ANTICANCER ACTIVITY OF GONIOTHALAMIN ON ORAL CANCER CELLS *IN VITRO* AND *IN VIVO*

FAHME ABDUL KALK SAEED KAID

FACULTY OF DENTISTRY
UNIVERSITY OF MALAYA
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FAHME ABDUL KALK SAEED KAID

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ANTICANCER ACTIVITY OF GONIOTHALAMIN ON ORAL CANCER CELLS *IN VITRO* AND *IN VIVO*

ABSTRACT

Goniothalamin (GTN) is one of styryl-lactons naturally available compounds isolated from Goniothalamus macro-phyllus. It shows a selective cytotoxicity against variety of cancer cell lines with no significant cytotoxicity toward non-malignant cells. The aim of this study was to evaluate the activity of GTN against oral cancer cells *in vitro* and *in vivo*. **Materials and Methods**: *in vitro* part of study, anti-proliferative effect and cytotoxic screening of GTN was conducted on H400 oral cancer cells using MTT assay. The mechanism of apoptosis was studied by mitochondrial membrane potential, cytochrome c detection and caspase-3/7, -8 and -9 assays. The blockage of apoptosis and cell proliferation was investigated using NF-kb assay. As for the in vivo part of study, the haematological, biochemical and histoathological evaluation of GTN was investigated on selected organs (kidney, liver, lung, heart, spleen and brain) of Sprague Dawley (SD) rats using Acute and sub-acute toxicity tests. The anticancer activity of GTN was evaluated using 4NQO-induced oral cancer rat model at different doses of GTN; 25, 50, 100, 150 mg/kg. The carcinogenic agent administered intraperitoneally into rats in drinking water for 12 weeks and the GTN treatment started at the 13th week for 10 weeks. At the end of the experiments the animals were anesthetized and then blood samples were collected for haematological and biochemical analysis. And then autopsy was carried out for all rats and tongue of rats were harvested for gene expression, histopathological and immunohistochemistry analysis. **Result:** As for *in vitro* study, MTT assay findings showed that GTN exhibited selective cytotoxicity and inhibited the growth of H400 oral cancer cells via apoptosis in dose and time-dependent manner. Mitochondrial membrane potential assay revealed the release of mitochondrial cytochrome c into cytosol. Caspases assays revealed the activation of initiator caspase-9 and executioner caspase-3/7 in dose-

dependent manners. This form of apoptosis was closely associated with the inhibition of NF-kb translocation from cytoplasm to nucleus. As for *in vivo* study, the acute toxicity revealed that the GTN lethal dose was 420 mg/kg and the LD_{50} was 42 mg/kg. The sub-acute test revealed no evidence of any treatment-related changes of the animals used in this study. As for the anticancer activity of GTN on rat oral cancer, the finding showed no significant differences between GTN different doses. In the GTN-treated animal, the tumor size was reduced comparing to the untreated animals. The findings showed that GTN inhibit the expression of Cyclin D1, Ki-67, Bcl-2 and p53 genes while a slight increase in the expression of β-catenin and E-cadherin genes that was observed. The findings also revealed that GTN induces apoptosis by upregulation of Bax and Casp3 genes and down-regulation of Tp53, Bcl-2, Cox-2, Cyclin D1 and EGFR. GTN and Cisplatin combination showed better results in inhibiting oral cancer than GTN or Cisplatin alone. **Conclusion:** This study provides scientific validation for the safety of GTN up to the highest dose level used in this study. The findings of current study indicated that GTN has the potential to act as an anticancer agent against oral cancer.

Key words: Oral cancer, Goniothalamin, Apoptosis, Toxicity, Chemotherapeutic.

