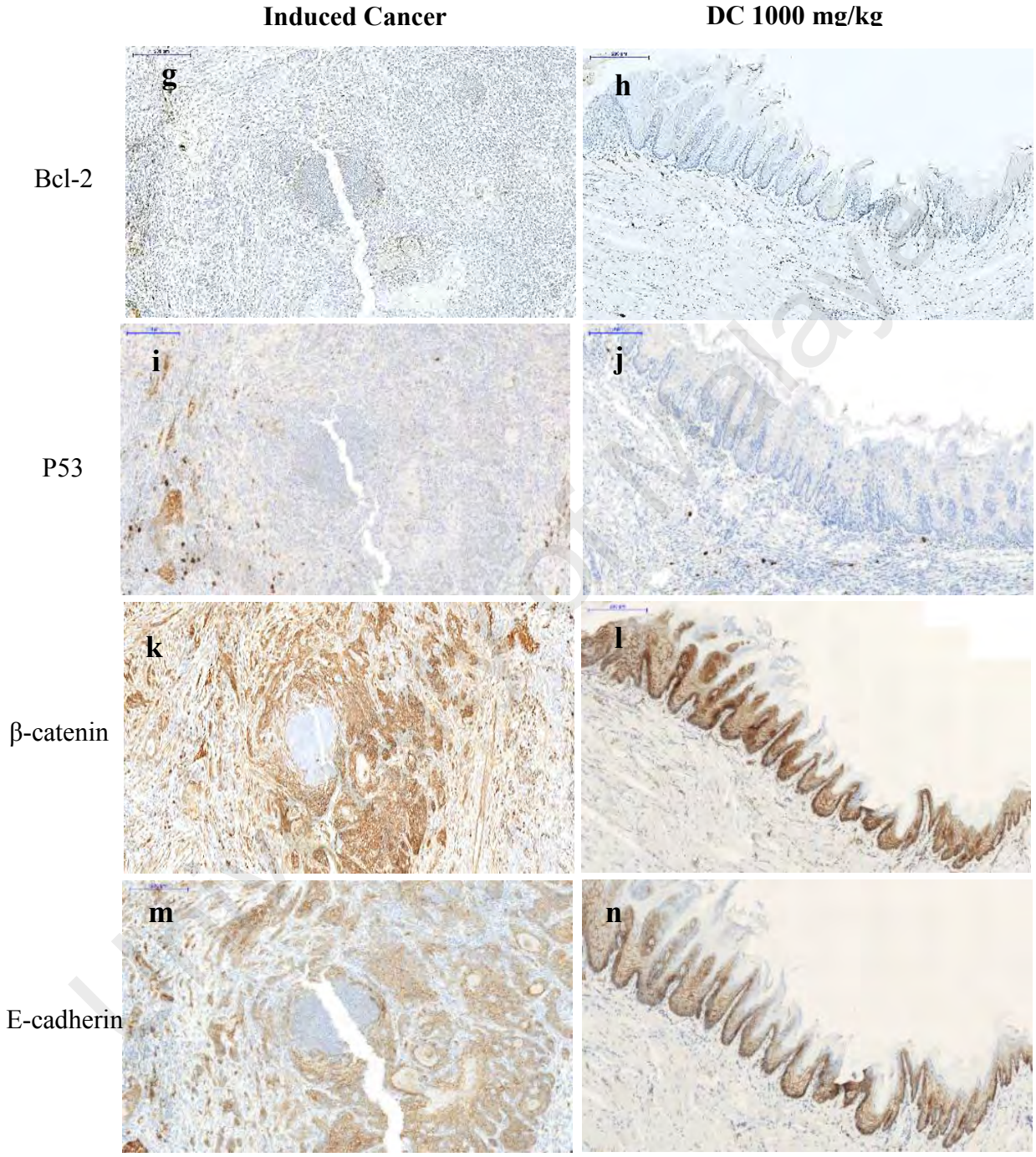

Figure 4. 16: Photomicrograph of induced cancer and *DC* 1000 mg/kg groups showing (a and b) H&E stained, immunohistochemical expression of (c and d) Ki-67, (e and f) Cyclin D1, (g and h) Bcl-2, (I and j) p53, (k and l) β-catenin and (m and n) E-cadherin, respectively, ×100 magnification

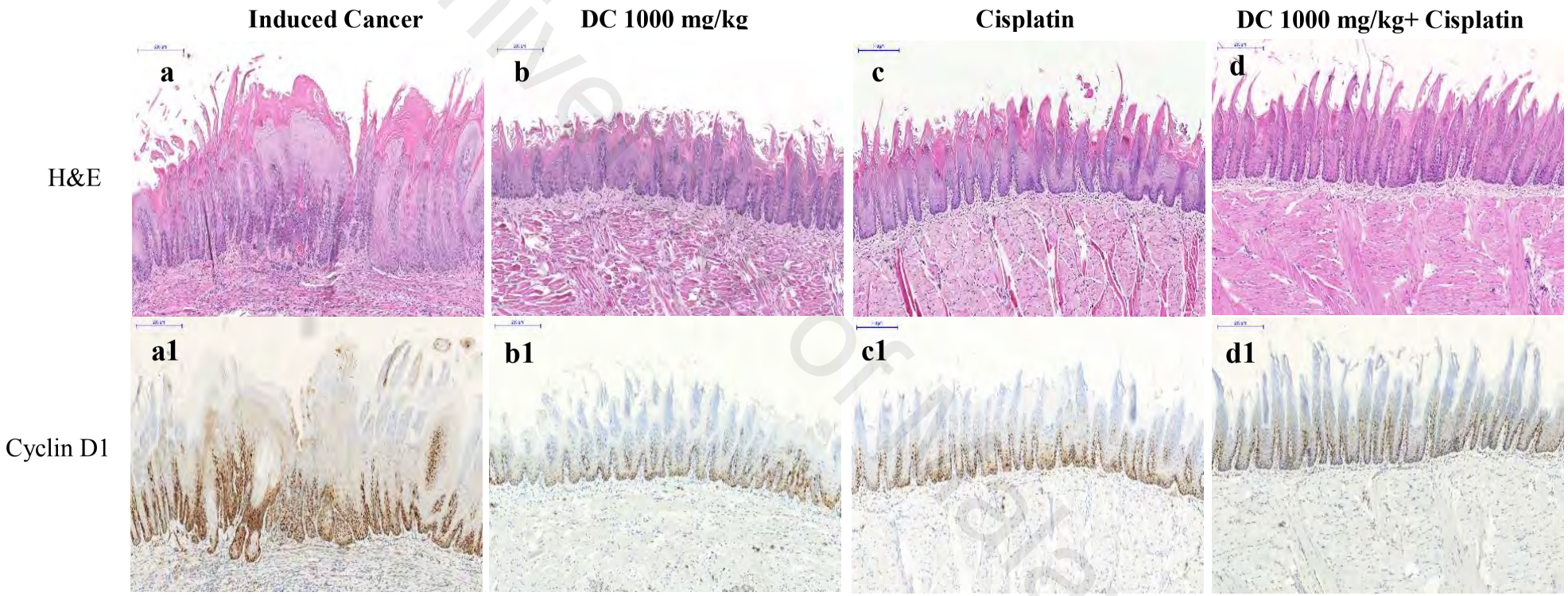

Figure 4.17, continued

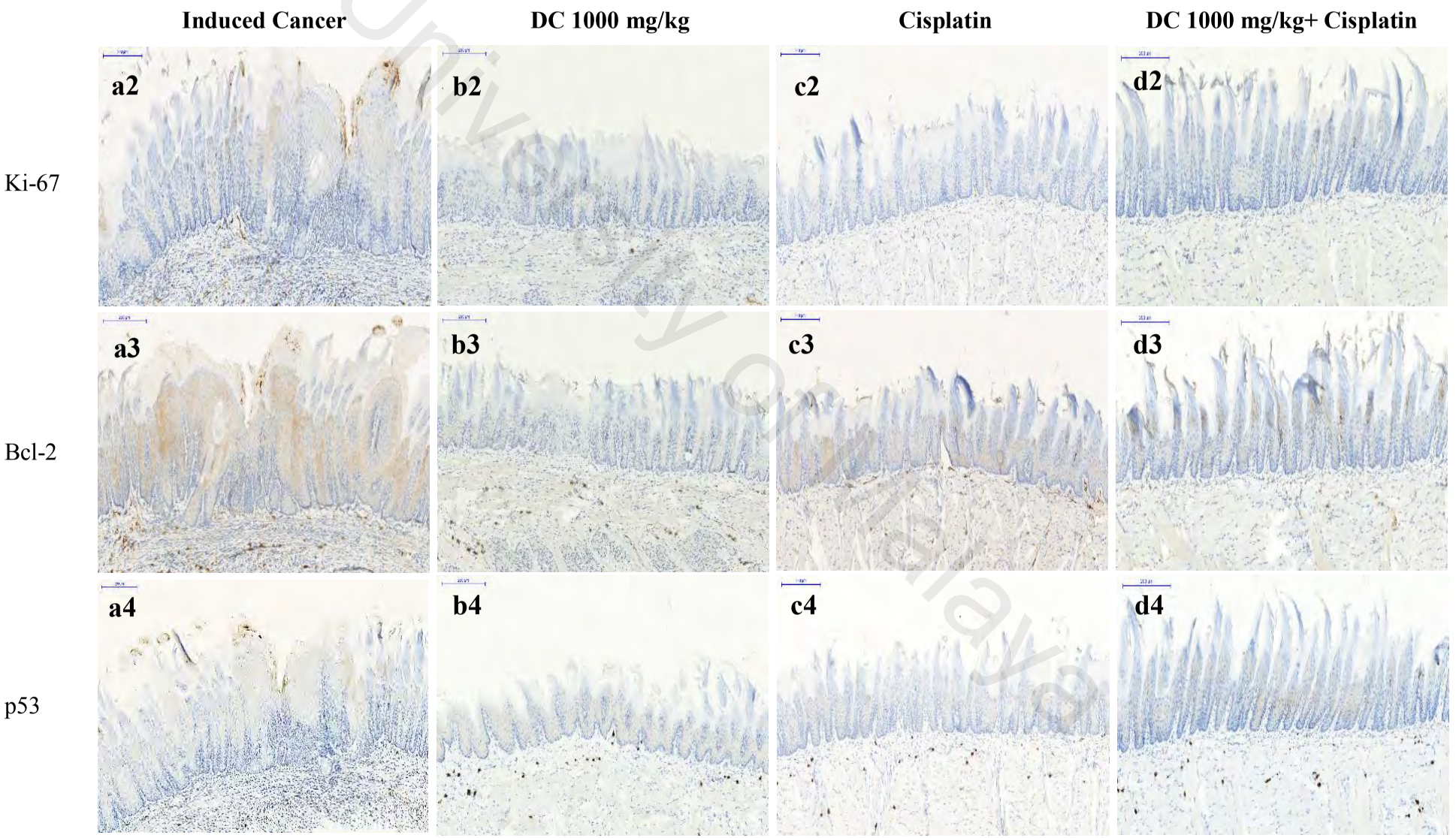

Figure 4.17, continued

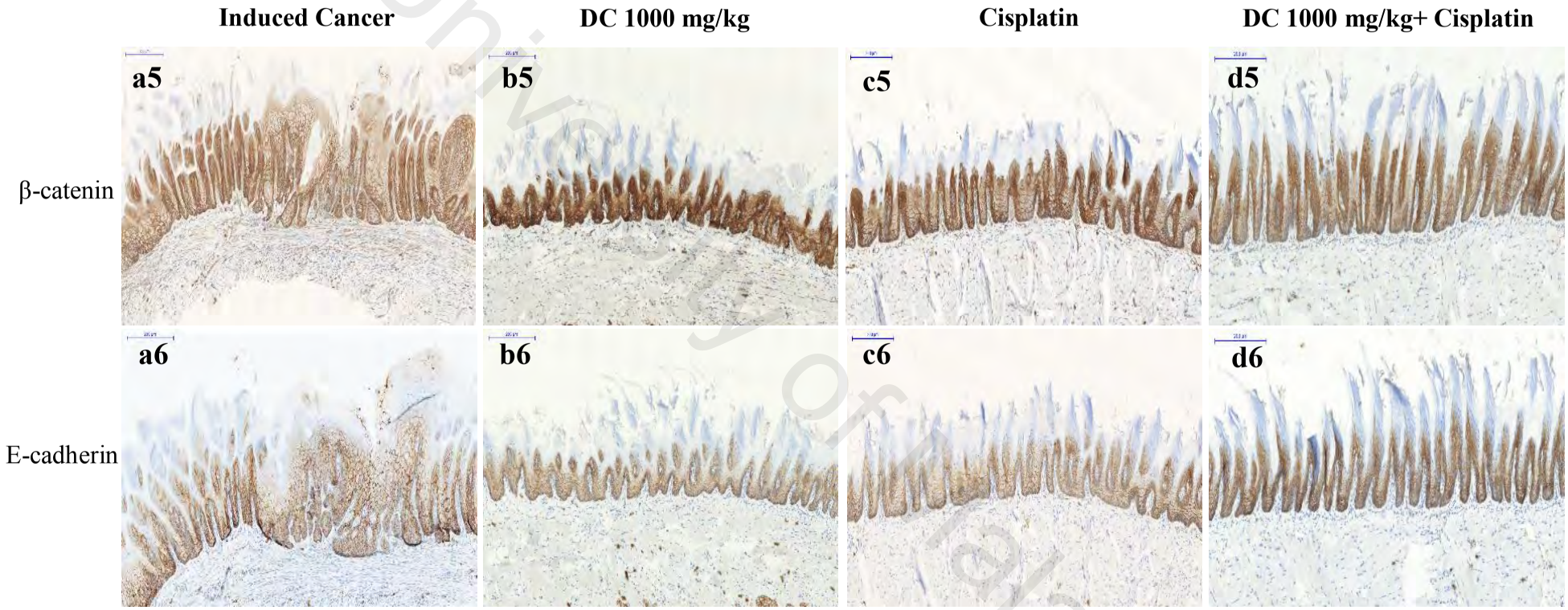

Figure 4. 17: Photomicrographs of H&E. (a) Induced cancer group. (b) *DC* 1000 mg/kg. (c) Cisplatin. (d) *DC* 1000 mg/kg + Cisplatin. Photomicrographs of immunohistochemical expression of Cyclin D1: (a1) Induced cancer group. (b1) *DC* 1000 mg/kg. (c1) Cisplatin. (d1) *DC* 1000 mg/kg + Cisplatin. Ki-67: (a2) Induced cancer group. (b2) *DC* 1000 mg/kg. (c2) Cisplatin. (d2) *DC* 1000 mg/kg + Cisplatin. Bcl-2: (a3) Induced cancer group. (b3) *DC* 1000 mg/kg. (c3) Cisplatin. (d3) *DC* 1000 mg/kg + Cisplatin. p53: (a4) Induced cancer group. (b4) *DC* 1000 mg/kg. (c4) Cisplatin. (d4) *DC* 1000 mg/kg + Cisplatin. β-catenin: (a5) Induced cancer group. (b5) *DC* 1000 mg/kg. (c5) Cisplatin. (d5) *DC* 1000 mg/kg + Cisplatin. E-cadherin: (a6) Induced cancer group. (b6) *DC* 1000 mg/kg. (c6) Cisplatin. (d6) *DC* 1000 mg/kg + Cisplatin, 100× magnification

4.3.8.4 Image Analysis of Immunohistochemical Stain Expression

The immunoreactivity for each tumour marker was measured in the form of area percent by using ImageJ software with an IHC toolbox plugin. In the chemopreventive study, *DC* 1000 mg/kg group showed low positive stained tumour cell percentage area with statistical significant difference for the following antibodies; Cyclin D1, Ki-67, Bcl-2 and p53 compared to Induced cancer group ($p < 0.05$). Whereas β-catenin and E-cadherin tumour marker antibodies showed slightly higher positive stained tumour cell percentage area (no significant difference) compared to the induced cancer group as $p > 0.05$ (Table 4.24).

Table 4. 24: The positive stained percentage area of tumour cell during oral carcinogenesis induced by 4NQO in rats for the chemopreventive study (Independent *t* test).

Tumour marker antibodies	Groups	Mean ±SD	*P* value
Cyclin D1	Induced cancer	12.86±4.35	0.040*
	DC 1000 mg/kg	2.67±1.96	
Ki-67	Induced cancer	1.96±1.49	0.016*
	DC 1000 mg/kg	0.68±0.68	
Bcl-2	Induced cancer	10.17±5.98	0.015*
	DC 1000 mg/kg	4.35±2.15	
p53	Induced cancer	0.51±0.41	0.000*
	DC 1000 mg/kg	0.09±0.05	
B-catenin	Induced cancer	21.77±6.28	0.909
	DC 1000 mg/kg	23.90±5.42	
E-cadherin	Induced cancer	19.09±6.28	0.478
	DC 1000 mg/kg	22.82±7.25	

*p value less than 0.05, ($p < 0.05$) significant value.