AKTIVITI ANTIKANSER GONIOTHALAMIN PADA SEL KANSER MULUT *DALAM VITRO* DAN *DALAM VIVO*

ABSTRAK

Goniothalamin (GTN) adalah sejenis sebatian yang boleh didapati secara semula jadi dari Goniothalamus macro-phyllus. Ia menunjukkan sitotoksisiti selektif terhadap pelbagai barisan sel kanser tanpa sitotoksis yang ketara terhadap sel-sel tidak malignan. Tujuan kajian ini adalah untuk menilai aktiviti GTN terhadap sel-sel kanser oral dalam *vitro* dan *vivo*. Bahan dan Kaedah: bahagian in vitro kajian, kesan anti-proliferatif dan penapisan sitotoksik GTN telah dijalankan ke atas sel-sel kanser mulut H400 menggunakan ujian MTT. Mekanisme apoptosis dikaji oleh potensi membran mitokondria, pengesanan cytochrome c dan caspase-3/7, -8 dan -9 assays. Blok apoptosis dan proliferasi sel telah disiasat menggunakan ujian NF-kb. Bagi bahagian dalam vivo kajian, penilaian Hematologi, biokimia dan histoathologi GTN disiasat pada organ terpilih (buah pinggang, hati, paru-paru, jantung, limpa dan otak) tikus Sprague Dawley (SD) yang menggunakan ketoksikan akut dan sub-akut ujian. Aktiviti antikanser GTN dinilai menggunakan model tikus mulut yang disebabkan oleh 4NQO pada dosis yang berlainan GTN; 25, 50, 100, 150 mg / kg. Ejen karsinogenik diberikan secara intraperitoneally ke dalam tikus dalam air minum selama 12 minggu dan rawatan GTN bermula pada minggu ke-13 selama 10 minggu. Pada akhir eksperimen haiwan itu dilumpuhkan dan kemudian sampel darah dikumpulkan untuk analisis hematologi dan biokimia. Dan kemudian bedah siasat dilakukan untuk semua tikus dan lidah tikus dituai untuk ekspresi gen, analisis histopatologi dan immunohistokimia. Hasilnya: Bagi kajian in vitro, penemuan MTT assay menunjukkan bahawa GTN mempamerkan sitotoksisiti selektif dan pertumbuhan sel H400 yang menghalangi melalui apoptosis dalam dos dan masa yang bergantung kepada masa. Ujian potensi membran mitokondria mendedahkan pembebasan cytochrome mitokondria c ke dalam sitosol. Pemeriksaan caspases

mendedahkan pengaktifan caspase-9 pemula dan caspase-3/7 dalam sikap yang bergantung kepada dos. Bentuk apoptosis ini bekaitk rapat dengan perencatan transplantasi NF-kb dari sitoplasma kepada nukleus. Bagi kajian vivo, ketoksikan akut menunjukkan bahawa dos mematikan GTN adalah 420 mg / kg dan LD_{50} adalah 42 mg / gk. Ujian sub-akut tidak menunjukkan sebarang bukti mengenai perubahan berkaitan dengan haiwan yang digunakan dalam kajian ini. Bagi aktiviti antikanser GTN pada kanser mulut tikus, penemuan ini tidak menunjukkan perbezaan yang signifikan antara GTN dos yang berbeza. Dalam haiwan yang diobati GTN, saiz tumor dikurangkan berbanding haiwan yang tidak dirawat. Penemuan menunjukkan GTN menghalang ekspresi gen Cyclin D1, Ki-67, Bcl-2 dan p53 sementara peningkatan sedikit dalam ekspresi gen β-katenin dan E-cadherin yang diperhatikan. Penemuan ini juga mendedahkan bahawa GTN menginduksi apoptosis dengan menaikkan gen Bax dan Casp3 dan turun peraturan Tp53, Bcl-2, Cox-2, Cyclin D1 dan EGFR. Kombinasi GTN dan Cisplatin menunjukkan hasil yang lebih baik dalam menghalang kanser mulut daripada GTN atau Cisplatin sahaja. Kesimpulan: Kajian ini menyediakan pengesahan saintifik untuk keselamatan GTN hingga tahap dos tertinggi yang digunakan dalam kajian ini. Penemuan kajian semasa menunjukkan bahawa GTN berpotensi untuk bertindak sebagai agen antikanser terhadap kanser mulut.

Kata kunci: Kanser mulut, Goniothalamin, Apoptosis, Ketoksikan, Kemoterapeutik.

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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
F	:	Female
g	:	Gram
G0 phase	:	Quiescence phase
G1 phase	:	Gap 1 phase
G2 phase	:	Gap 2 phase
GTN	:	Goniothalamin
H&E	:	Haematoxylin and eosin
Hb	:	Haemoglobin
IHC	:	Immunohistochemical
M phase	:	mitotic phase
mg/kg	:	Milligram per kilogram
nm	:	Nanometre
OSCC	:	Oral Squamous Cell Carcinoma
$P < 0.05$	:	p value less than 0.05
RNA	:	Ribonucleic acid
S phase	:	Synthesis phase
SD	:	Sprague-Dawley
WBC	:	White blood cell