Similarly, In the chemotherapeutic study, *DC* 1000 mg/kg group showed low positive stained tumour cell percentage area for the following antibodies; Cyclin D1, Ki-67, Bcl-2 and p53 compared to induced cancer group. Whereas, β-catenin and E-cadherin tumour marker antibodies showed slightly higher positive stained tumour cell percentage area when compared with the induced cancer group (Table 4.25). Statistical significant difference ($p < 0.05$) was observed between the groups in all selected antibodies except in β-catenin and E-cadherin. A post hoc Dunnett's test was used to detect the differences between the groups as presented in Table 4.26. However, Cyclin D1 positive stained tumour cell percentage area was slightly higher in the Cisplatin group than *DC* 1000 mg/kg with or without Cisplatin. Interestingly, the combination group of *DC* 1000 mg/kg + Cisplatin showed superior result than either *DC* 1000 mg/kg or Cisplatin group alone.

Table 4. 25: The positive stained percentage area of the tumour cell after oral carcinogenesis induced by 4NQO in rats for the chemotherapeutic study (one-way ANOVA).

Tumour marker antibodies	Induced cancer (%) area	*DC* 1000 mg/kg (%) area	Cisplatin (%) area	*DC* 1000 mg/kg + Cisplatin (%) area	*P* value
Cyclin D1	10.30±4.62	0.94±0.91	1.85±1.13	0.70±0.33	0.000*
Ki-67	1.76±2.19	0.13±0.09	0.12±0.11	0.10±0.10	0.000*
Bcl-2	11.90±7.40	4.34±4.32	2.30±1.23	0.71±0.82	0.000*
p53	0.18±0.19	0.05±0.11	0.04±0.02	0.02±0.02	0.001*
β-catenin	22.16±3.06	24.40±7.25	25.00±5.14	26.59±4.47	0.146
E-cadherin	20.80±3.86	22.44±6.55	21.32±5.86	25.50±7.97	0.176

Values are expressed as mean ± standard deviation. **p* value less than 0.05, ($p < 0.05$) significant value.

Table 4. 26: Immunohistochemical analysis for chemotherapeutic study. A post hoc Dunnett's test.

Dependent Variable	(I) IHC expression	(J) IHC expression	Mean Difference (I-J)	Std. Error	*P* value
Cyclin D1	*DC* 1000 mg/kg	Induced cancer	-9.36	0.89	0.000*
	Cisplatin		-8.45	0.89	0.000*
	DC 1000 mg + Cisplatin		-9.60	0.89	0.000*
Ki-67	*DC* 1000 mg/kg	Induced cancer	-1.63	0.40	0.000*
	Cisplatin		-1.64	0.40	0.000*
	DC 1000 mg + Cisplatin		-1.66	0.40	0.000*
Bcl-2	*DC* 1000 mg/kg	Induced cancer	-7.56	1.59	0.000*
	Cisplatin		-9.60	1.59	0.000*
	DC 1000 mg + Cisplatin		-11.19	1.59	0.000*
p53	*DC* 1000 mg/kg	Induced cancer	-0.12	0.04	0.008*
	Cisplatin		-0.13	0.04	0.004*
	DC 1000 mg + Cisplatin		-0.15	0.04	0.001*
β-catenin	*DC* 1000 mg/kg	Induced cancer	2.24	1.90	0.506
	Cisplatin		2.84	1.90	0.317
	DC 1000 mg + Cisplatin		4.43	1.90	0.060
E-cadherin	*DC* 1000 mg/kg	Induced cancer	1.64	2.28	0.813
	Cisplatin		0.52	2.28	0.992
	DC 1000 mg + Cisplatin		4.70	2.28	0.110

**p* value less than 0.05, ($p < 0.05$) significant value.

4.3.9 Gene Expression Analysis

A custom RT² PCR array was performed to assess and compare the expression of some selected gene in the induced cancer group that given 4NQO alone and *DC* treated group that given *DC* 1000 mg/kg during (as in the chemopreventive study) or after secession (as in the chemotherapeutic study) of 4NQO administration. In addition, groups of Cisplatin and combination of *DC* 1000 mg/kg + Cisplatin were added to analysed the gene expression in the chemotherapeutic study. Gene expression analysis of the selected genes are shown in Table 4.27 for chemopreventive study and in Table 4.28 for chemotherapeutic study.

The group of *DC* 1000 mg/kg showed statistically significant increase in expression of both Bax and Casp3 genes compared to Induced cancer group. Whereas Tp53, Bcl-2,

Cox-2, Cyclin D1 and EGFR genes were decreased when compared with the induced cancer group. The statistical significant difference ($p < 0.05$) was observed between the groups in all selected genes except in Cyclin D1 and EGFR for both chemopreventive and chemotherapeutic studies. Interestingly, the combination of *DC* 1000 mg/kg + Cisplatin group showed superior result than either *DC* 1000 mg/kg or Cisplatin alone.

Table 4. 27: Gene expression analysis during oral carcinogenesis induced by 4NQO in rats for the chemopreventive study

	***DC* 1000 mg/kg (2^-ΔCt)**	**Induced cancer (2^-ΔCt)**	**Fold Regulation**	***P* value**
Bax	0.042	0.014	2.98	0.010*
Bcl-2	0.021	0.043	-2.06	0.035*
Casp3	0.031	0.011	2.71	0.039*
Tp53	0.016	0.040	-2.43	0.002*
Ptgs2	0.184	0.437	-2.37	0.029*
Ccnd1	0.054	0.120	-2.23	0.060
EGFR	0.139	0.302	-2.17	0.119

*p value less than 0.05, ($p < 0.05$) significant value. Red colure means overexpression and blue colure means under-expression.

Table 4. 28: Gene expression analysis during oral carcinogenesis induced by 4NQO in rats for the chemotherapeutic study.

	Bax	**Bcl-2**	**Casp3**	**Tp53**	**Ptgs2**	**Ccnd1**	**EGFR**
Induced cancer (2^-ΔCt)	0.009	0.039	0.011	0.034	0.357	0.121	0.290
***DC* 1000 mg/kg (2^-ΔCt)**	0.033	0.012	0.033	0.011	0.174	0.046	0.122
Fold Regulation	3.57	-3.17	3.10	-3.14	-2.06	-2.62	-2.37
***P* value**	0.018*	0.040*	0.038*	0.008*	0.015*	0.087	0.123
Cisplatin (2^-ΔCt)	0.036	0.009	0.033	0.009	0.124	0.057	0.129
Fold Regulation	3.85	-4.38	3.15	-3.80	-2.87	-2.12	-2.25
***P* value**	0.001*	0.027*	0.012*	0.005*	0.003*	0.111	0.117
Cisplatin + *DC* 1000 mg/kg (2^-ΔCt)	0.048	0.008	0.047	0.007	0.111	0.039	0.111
Fold Regulation	5.15	-4.68	4.41	-4.59	-3.20	-3.07	-2.61
***P* value**	0.000*	0.025*	0.033*	0.002*	0.004*	0.069	0.105

*p value less than 0.05, ($p < 0.05$) significant value. Red colure means overexpression and blue colure means under-expression.

4.4 Discussion

Head and neck SCC animal models have been achieved by using carcinogenic agents such as 7,12-dimethylbenz(*a*)anthracene or 4NQO. The induction of OSCC using 4NQO has been confirmed in several different rodent species, including mice, hamsters, and rats. This carcinogenic agent has been found to be superior on other agent for several reasons. First, the 4NQO induced tongue carcinogenesis model has been considered quite useful to investigate oral carcinogenesis and identify cancer chemopreventive agents because the most common site for oral carcinoma is the tongue and the administration of drinking water containing 4NQO is a straightforward and easy method (Tanaka et al., 2011). Second, 4NQO induced lesions that developed in the absence of nonspecific inflammatory changes. This is a very important because substances such as 7,12-dimethylbenz(*a*)anthracene can be significant irritants, resulting in chronic inflammation, sloughing of tissue, necrosis, and the formation of organizing granulation tissue. These factors can produce lesions that are differ in the cytology and morphology from the dysplasia (Hasina et al., 2009). Finally, oral lesions produced by 4NQO are similar to human lesions because many ulcerated and endophytic or exophytic tongue tumours and dysplasia developed (Yoshida et al., 2005). In addition, the molecular alterations induced in rat tissue by 4NQO closely mimic the human disease. For example, altered expression of p53 as well as mutation of p53 have been shown in 4-NQO models are similar to humans (Takeuchi et al., 2000).

4NQO is a water soluble quinoline derivative that produce DNA adducts and also can undergo redox cycling to produce reactive oxygen species that result in mutations and DNA strand breaks (Kanojia & Vaidya, 2006). A study that compared 4NQO painting of tongues and delivery of 4NQO in the drinking water in two mouse strains revealed that the latter method, resulted in a much higher incidence of oral cavity carcinogenesis (Tang et al., 2004).

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 chemopreventive intervention (McCormick et al., 2015).

Cisplatin is one of the main standard anticancer drugs, which still has an essential role in cancer chemotherapy (Ravindra et al., 2010). The common component of chemotherapy for the treatment of solid organ cancers was Cisplatin that was first described by Michele Peyrone as early as in 1845 (Kim et al., 2015). Cisplatin and other platinum drugs are the common drugs in the contemporary medical oncology, which considered to have a major impact in management of tumours of the ovary, testes, head and neck and other cancers (Kelland, 2007). However, the dose toxicities associated with platinum therapy has presented a serious concern where Cisplatin can affect organs such as nervous system and kidneys among the clinically established platinum compound (Hartmann et al., 1999; Hartmann & Lipp, 2003). For this reason, in the present study a combination of *DC* extract with Cisplatin has been evaluated.

The present study was performed to evaluate the null hypothesis that carcinogenesis in the oral cavity cannot be inhibited or delayed by pharmacologic activation of medicinal plant. To address this null hypothesis, *in vivo* study was performed to determine the chemopreventive and chemotherapeutic activity of *DC* extract. In this study, 4NQO was used to induce oral cancer in rat, 4NQO is a potent chemical carcinogen. This material has been used in previous studies to induce oral cancer in rat tongues (Lee & Choi, 2011; McCormick et al., 2015; Patel & Damle, 2013; Ribeiro et al., 2007). Clinical and histopathologic studies have shown that squamous cell carcinoma due to 4NQO is considered equivalent to human SCC (Arima et al., 2006; Kitakawa et al., 2006). In many

oral cancer chemoprevention studies, the 4NQO/rat models have been performed using F344 rats, however, the induction of OSCC in rats by 4NQO has also been studied in a variety of other 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 considerable inter-strain differences in OSCC responses to 4NQO; Dark-Agouti rats were the most sensitive to the induction of oral carcinogenesis by 4-NQO, while Wistar/Furth rats were the least sensitive (Kitano et al., 1992).

4.4.1 Food Consumption and Body Weight Gain

Regarding to the food consumption, it is important as proper intake of nutrients is essential to the physiological status of the animal and give a good impression on the proper response to the treatment (Sathish et al., 2012). Administration of 4NQO produced a significant body weight loss in animals due to the intra oral cancer development accompanying with malnutrition that caused by lack of appetite, unable to eat, increased metabolic rate (Thandavamoorthy et al., 2014).

In the present study (chemopreventive study), 4NQO administration produced decreased food consumption in the induced cancer and vehicle groups from 12 through 22 weeks which controlled and returned to nearly normal level in the groups that given *DC* resin methanol extract especially at doses of 500 and 1000 mg/kg except at the 21 and 22 weeks compared to normal control group. In chemotherapeutic study, 4NQO administration and Cisplatin reduced food consumption which similarly controlled by *DC* resin methanol extract especially at doses of 500 and 1000 mg/kg throughout the experimental period except in the last three weeks for *DC* 500 mg/kg group and in the last two weeks for *DC* 500 mg/kg + Cisplatin and *DC* 1000 mg/kg with or without Cisplatin compared to normal control group. The effect of 4NQO on the food consumption reduction has been confirmed in several studies (Patel & Damle, 2013;

Sohrabi et al., 2009). Inversely, another study that used a mice as 4NQO animal model to study the chemopreventive effect of ABT-510 (a synthetic peptide) reported that there is no significant difference in food consumption between the control and treated groups when 4NQO administered in the drinking water for 8 weeks (Hasina et al., 2009).

Patient with cancer cachexia usually suffer from body weight loss which contribute to patient morbidity and mortality (Fearon & Preston, 1990). In the current study, 4NQO produced a significant reduction of body weight among the induced cancer and vehicle groups which were controlled by treatment with *DC* extract with or without a combination of Cisplatin not with Cisplatin alone. Herein, the result based on the body weight gain revealed that the combination of *DC* extract (*DC* 500 mg/kg and *DC* 1000 mg/kg) with Cisplatin reduce the toxic effect of Cisplatin by gaining the body weight as nearly as that in normal control group. This result is in a like manner with study performed by Patel and Damle (2013), who demonstrated that loss of body weight caused by Cisplatin could be controlled by treatment with combination of telmisartan with Cisplatin.

4.4.2 Haematological and Biochemical Parameters

The administration of 4NQO in the drinking water in animals produces a significant alteration in the haematological and biochemical parameters. The dangerous effects of 4NQO on liver and kidney are well identified and appear to be directly linked to increasing the concentration of hepatic and renal enzymes in the serum of experimental animals (Viswanadha et al., 2011). Furthermore, the observed changes in serum ALP, ALT, AST activity and bilirubin levels are good indicators of hepatic injury resulting from cellular leakage and the loss of functional integrity of the cell membrane in liver (Kadir et al., 2013). A study performed by Barcessat et al. (2014), who studied the systemic toxicity effect of 4NQO during oral carcinogenesis in rats, showed a significant reduction in WBC and monocyte counts as well as significant increase in serum ALT and

AST when 4NQO topically applied to the tongue of the rat (Barcessat et al., 2014). In the present study, a significant reduction in WBC, neutrophil and monocyte counts presented in the induced cancer and vehicle groups. This reduction could be attributed to the effect of 4NQO on the immune system. In this regard, a study performed by Gannot et al. (2004), who analysed the spleens in mice that developed OSCC, discussed the splenic alterations regarding cytokines released by the tumour cells affecting the immune system.

Chemotherapy is one of the most important causes of anaemia in cancer patients, and the association between dose and duration of chemotherapy with anaemia is well known (Coiffier et al., 2001). The negative impact of anaemia on the cancer patient regarding survival and quality of life deserves attention, and this association has been established for almost all types of cancer studied (Caro et al., 2001; Spivak et al., 2009). In the previous study, when Cisplatin given to the rats twice a week at 2 mg/kg body weight, the lowest haemoglobin value recorded at week 4 after therapy was 5.9 g/dl. This progressive anaemia has been associated with a slight decline in total WBC and platelet counts. However, this reduction was within the normal range (Wood & Hrushesky, 1995).

Cisplatin-induced renal dysfunction and the degree of therapy-induced anaemia is correlated with the amount of Cisplatin-induced renal damage (Wood & Hrushesky, 1995). In the same manner, the Cisplatin-treated rats (Cisplatin group) in the chemotherapeutic study showed a significantly lower level of haemoglobin accompanied with lower WBC (with no significant difference) than that of normal control group. In a study performed by Wood and Hrushesky (1995), he found that during the administration of eight doses of Cisplatin over 25 days (total 16 mg/kg), rats developed a progressive decline in renal function measured by a three to fourfold rise in serum creatinine. Herein, in the groups of induced cancer, vehicle, Cisplatin and *DC* 100 mg/kg + Cisplatin, the serum creatinine showed significantly higher compared to normal control group.

However, the increase in serum creatinine was controlled when *DC* extract at doses of 500 and 1000 mg/kg have been administered to the rats.

Previous studies have shown that administration of 4NQO in drinking water for 8 weeks significantly increased the level of liver and renal markers (ALP, AST, ALT, urea, creatinine and uric acid) which reflected a liver and renal toxicity (Viswanadha et al., 2011). It is found that when the rats were given 4NQO in their drinking water, the level of liver markers (AST, ALT, ALP) and creatinine level were significantly increased, and conversely, albumin level was reduced. However, after the rats have been treated with *Oroxylum indicum* (L.) leaf extract, all changes brought back to near normal level (Mohan et al., 2016).

According to previous studies performed by Palipoch and Punsawad (2013) and Yadav (2015), reported that Cisplatin administration in a single dose at 7.5, 10, 25 and 50 mg/kg induced significant increase in serum ALT, AST and ALP and significant decrease in serum albumin. These results were in agreement with the present study where the induced cancer, vehicle and Cisplatin groups presented with higher level of liver markers (AST, ALT, ALP) and creatinine, and low albumin level, however, Cisplatin 3 mg/kg was used in the present study. The ability of Cisplatin to cause alterations in the activity of these enzymes could be a secondary event following Cisplatin-induced liver damage with the consequent leakage from hepatocytes (Yadav, 2015).