CHAPTER 1: INTRODUCTION

1.1 Introduction

Cancer, an abnormal or out-of-control cell growth with the possibility to invade or spread to other parts of the body, is one of the most common causes of death in developed and developing countries. Globally, in 2012, there were about 14.1 million new cancer cases and 8.2 million cancer-related deaths being reported, compared to the previously reported figures of 12.7 million and 7.6 million for both categories in 2008 (Ferlay et al., 2014). Cancer is generally increasing in non-industrialized regions due to population aging and growth as well as an adoption of cancer-related lifestyle choices. It is estimated that about 43% of cancer-related deaths are associated with tobacco use, alcohol consumption, unhealthy diets, physical inactivity and infection (Jemal et al., 2011; Petersen, 2009). So far, over one hundred types of cancer have been recognized and the classification of cancer will depend according to the type of affected tissue or cells (Ferlay et al., 2014).

Head and neck cancer is an abnormal growth in any parts of the mouth or the oral cavity like lips, gum, and tongue and is considered the third most common cancer in the developing countries and the sixth most common cancer worldwide (Ferlay et al., 2010; Ogbureke & Bingham, 2012). Oral cancer is a major public health problem and has been considered as one of the most highly prevalent cancers with 300,373 new cancer cases worldwide. More than half of the world cases of this disease occur in Asia alone with about 168,850 new cases and about 11% of these cases were diagnosed in South East Asia (Cheong et al., 2017).

In Malaysia, oral cancer incidence was about 776 with and the mortality was 253 in 2012 (Cheong et al., 2017). According to the World Health Organization (WHO), oral cancer causes 1.55% of the total deaths in Malaysia (Zainal Ariffin & Nor Saleha, 2011).

In 2007, the National Cancer Registry (NCR) revealed that oral cancer ranks the second (13.2%) of the top five leading cancers in Malaysia (Zainal Ariffin & Nor Saleha, 2011).

In the last few decades, great efforts have been done to improve the chemotherapy, radiotherapy and surgical therapy. However, cancer treatment using these therapies techniques are still not sufficiently effective to reduce the 5-year survival rate and the high incidence of oral cancer (Moongkarndi et al., 2004). Moreover, large tissue defects can occur as a result of the radical removal of the cancer and its surrounding tissues. Radiation therapy of squamous cell carcinoma of oral and pharyngeal regions can result in side effects such as oral mucositis or xerostomia (Silverman Jr, 2007). A previous study has reported that there is no intervention that can treat or prevent oral mucositis on its own and the strategies that can reduce oral mucositis is still unclear as there is not enough evidence pertaining to a treatment that is efficient and superior when compared to other existing treatments for this condition (Rodriguez-Caballero et al., 2012). Recently, researchers in the field of cancer have been focusing on identifying new anticancer agents isolated from medicinal plants that can be used as chemotherapeutic or chemopreventive agents and many novel anticancer agents have been discovered but the side effects and toxicity are still intolerable. Therefore, new anticancer agents that can preserve tissues and organs with low side effects are needed.

Oral cancer research development has involved many animal models such as rats, mice, and hamsters which have been utilized to study and evaluate many natural and synthetic anticancer agents and their chemotherapeutic potential (Suzuki et al., 2005). There are several carcinogenic agents like coal tar, 20 methylcholanthrene (20MC), 9,10-dimethyl-1,2-benzanthracene (DMBA) and 4-nitroquinoline-1-oxide (4NQO) that have been used in experimental oral carcinogenesis. However, 4NQO is the preferred carcinogen in the development of experimental oral carcinogenesis (Kanojia & Vaidya,

2006). 4NQO is a water-soluble carcinogen, which encourages tumor development mainly in the oral cavity. It produces all stages of oral carcinogenesis like hyperplasia, dysplasia, severe dysplasia, *carcinoma in-situ* and squamous cell carcinoma. In addition, there have been a number of evidence to suggest that the histological, as well as molecular changes, induced by 4NQO, is similar to that observed in the human oral cancer (Kanojia & Vaidya, 2006).