In the present study, a similar effect of 4NQO on rats was showed, i.e. an alteration in the level of the ALP, ALT, AST serum was evident in the groups of induced cancer, vehicle and Cisplatin. Treatment of the rats with *DC* extract at 100, 500 and 1000 mg/kg with or without the combination of Cisplatin restored the biochemical parameters (ALP, ALT and AST) towards normal. Decreased level of ALT, ALP and AST in blood serum of the rats could be attributed due to the antioxidant effect of *DC* and its ability to act as

a free radical scavenger (Machala et al., 2001; Yehia et al., 2013), thereby protecting membrane permeability after treatment with *DC* extract indicate hepatoprotective and curative effect. The homoisoflavonoids of *DC* exhibited a strong antioxidant activity comparable to that of the strongest flavonol antioxidant, quercetin (Machala et al., 2001). The *DC* extract returned the levels of haematological and biochemical parameters to nearly normal level compared to the normal control group in both chemopreventive and chemotherapeutic studies.

4.4.3 Survival Rate and Tongue Tumour Volume

The survival rates of the rats in both chemopreventive and chemotherapeutic studies that treated with *DC* extracts was 100% in both *DC* 500 and *DC* 1000 mg/kg groups and it was 85.7 in *DC* 100 mg/kg group, the survival rate seen to be dose-dependant. The rats that given 4NQO alone (induced cancer group) or with 10%DMSO (vehicle group) exhibited survival rates of 71.4%. The survival rate of 71.4% was showed in the Cisplatin as well as in the combination of *DC* 100 mg/kg + Cisplatin groups. The administration of *DC* extract for 10 consecutive weeks especially at doses of *DC* 500 and 1000 mg/kg during or after the secession of 4NQO increased the survival rate. This result was in agreement with a study performed by McCormick et al. (2015), who reported that when the rats treated with rosiglitazone (800 mg/kg diet) after 10 weeks administration of 4NQO, the survival rate increased from 73% to 90%. Inversely, in a study performed by Droguett et al. (2015), who studied the efficacy of quercetin on 4NQO-mice model, they found that the survival rate was 65% in the animals that given 4NQO alone compared to that groups combined with quercetin at doses of 10 or 100 mg/kg where the survival rate presented were 60% and 45%, respectively. However, the significant difference was showed between the normal control and other groups. Quercetin does not improve survival rate in mice given 4-NQO (Droguett et al., 2015).

The survival rate could be linked to the tumour volume and general health of the animals as the administration of 4NQO produced a significant body weight loss due to the intra oral cancer development accompanying with malnutrition that caused by lack of appetite, unable to eat, increased metabolic rate (Thandavamoorthy et al., 2014). Similarly, in the present study, the tumour volume was statistically increased in the induced cancer and vehicle groups compared to that groups treated with *DC* extract at all doses (100, 500 and 1000 mg/kg) during or after 4NQO administration. This was in agreement with a study performed by Wali et al. (2012), who reported that the administration of PEG-8000 for 14 weeks after the 14 weeks of 4NQO administration significantly developed smaller tumours volume than 4NQO group. Furthermore, the groups treated with Cisplatin alone or in combination with *DC* extract also showed smaller tumour volume compared to the induced cancer and vehicle groups.

4.4.4 Histopathological Changes in the Tongue of the Rat

4NQO produces histopathological changes in oral tongue from normal epithelium, hyperplasia, premalignant dysplasia, and carcinoma in situ to invasive squamous cell carcinoma similar to those in humans (Ribeiro et al., 2004). The results of the present study, showed that none of the normal control animals developed any visible tongue epithelia lesions and histopathological changes through the end of the study. In addition, 4NQO at 20 ppm for 8 weeks induced typical precancerous signs and morphological alterations on the tongue epithelium after 22 weeks from the start of carcinogen administration. None of the induced cancer and vehicle groups showed with hyperplasia. There were sever epithelial dysplasia and SCC of well-differentiated type among the induced cancer and vehicle animals which were reduced with the treatment by *DC* extract especially at doses of *DC* 500 and *DC* 1000 mg/kg, in chemopreventive study. Similarly, in the chemotherapeutic study, the sever epithelial dysplasia and SCC controlled by *DC* extract especially at doses of *DC* 500 and *DC* 1000 mg/kg with or without Cisplatin and

with Cisplatin alone. However, combination of *DC* extract (*DC* 500 mg/kg and *DC* 1000 mg/kg) with Cisplatin showed better improvement in histology of tongue. The combination of *DC* 1000 mg/kg + Cisplatin reduced the incidence of SCC to 0%. Similarly, in previous study, the combination of telmisartan with Cisplatin reduced 4NQO induced invasive SCC in the tongue (Patel & Damle, 2013). The histopathological grade was usually SCC of a well-differentiated type. The tumours spread into the submucosa and underlying muscle layer, forming small nests with typical keratin pearl formation (Zhao et al., 2014). In like manner, a study performed to evaluate the effects of a etodolac (Cox-2 inhibitor) on 4NQO-induced rat tongue carcinogenesis, showed that dysplasia, papilloma and SCC developed at incidences of 21.4, 7.2 and 100%, respectively, whereas hyperplasia was not detected. However, the 4NQO was administered in the drinking water for 8 weeks at 20-30 ppm and the experiment was completed at 28 weeks (Yamamoto et al., 2004).

In another study, when the rats received 4NQO at 20 ppm in the drinking water for 10 weeks, the incidence of invasive OSCC in the rat tongue was highly reproducible which was 83% and 75% in the two experiments demonstrated invasive oral cancers at 26 weeks after the start of 4NQO administration (Peng et al., 2015). Generally, 4NQO induced a variety of dysplastic and neoplastic lesions with morphological and molecular alterations in the oro-esophageal epithelium that mimic those occurring in human oral epithelial pre-neoplastic and neoplastic lesions depend on the dose and duration of administration (Tang et al., 2004).

4.4.5 Oral Cancer Metastasis

Prognosis of cancer patients and success or failure of treatment is associated with the presence or absence of metastasis (Kawashiri et al., 2001). The effect of chemotherapy on the metastasis is expected when it achieved in combination with either surgery and/or

radiotherapy (local therapies). However, there were few reports in which the inhibition of metastasis using chemotherapy was investigated, and the evaluations were not consistent (Kawashiri et al., 2009). Blocking metastatic spread measured as the greatest challenge of metastatic cancer that has proven additional worrying. Certainly, most of the current anticancer treatments have been developed to treat primary disease; they often fail in blocking metastasis (Desmet et al., 2013).

Detection of metastatic foci, whether in lymph nodes or distant organs, can present a significant challenge. Classically, the presence of metastasis has been evaluated at necropsy in euthanised mice after completion of the experimental protocol. In OSCC, most investigators resect both the draining lymph node basins and the lungs for pathological evaluation (Milas et al., 2010). In other studies performed to investigate the effects of anticancer agents on cancer invasion and metastasis in mice, concluded that both Cisplatin and peplomycin are effectively inhibiting invasion and metastasis (Kawashiri et al., 2001; Kawashiri et al., 2009). Similarly, in the current chemotherapeutic study, metastatic tumour in the cervical lymph node was presented in two rats from the induced cancer and vehicle groups, while *DC* treated groups with or without Cisplatin as well as Cisplatin group inhibited cervical lymph node metastasis.

4.4.6 Immunohistochemical Analysis

Immunohistochemistry is a process of detecting targeted antigens (proteins) in tissue sections by the use of labelled antibodies through antigen-antibody interactions and is applied in paraffin embedded tissues (Van Eycke et al., 2017). Over the past decade, in clinical pathology diaminobenzidine (DAB) has been widely used as a (brown) chromogen for revealing protein expression using IHC together with haematoxylin for tissue counterstaining (Helps et al., 2012). Discrepancies in the decision of the accurate colour intensity turn out to be a problem and thus often leads to inter-observer variations.

Calculation of the stained area percentage has a varying (poor to good) rate of reproducibility, even if the data provided by the pathologist have good to excellent inter-observer reproducibility (Jaraj et al., 2009). Because of the limitations mentioned above of visual IHC stained assessment, means of automated quantitation of IHC images may provide the necessary detailing to improve IHC data quality across the world. Recently, advanced digital image processing systems have been introduced to gather the high volume IHC analysis and scoring (Varghese et al., 2014).