Goniothalamin (GTN) is a novel styryl lactone derivative compound with putative anti-cancer properties isolated from Goniothalamus species which are distributed in many parts of Malaysia (Al-Qubaisi et al., 2011; Chen et al., 2005). Previous studies revealed that GTN has cytotoxic activity and induces apoptosis in various cancer cell lines; such as cervical (HeLa), gastric (HGC-27), kidney (768- 0), breast carcinomas (MCF-7, T47D and MDA-MB-231) and leukemia (HL-60, Jurkat and CEM-SS) (Alabsi et al., 2012; Inayat-Hussain et al., 2010; Rajab et al., 2005). As compared with tamoxifen (breast cancer drug) or taxol (anticancer drug) treated cells, GTN has shown cytotoxicity against ovarian cancer cell lines (Caov-3) and there was no cell death in normal kidney cells (MDBK) as the case with tamoxifen or taxol (Lin & Pihie, 2003). Lower toxicity to normal Chang liver cell line has been reported when compared with the chemotherapeutic drug doxorubicin (Al-Qubaisi et al., 2011). A previous study investigated the effect of GTN on the development of Ehrlich solid tumor in mice reported that GTN has anti-inflammatory and antiproliferative activity and its potential as a therapeutic agent has been confirmed (Vendramini-Costa et al., 2010). Cytotoxicity of GTN in human leukemia (HL-60 and Jurkat), human breast carcinoma (MDA-MB-231) and cervical (HeLa) occurs via apoptosis after treated with GTN (Alabsi et al., 2012; Chen et al., 2005; Inayat-Hussain et al., 2010). GTN induces apoptosis in which oxidative stress and mitochondria-mediated pathway involve in its mechanism of action (Chan et al., 2010; Inayat-Hussain et al., 1999). Moreover, GTN induces DNA damage, generation of reactive oxygen

species (ROS), loss of mitochondria membrane potential, the release of cytochrome c and activation of caspases (Chen et al., 2005; Inayat-Hussain et al., 2003; Rajab et al., 2005). However, data from previous studies is still insufficient and less convincing because most are done *in vitro*. Hence, *in vivo* studies using an animal model or human body are still needed. Moreover, there has been no *in vitro* or *in vivo* study conducted to investigate the anticancer activity of GTN in oral cancer. Thus, more *in vitro* and *in vivo* studies using various experimental oral cancer cell lines and animal models are needed to determine the anticancer activity of GTN in oral cancer.

1.2 Study Justification

Head and neck cancer, which include oral cancer, is classified as the sixth most common type of reported cancer globally (Ferlay et al., 2010). Chemotherapy is one of the most broadly studied methods in the context of anticancer therapies, however, its side effects and toxicity is quite well-known, which somewhat diminishes its effectiveness (Newman et al., 2003). Several natural plant compounds with a potent cytotoxic effect have been used as anticancer drugs. GTN, is a natural plant-derived compound, has been reported as an antitumor agent; an apoptosis inducer, antiproliferative and cytotoxic agent against a variety of cancer cell lines including: vascular smooth muscle cells (VSMCs), Chinese hamster ovary cells, renal cells, hepatoblastoma, gastric, kidney cells, breast carcinomas, leukemia, Jurkat cells, hepatocellular carcinoma, lung cancer cells and HeLa cells (Al-Qubaisi et al., 2011; Alabsi et al., 2012; Orlikova et al., 2013; Yen et al., 2012). Previous studies revealed a possible selectivity of GTN against tumor cell lines with no confirmed activity against normal cells, which made a great interest in GTN in the last two decades (Azimahtol Hawariah et al., 1998; Orlikova et al., 2013; Tian et al., 2006; Wattanapiromsakul et al., 2005). However, the available data on GTN anticancer activity is insufficient and less convincing due to its limitation as most of the experiments are done *in vitro*. Moreover, there are no *in vitro* studies investigated the anticancer activity

of GTN in oral cancer cell lines have been carried out yet and there are no *in vivo* studies that have been done with GTN in 4NQO oral cancer induced rat model. Thus, *in vitro* and *in vivo* studies using various experimental oral cancer cell lines and oral cancer animal models to investigate the anticancer activity of GTN are needed.

1.3 Hypothesis

GTN has anticancer activity against oral cancer and could be further developed as chemotherapeutic drugs for head and neck cancer.

1.4 Objectives

1.4.1 The general objective of the study

To investigates the anticancer activity of GTN on oral cancer *in vitro* and *in vivo.*

1.4.2 Specific Objectives of the study

1. To investigate the anticancer activity of GTN on oral cancer and normal cell lines.
2. To study the mechanism of apoptosis induced by GTN in oral cancer cells.
3. To investigate the histological biochemical and hematological changes in selective internal organs of rats due to GTN compound.
4. To evaluate the chemotherapeutic effect of GTN at different doses on oral cancer *in vivo* using oral cancer animal model.
5. to investigate the effects of GTN on gene expression profiles in 4NQO-induced oral cancer model using RT2 profile PCR array and immunohistochemistry.

CHAPTER 2: LITERATURE REVIEW

2.1 Cancer

Cancer is a multifactorial disease that consists of the alteration of cellular processes that lead to the uncontrolled proliferation of a certain group of cells. In many cases, these cells are able to migrate and colonize other organs, establishing and generating an often-irreversible damage in the tissue, and may even cause the death of the individual (Stewart & Wild, 2017). These alterations are produced by genetic and epigenetic factors that generate a dysregulation of the cell cycle, giving rise to cells with an increased proliferative potential (Hanahan & Weinberg, 2011). There are eight biological capabilities linked to cancer via multi-stage growth of tumors, which are maintaining proliferative signaling, evading growth suppressors, resisting cell death (apoptosis), enabling replicative immortality, causing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction (Hanahan & Weinberg, 2011). Cancer represents one of the major global cause of death and fatalities (Stewart & Wild, 2017). According to the world cancer report by IARC, GLOBOCAN (2012), cancer is the second leading cause of death worldwide after cardiovascular diseases, with a mortality rate of 113 per 100,000 inhabitants. Approximately 14.1 million new cases each year, 8.2 million cancer deaths and an estimated 32 million people are living with the disease (Ferlay et al., 2014). The most common cancers in men are lung, prostate, and colorectal cancers, whereas in women they are the breast, colorectal, and cervix (Ferlay et al., 2014). Based on the Malaysian Ministry of Health, a total of 18,219 new cancer cases were diagnosed in Malaysia in 2007. It comprises of 8,123 (44.6%) males and 10,096 (55.4%) females. The five most common cancers in Malaysia in 2007 include breast, colorectal, lung, nasopharynx and cervix (Zainal Ariffin & Nor Saleha, 2011)

Some of the major causes of cancer globally include tobacco use, alcoholic beverages consumption, virus, unhealthy dietary habits and physical inactivity. Furthermore, some chronic infections represent cancerous risk factors and are quite prevalent in developing countries (Stewart & Wild, 2017).

2.1.1 Cancer biology

Cancer begins with epigenetic and genetic alterations, such as amplification, chromosomal translocations or point mutations, both in the coding and regulatory regions of certain genes. These genes correspond to two broad groups: oncogenes and tumor suppressors. Oncogenes promote cell proliferation and/or inhibit apoptosis, such as Ras, c-Myc, cyclin-D1, b-catenin and survivin. Tumor suppressors are those that slow down proliferation and/or promote apoptosis, such as APC, p53, and E-cadherin (Hanahan & Weinberg, 2011; Lee & Muller, 2010). In cancer, oncogenes are characterized by an increase in function and tumor suppressors due to loss of function. These characteristics may be caused by changes in protein activity, conformational changes, variations in their levels and alterations in subcellular localization (Lee & Muller, 2010). There is also another set of genes that encode DNA repair machinery proteins, which being defective can increase the rate of accumulation of mutations as cell divisions occur, making the cell even more susceptible to inherit and generate genetic alterations. All of these alterations can result in a cell with high genetic instability, causing a deregulation of the cell cycle, giving rise to cells with an increased proliferative potential (Lee & Muller, 2010). As the tumor develops, cells may acquire a new phenotype with characteristics such as growth signal self-sufficiency, resistance to growth inhibitory signals, unlimited replicative potential, apoptosis evasion ability, sustained angiogenesis, deregulation of cellular energy, immune system evasion and the ability to invade other tissues (Hanahan & Weinberg, 2011).

2.1.2 Carcinogenesis

Carcinogenesis is the process by which cancer is generated (Robbins et al., 2005). There are a number of agents capable of causing genetic damage and induce the transformation of normal cells to malignant neoplastic cells. The carcinogenic chemicals that are involved in the development of human tumors have been identified. The most important carcinogenic chemicals are polycyclic hydrocarbons that are present in cigarette smoke, aromatic amines that are present in industrial products such as rubber and dyes, nitroamides which are nitrites and nitrates of normal diet transformed by intestinal bacteria, and alkylating agents which bind directly to DNA and can induce mutations (Stevens et al., 2008).