ImageJ released in 1997 and is public domain, Java-based image processing program, developed at National Institute of Mental Health, Bethesda, MD, USA (Schneider et al., 2012). ImageJ with colour deconvolution and Immunoratio plugins, are freely available web-based applications, enabling their routine use for quantitative IHC analysis (Sysel et al., 2013). Another study used ImageJ with a plugin called IHC-toolbox as a method of automated digital image analysis for quantification of histological biomarkers, concluded that the ImageJ with IHC-toolbox plugin could generate a visual illustration of the statistical model and detect a specified colour automatically (Shu et al., 2016). Therefore, in the present study, ImageJ software with IHC-toolbox plugin was used to assess the quantification of tumour marker staining in tissue sections.

Cyclin D1 considered as a key regulator of the cell-cycle progression and works as a transcriptional co-regulator. The overexpression of Cyclin D1 encourages transformation to a malignant phenotype, and it has been related to the development and progression of different types of cancer (Shan et al., 2009). Overexpression of cyclin D1 has a direct link to histopathological differentiation (Saawarn et al., 2012) and also functioned as an independent prognostic factor of oral cancers aggressiveness (Huang et al., 2012). In the present study, for both the chemopreventive and chemotherapeutic experiments, oral administration of *DC* 1000 mg/kg reduced the expression of Cyclin D1 that is usually

overexpressed in head and neck SCC (Perez-Ordonez et al., 2006), as shown in the induced cancer group of this study. Administration of *DC* 1000 mg/kg also reduced the expression of Ki-67. This result is in agreement with a study performed by Wali et al. (2012), who demonstrated that the topical application of Polyethylene Glycol decreased proliferation (number of Ki-67 positive cells) and reduced expression of Cyclin D1 in the tongue mucosa of the 4NQO-rats group. Ki-67 protein is a reliable marker of proliferating cells because it can be detected during cell cycle especially in phases G1, S, G2, and M, however, cannot be detected in G0 phase. There was a direct connection between increasing Ki-67 labelling index and increasing Broders' grading of OSCC (Tumuluri et al., 2002). In a study that analysed the synergistic antitumor effect of Cisplatin with Andrographolide in human OSCC (CAL-27 cells *in vitro* and *in vivo*), the results demonstrated that this combination decreased Ki-67 expression *in vivo* and can be used as a potential therapeutic strategy for OSCC (Chen et al., 2017).

In the present study (both chemopreventive and chemotherapeutic studies), a higher expression of Ki-67 was observed in the induced cancer group compared to the *DC* 1000 mg/kg with or without Cisplatin. The combination of *DC* 1000 mg/kg + Cisplatin resulted in more reduction in the expression of Ki-67 than other groups. This result indicates that the *DC* 1000 mg/kg in combination with Cisplatin produced a synergistic anti-tumour growth activity against OSCC. In addition, this result indicated that *DC* 1000 mg/kg reduced the Ki-67 and Cyclin D1 expression, which in turn decreases the proliferation and the aggressiveness toward OSCC.

The previous study presented that the expression of the antiapoptotic Bcl-2 protein closely correlated to the expression of p53 (Ravi et al., 1996). Furthermore, it is reported in North Indian patients, p53 was positivity expressed in 55% of oral dysplasia lesions, in 75% of oral carcinoma lesions and no positive staining was detected in normal oral

mucosa (Kaur et al., 1994). In another study, when the 4NQO topically applied on the rat tongue for 12 weeks, the expressions of Ki-67, Cyclin D1, p63, Bcl2 and p53 showed increased expression according to the grade of the dysplastic lesion in oral mucosal tissues of the rats (Scrobota et al., 2016). The result of the current study is in line with these findings where the Bcl-2 and p53 proteins expressed more in the induced cancer group that was given 4NQO alone for 8 weeks than the treated group that was given *DC* 1000 mg/kg. Down-expression of Bcl-2 and high expression and activation of Bax, as well as caspase-3, were reported to enhance apoptotic effect (Kang et al., 2010).

Overexpression of p53 mutant protein has been demonstrated in precancerous and cancerous lesions of the oral cavity and associated with poor clinical outcome (Chin et al., 2004). However, the present study indicated that *DC* 1000 mg/kg reduced the Bcl-2 and p53 levels, successively enhanced the apoptotic effect during oral carcinogenesis. In head and neck SCC cell lines, high expression of endogenous Bcl-2 was linked to increase Cisplatin resistance, and experimental overexpression of Bcl-2 promoted Cisplatin resistance (Michaud et al., 2009). For this reason, during treatment of OSCC with Cisplatin, an adjunctive treatment that targeting Bcl-2 and its family members could be helpful. In the present study, in the chemotherapeutic experiment, the combination of *DC* 1000 mg/kg with Cisplatin reduced the expression of Bcl-2 more than either *DC* 1000 mg/kg or Cisplatin alone. This indicated that *DC* 1000 mg/kg augments the inhibitory effects of Cisplatin *in vivo*.

β-catenin distribution is different in normal mucosal cells than in OSCC cells. In normal oral epithelium, β-catenin show mainly membranous staining (Pannone et al., 1998), whereas in OSCC there is leaks of membrane-bound β-catenin and this results in a corresponding increase in cytoplasmic localisation and an irregular nuclear accumulation (Lo et al., 1999). β-catenin is a transcription factor in the Wnt pathway,

which promotes the transcription of genes involved in cellular proliferation and apoptosis inhibition (Van de Wetering et al., 1997). Overexpression in β-catenin protein level can lead to increased cell proliferation in human head and neck cancer cells (Song et al., 2010) and has been detected during human OSCC development (Pannone et al., 2010). Another study demonstrated that abnormal expression of β-catenin is expressively associated with invasion and poor prognosis (Mahomed et al., 2007).

In a study performed by Osei-Sarfo et al. (2013), the level of β-catenin in the female mice tongue was increased by 2- to 3-fold after 4NQO treatment compared to untreated vehicle group. Hence, 4NQO treatment is associated with an increase in total β-catenin levels (Osei-Sarfo et al., 2013). Another study showed that β-catenin protein primarily presented in the basal layer of tongue epithelium in the normal control group samples, whereas 4NQO administration resulted in both an increase in the β-catenin level and an expansion of β-catenin staining to the suprabasal layers of the tongue epithelium and the tumour cells invading the underlying connective tissue. All 4NQO plus drug treatment groups (bexarotene, retinoid CD1530 and the combination of both of them) showed lower β-catenin protein levels in the tongue epithelium, primarily limited to the basal layer, even in the regions of the tumour (Tang et al., 2014).

Correspondingly, in the present study, the expression of β-catenin in the induced cancer (4NQO alone) group showed an aberrant pattern with the leak of membrane-bound β-catenin and revealed a corresponding increase in cytoplasmic localisation of β-catenin as compared to that in the *DC* 1000 mg/kg group for both chemopreventive and chemotherapeutic studies. It has been suggested that the cytoplasmic expression pattern instead of membrane staining of β-Catenin is a common abnormal tumour-related alteration (Gao et al., 2005) rather than loss or reduction of expression, which has been described previously (Bankfalvi et al., 2002). In a study performed by Li et al. (2016),

who examined the role of β-catenin in the development of Cisplatin resistance in OSCC cell lines, found that the overexpression of β-catenin can result in Cisplatin resistance. In the present study, level of β-catenin in the Cisplatin group was higher than that in induced cancer group; however, the highest expression was presented in the combination of *DC* 1000 mg/kg + Cisplatin group. This result indicates that *DC* 1000 mg/kg could restore the β-catenin level to the membranous layer and enhance role of Cisplatin in regulating level of β-catenin, which in turn decrease the cell proliferation in the cancer cells, and maintains normal epithelial polarity and inhibits invasion.

E-cadherin is a calcium-dependent transmembrane glycoprotein, and it is expressed in most epithelial cells and is essential for the establishment of adherence junction between cells (Gall & Frampton, 2013). It has been reported that after topical application of 4NQO on the mouse tongue for 24 weeks, staining of E-cadherin was expressed strongly in the parabasal cell layer, but not on the surface of basal cells and after 40 weeks of 4NQO application, E-cadherin expression was lost in the invasive front, and a positive staining of tumour nests was noticed (Schoop et al., 2009).