The current knowledge of the process of cancer development comprises more than half a century of research. Evidence for promoting chemical carcinogenesis initially provided by Berenblum in 1947 on a model of skin cancer in rats, where croton oil and benzopyrene was used. These investigations, as well as a number of others, are responsible in the definition of many important aspects of initiation and promotion of carcinogenesis (Slaga, 1983). The induction of genetic alterations of chemical carcinogenesis involves three stages; initiation, promotion, and progression (Figure 2.1).

Initiation**:** The response to the exposure of cells to sufficient doses of carcinogen (initiator); a cell-initiated is altered, making it potentially capable of giving rise to a tumor. However, initiation, by itself, it is not sufficient for tumor formation. Initiation produces permanent damage of DNA (mutations) therefore it is irreversible and has memory (Robbins et al., 2005). The nature of the initial changes of cells is still unknown in many human cancers and few genetic mutations in carcinogenesis models have been recognized (Stevens et al., 2008).

Promotion**:** This phase is mediated by so-called promoters which are agents that can induce tumors in cells initiated. However, these agents themselves are not tumorigenic and can act on cells initiated until several months after the application of the initiator. Unlike the effects of the initiators, the cellular changes resulting from the application of promoters does not directly affect DNA and are reversible. For example, they can increase the proliferation of initiated cells which is an effect that can contribute to the development of additional mutations (Robbins et al., 2005). Promotion is reversible in early stages if the promoter agent is removed (Stevens et al., 2008).

Progression**:** If cell proliferation persists, new initiated cells acquire genetic alterations in oncogenes that result in dysregulation of growth and sometimes in autonomous growth. The endpoint of this progression is the development of the invasive tumor. Although many agents are initiators or promoters, some behave like both, what defines them as a complete carcinogen (Stevens et al., 2008).

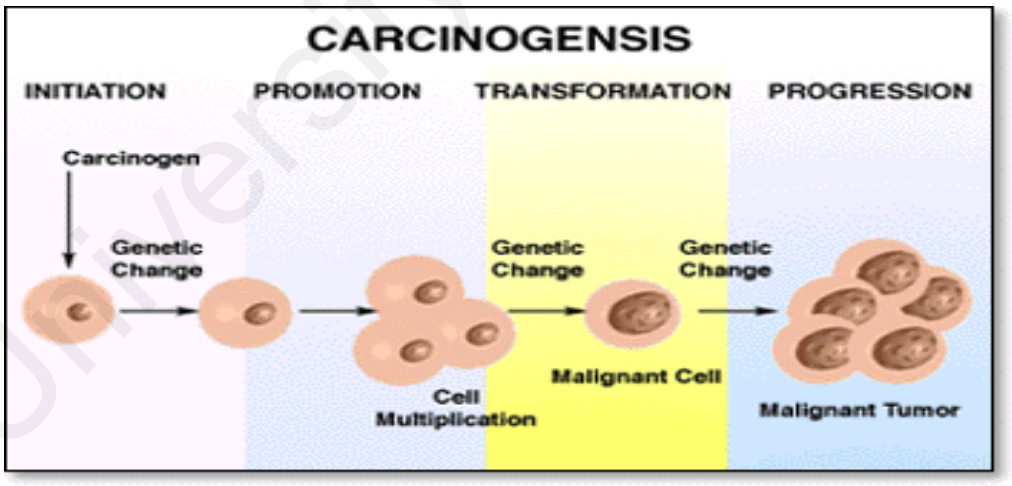


Figure 2.1: Step in carcinogenesis (Oliveira et al., 2007).