The expression of E-cadherin and β-catenin in OSCC depended on the degree of tumour differentiation as reported by Mahomed et al. (2007), where well-differentiated tumours showed significantly higher expression of E-cadherin and β-catenin compared with the moderate and poor differentiated tumours. Kudo et al. (2004) have linked this finding due to methylation of the E-cadherin gene promoter region and to degradation of the membranous β-catenin protein. However, a positive E-cadherin expression was observed in the cell membrane of the normal oral mucosa epithelial tissues, and it was also detected in poor- and moderate-differentiation OSCCs with the highest expression seen in well-differentiation OSCC (Zhou et al., 2015). In the present study, the expression of E-cadherin in the induced cancer group showed an aberrant pattern with leaks of

membrane-bound E-cadherin and revealed a corresponding increase in cytoplasmic localisation as opposed to that in the *DC* 1000 mg/kg group for both chemopreventive and chemotherapeutic experiments. The result of the present study is in agreement with another study performed by Gao et al. (2005), who reported a mixture of cytoplasmic and membrane staining expression pattern of E-cadherin and β-catenin in the tumour cells. Therefore, the result of the present study indicated that *DC* 1000 mg/kg could restore the E-cadherin to the membranous layer, which in turn decreases the cell proliferation in the cancer cells and maintain normal epithelial polarity and inhibit invasion.

Drug resistance considered the main problem associated with Cisplatin application as well as toxic side effects, which lead to termination or limited therapeutic efficacy. To overcome these problems, combination therapy with other agents is recommended (Jo et al., 2016). For this reason, *DC* 1000 mg/kg was used as an adjunctive agent with Cisplatin to treat OSCC *in vivo*.

4.4.7 Gene Expression Analysis

The findings of RT^2 profiler PCR array in the present study showed a significant alteration in the genes; Bax, Bcl-2, Casp3, Tp53 and Cox-2, however, Cyclin D1 and EGFR genes showed an alteration but without significant difference between the induced cancer and *DC* 1000 mg/kg groups for both experiments (chemopreventive and chemotherapeutic). In addition, a significant difference showed in the induced cancer group compared to both Cisplatin and Cisplatin + *DC* 1000 mg/kg groups in the chemotherapeutic experiment.

Apoptosis is a strongly regulated pathway of cell death not only during development but also in adult multicellular organisms, in which it partially controls cell numbers. Apoptosis is characterised by cell shrinkage, chromatin condensation, and nuclear and cell fragmentation. These features result in the formation of apoptotic bodies that are then

engulfed by adjacent phagocytic cells (Cotter, 2009). Severe DNA damage, such as that found in precancerous lesions can induce apoptosis via activation of the DNA damage checkpoint pathway which can serve to remove potentially harmful DNA-damaged cells and thereby block cancer development (Plati et al., 2011).

The Bcl-2 proteins i.e. Anti-apoptotic proteins (such as Bcl-2 and Bcl-XL) and pro-apoptotic (such as Bad, Bax or Bid) are a family of proteins involved in the response to apoptosis. The sensitivity of cells to apoptotic stimuli can depend on the balance of pro- and anti-apoptotic Bcl-2 proteins. When there is an excess of pro-apoptotic proteins, the cells are more sensitive to apoptosis, while an excess of anti-apoptotic proteins make the cells tend to be more resistant (Youle & Strasser, 2008). In the present study, the results revealed that the *DC* 1000 mg/kg induced apoptosis associated with a significant up-regulation of Bax, activation of caspase 3, and the down-regulation of Bcl-2 in both chemopreventive and chemotherapeutic studies. This result is in agreement with a study conducted by Wang et al. (2014), who reported that the injection of Clotrimazole I.P at 150 mg/kg per day for two weeks markedly reduced Bcl-2 expression and increased the protein level of Bax in tumour tissues of xenograft mice model. Anti-apoptotic Bcl-2 family members function at least in part by inhibiting cytochrome c release from the mitochondria. They perform this task by preventing translocation and/or activation of Bax-like proteins on the mitochondria (Choi et al., 2005). This may explain the gene alterations of the present study where the induced cancer group showed with overexpression of Bcl-2 and down-expression of Bax genes.

When the ratio of Bax to Bcl-2 increases, the mitochondrial permeability transition pore opens and in turn results in releasing apoptogenic mitochondrial proteins to activate caspases and induce cell apoptosis (Tait & Green, 2013). This could be the reason, in the present study, the caspase 3 was overexpressed in *DC* 1000 mg/kg group compared to the

induced cancer group in both chemopreventive and chemotherapeutic studies. Recently, the effect of *DC* extract on H400 cells line was studied by Alabsi et al. (2016), and they found that the *DC* extract inhibited cells proliferation via apoptosis through activation of caspases 9 and 3/7 and demonstrated an up-regulation of Bax, Bad, and Bid as well as down-regulation of Bcl-2.

Clinically, patients with high Bax expression had better disease specific survival compared with those who had low Bax expression, while high Bcl-2 expression resulted in poorer disease specific survival for patients (Xie et al., 1999). In the current study, the expression of Bax and Casps3 genes were overexpressed in Cisplatin group and were more in the combination group of *DC* 1000 mg/kg + Cisplatin, whereas Bcl-2 was down-expressed with a greater reduction showed in the combination group. In another study that used combination therapy with Bax gene transfer and Cisplatin to increase the sensitivity of SCC to Cisplatin reported that a Bax gene administration to SCC by gene gun system has therapeutic applications for enhancing chemotherapy in SCC (Sugimoto et al., 1999). It has been reported that the combination of Ad-ING4-P53 and Cisplatin in an attempt to enhance chemosensitivity of hypopharyngeal cancer to Cisplatin, resulted in increased apoptosis induction through overexpression of Bax and down-expression of Bcl-2 (Ren et al., 2016). This could be the reason that the *DC* 1000 mg/kg enhanced the effect of Cisplatin during chemotherapy treatment of oral cancer.

The tumour suppressor gene p53 produced a p53 protein which functions in the G1-S phase of the cell cycle to allow repair of damaged DNA and to prevent the cell from entering S phase, or alternatively, in guiding the damaged cells to apoptosis (Patil et al., 2016). In its wild-type form, p53 is a major tumour suppressor whose function is critical for protection against cancer (Mercer, 1992). Inversely, the mutant p53 protein loses its tumour suppressor function and becomes a tumour-promoting factor and promotes the

process of the tumour (Rivlin et al., 2011). Moreover, mutation of the p53 gene precedes the process of carcinogenesis (Osugi, 1996; Strano et al., 2007). Therefore, the level of p53 in 4NQO-treated mouse cells was dramatically increased compared with the untreated control (Maltzman & Czyzyk, 1984). From a clinical view, a high positivity p53 in patients with tumours make them tended to have a poor prognosis when compared with those who had tumours with positivity low p53 (Xie et al., 1999). Another study conducted to assess the effect of Zinc supplementation on 4NQO-induced rat oral carcinogenesis, found that Zinc supplementation can decrease the expression of the tumour markers like p53, COX-2 and cyclin D1 compared to 4NQO-treated rats (Fong et al., 2011).

In consistent with previous studies, the result of the current study demonstrated an overexpression of Tp53 in the induced cancer (4NQO-treated rats) compared to *DC* 1000 mg/kg group in both chemopreventive and chemotherapeutic studies. Cisplatin also reduced the expression of Tp53, and this was further reduced when *DC* 1000 mg/kg used with the Cisplatin in *DC* 1000 mg/kg + Cisplatin combination group. It has been reported that in the xenograft mice model with p53 mutation a higher resistance to Cisplatin has been detected (Henriksson et al., 2006). So, from this point of view, the combination therapy with Cisplatin is necessary (Jo et al., 2016).

Overexpression of the COX-2 gene is associated with oral cancer and oral premalignant lesions and appears to be greater in high-risk oral lesions (Wang, 2005). This concept was confirmed in a study performed by Ribeiro et al. (2009), who concluded that the expression of COX-2 showed abnormally in the initial stage of oral carcinogenesis and up-regulated with transformation to the oral malignant stage. In another study, the mean expression of COX-2 in OSCC was 15.9 ± 6.7-fold higher than that in adjacent phenotypically normal oral tissues harvested from the same animal

(McCormick et al., 2010). In the present study, the expression of COX-2 was decreased when the rats treated with *DC* extract compared to the induced cancer group in both studies (chemopreventive and chemotherapeutic). This result was in agreement with a study that conducted to evaluate the inhibitory effect of apple extract in which the groups that treated with apple extract showed decreased COX-2 expression when compared with the induced cancer (4NQO-treated) group (Ribeiro et al., 2014). For the chemotherapeutic study, the Cisplatin and combination of Cisplatin + *DC* 1000 mg/kg groups presented with down-expression of COX-2 and the highest inhibition exhibited in the combination group.

Cyclin D1 (CCND1) located on chromosome 11q13 is a positive regulator of the cell cycle (Huang et al., 2012). Previous studies have approved the overexpression of cyclin D1 (CCND1) in OSCC and shortened overall survival (Kaminagakura et al., 2011; Michalides et al., 1995). In 2003, Yoshida and his colleagues investigated the inhibitory effect of troglitazone on 4NQO-rat tongue carcinogenesis and found that the supplementation of troglitazone in the diet reduced expression of CCND1 after 4NQO administration (Yoshida et al., 2003). Moreover, a study performed by Wilkey et al. (2009) reported that CCND1 overexpression increases susceptibility to 4NQO-induced oral tumorigenesis in mice.

Similarly, Naoi et al. (2010) found that Nimesulide (a selective COX2 inhibitor) significantly reduced the expression of CCND1 and COX2 in 4NQO-induce tongue tumours of rats using RT-PCR analysis. In the same manner, the present studies (chemopreventive and chemotherapeutic) showed that the *DC* 1000 mg/kg reduced the expression of CCND1 compared to the induced cancer group, however, no significant difference was detected. The overexpression of cyclin D1 is usually associated with resistance to chemotherapy and radiotherapy in several types of cancer including SCC of

head and neck (Kothari & Mulherkar, 2012). This could explain the result of the present study where the expression of CCND1 in the Cisplatin group was a bit higher than that in both *DC* 1000 mg/kg and combination groups. Consequently, in another study, an attempt to increase the Cisplatin sensitivity by inhibition of Cyclin D1 via RNAi (shRNA) against cyclin D1 has been done, and this demonstrate the potential of combining vector-based cyclin D1 silencing with chemotherapy to achieve maximum tumour regression. (Kothari & Mulherkar, 2012). In the present study, the combination of *DC* 1000 mg/kg with Cisplatin resulted in a significant inhibition of cyclin D1 which may increase the sensitivity of the chemotherapy.

Epidermal growth factor receptor (EGFR) is a critical early event in HNSCC and is overexpressed in > 80% of head and neck SCC (Benchekroun et al., 2010). It is a member of the ErbB family of receptors, and its overexpression has been associated with some cancers, including lung cancer, anal cancers and glioblastoma multiforme. A blockade of EGFR may lead the cancer cells to enter apoptosis (Khan et al., 2006). Therefore, inhibition of EGFR eliminates the invasive potential of the cancer cells (Singh et al., 2011). Topical application of polyethylene glycol-8000 by painting the oral cavity of the rat using a sable brush (#4) for up to 3–4 minutes significantly lowered the intensity as well as the number of areas overexpressing EGFR in 4NQO-treated rats where baseline EGFR expression was much higher than the tongue/oral mucosa of healthy control rats (Wali et al., 2012). In the present study, the expression of EGFR was reduced in *DC* 1000 mg/kg compared to the induced cancer groups in both chemopreventive and chemotherapeutic studies. In the chemotherapeutic study, the Cisplatin group exhibited an inhibition rate but a bit higher than *DC* 1000 mg/kg and this could be attributed to the chemotherapy resistance. A study performed by (Hiraishi et al., 2008) suggested that the changes in EGFR expression play a significant role in controlling the drug-resistant

phenotype of cells in response to Cisplatin. It is therefore suggested that the *DC* 1000 mg/kg could be used as an adjunctive agent with Cisplatin to treat OSCC.

Generally, in the present study, the results suggest that *DC* 1000 mg/kg combined with Cisplatin treatment can indeed enhance chemotherapy in OSCC by regulation of various inflammatory and proliferative genes.

4.5 Conclusion

The present study reported that *DC* extract especially at doses of *DC* 500 mg/kg and *DC* 1000 mg/kg both confer statistically significant protection against carcinogenesis in a well-studied rat model for OSCC. Chemopreventive and chemotherapeutic efficacy were expressed as a significant increase in the food consumption, body weight gain, regulation the haematological and biochemical parameters, reduction in oral cancer-related mortality and reduction in oral cancer incidence. The combination of *DC* extract and Cisplatin may be useful as a rational strategy for the treatment of patients with oral cancer.

CHAPTER 5: CONCLUSION

5.1 Introduction

The current study provided an insight into the toxicity profile of *DC* resin methanol extract on the food consumption, water intake, body weight, organ weight, haematological parameters, biochemical parameters and histopathological of the vital organs (liver, kidney, heart, spleen and lung) in SD rats by using acute and subacute oral toxicity tests. In addition, the anticancer activity of *DC* resin methanol extract in the 4NQO-induce oral tongue cancer animal model in both chemopreventive and chemotherapeutic studies by assessing food consumption, water intake, body weight, survival rate, tumour tongue volume and the incidence of OSCC in SD rats. Furthermore, the metastasis obstruction of *DC* resin methanol extract in the chemotherapeutic study by assessing the changed in regional cervical lymph node histopathologically.

5.2 Summary of findings

Results from the present study provided clear evidence into the following:

1. *DC* resin methanol extract did not cause any lethality or produce any treatment-related changes in haematological and serum biochemical parameters or important histopathological signs.
2. *DC* resin methanol extract could be well tolerated up to the dose 2000 mg/kg body weight and could be classified as Category 5.
3. *DC* resin methanol extract is well tolerated up to the dose of 1500 mg/kg body weight administered daily for up to 28 days.
4. *DC* resin methanol extract increased water intake in SD rats compared to the control group. This result could be referred to the ability of *DC* to produce vasodilatation (hypotension) due to relaxation of smooth muscles of blood vessels which in turn stimulated thirst and increased water intake.

5. The present investigation demonstrates, at least in part, the safety of *DC* resin methanol extract suggesting its promising potential for pharmaceutical uses.
6. *DC* resin methanol extract confer statistically significant protection against carcinogenesis in a well-studied rat model for OSCC as a marked increase in the food consumption, body weight gain, regulation the haematological and biochemical parameters, reduction in oral cancer-related mortality and reduction in oral cancer incidence especially at doses of *DC* 500 mg/kg and *DC* 1000 mg/kg.
7. *DC* resin methanol extract at dose 1000 mg/kg reduced the incidence of OSCC into 0 % when used in combination with the commercially used anticancer drug (Cisplatin).
8. *DC* 1000 mg/kg inhibited the proliferation of the cancer cells, maintained epithelial polarity and discouraged the aggressiveness toward OSCC by decreasing the expression of Cyclin D1, Ki-67, Bcl-2 and p53, and slightly increasing the expression of β-catenin and E-cadherin.
9. *DC* 1000 mg/kg induced apoptosis by upregulation of Bax and Casp3 genes, and down-regulation of Tp53, Bcl-2, Cox-2, Cyclin D1 and EGFR genes compared to the induced cancer group.
10. *DC* resin methanol extract can be used as an add-on therapy with Cisplatin for treatment of oral cancer and control Cisplatin toxicity, thus could increase the quality of life of cancer patients.

Taken all together, the results highlighted that the *Dracaena cinnabari* is safe to be used systemically via oral administration and possesses anticarcinogenic potency on the oral carcinogenesis and has the potential to be developed as an anticancer agent for oral cancer. This makes *Dracaena cinnabari* a promising candidate for further investigation as a natural chemopreventive and/or chemotherapeutic agent.

5.3 Recommendation for future study

Though oral administration of *DC* resin methanol extract may potentially represent a significant advance in chemopreventive and chemotherapy of OSCC, some questions remain to be answered. Future studies will need to assess the followings:

1. Identification of pure compound(s) and chemical characterization of the cytotoxically active compounds of Dracaena cinnabari.
2. We chose a maximum dose of *DC* resin methanol extract 1000 mg/kg orally administered for chemopreventive and chemotherapeutic studies based on data from acute and subacute oral toxicity tests. However, this will need to be increased to elucidate the most effective dose to treat OSCC.
3. While our studies with 4NQO performed at initiation and post-initiation stages, studies of the therapeutic effect of *Dracaena cinnabari* with other models of OSCC (transgenic and orthotopic xenograft models) are helpful.
4. It is useful to determine dosing and timing for introduction of *Dracaena cinnabari* treatment for achieving maximal effects depending on the stage of oral cancer.
5. An animal model that can produce a high incidence of cancer metastasis is necessary to verify the metastasis obstruction effect of Dracaena cinnabari.
6. Development a topically applied formulation of *Dracaena cinnabari* (via toothpaste, oral rinse, lozenge, chewing gum, etc.) may potentially represent a significant advance in oral cancer prevention strategy.

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