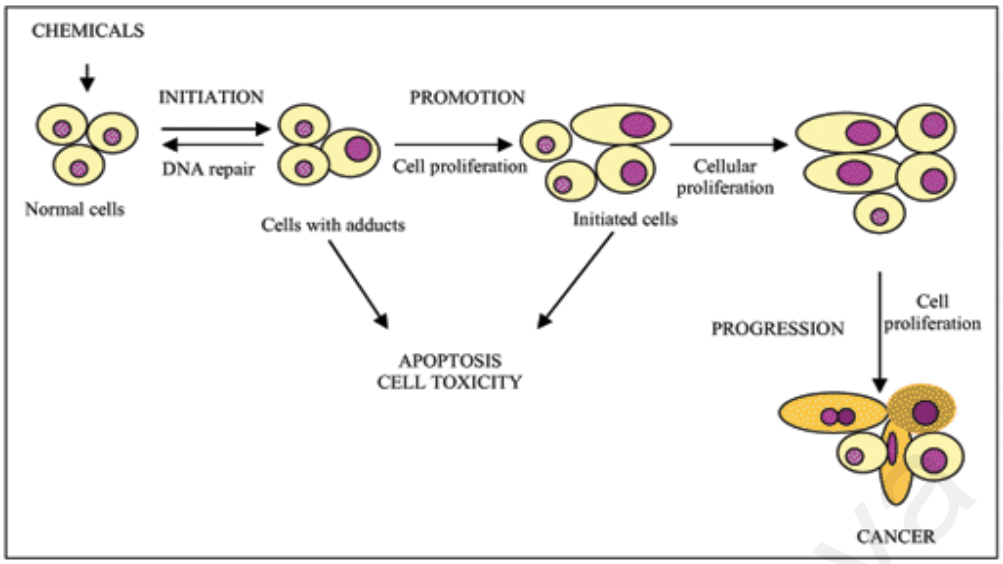


Figure 2. 2: Step in carcinogenesis (Oliveira et al., 2007).

2.2 Oral Cancer

2.2.1 Definition

Oral cancer is defined as a malignancy derived from the stratified squamous epithelium of the oral mucosa, high invasiveness, low survival and poor prognosis (Tumuluri et al., 2002; Vairaktaris et al., 2008). The most important risk factors that are related to the pathogenesis of oral cancer are smoking and alcohol (Wilkey et al., 2009), in addition to the habit of chewing betel (Neville et al., 2009). Oral cancer is more common in the lower lip, the lateral side of the tongue and the floor of the mouth. Its incidence increases with age, most cases occurring after the age of 40 years (Neville et al., 2009).

2.2.2 Oral Potentially Malignant Disorders

Histopathological examination of the affected squamous epithelium may reveal a spectrum of changes, ranging from squamous cell hyperplasia, through mild, moderate, and severe epithelial dysplasia to *carcinoma in-situ* (Table 2.1).

Although 2005 WHO classification is widely recognized and applied by both clinicians and histopathologists, there is continuing debate as to how precancerous epithelial changes should be conceptualized and classified. Two alternative classification systems are currently recognized by the WHO (Barnes, 2005). In addition to these, a binary classification system has also been proposed (Kujan et al., 2006)). The binary grading system complements the WHO Classification by excluding the intermediate category of 'moderate epithelial dysplasia', which confounds inter-observer agreement. The histopathologist assigns a grade according to the overall perceived 'risk' of the squamous epithelium. The binary system may, therefore, guide clinicians and facilitate critical management decisions (Kujan et al., 2006).

The debate surrounding the classification of epithelial dysplasia highlights the subjective nature of interpreting the histological features of precancerous lesions and assigning them to precise diagnostic categories. It is recognized that this interpretative process is liable to both intra- and inter-observer variation (Kujan et al., 2007). So far, no histological features have been identified that accurately predict which oral potentially malignant disorders will progress to oral squamous cell carcinoma (Lodi et al., 2006). There is, therefore, a need to develop biomarkers that enhance prognostication and direct treatment of oral cancer (Mishra, 2012).

Table 2. 1: classification systems for oral epithelial dysplasia

Classification of WHO	Intra-epithelial Neoplasia Classification	Ljubljana Classification of squamous Intra-epithelial lesions	Binary Classification
Hyperplasia	-	hyperplasia	-
Mild dysplasia	SIN 1	Basal/parabasal cell hyperplasia	Low-grade dysplasia
Moderate dysplasia	SIN 2	Atypical hyperplasia	Low-grade OR high-grade dysplasia
Severe dysplasia	SIN 3	Atypical hyperplasia	High-grade dysplasia
Carcinoma-in-situ	SIN 3	Carcinoma-in-situ	High-grade dysplasia

2.2.3 Epidemiology of Oral Cancer

According to global statistics (GLOBOCAN 2012), there were estimated 300,400 new cases and 145,400 deaths from oral cancer occurring in 2012 around the world. There is an estimated annual incidence of 275,000 cases, of which most occur in developing countries (Cheong et al., 2017; Nagini et al., 2009).

In Malaysia, oral cancer is one of the top 20 most common types of cancer. Based on data from the National Cancer Registry, there is one new case being diagnosed daily. The practices of tobacco use and habitual betel quid chewing are believed to be the most important factors for the high burden of oral cancer in the South-East Asian Region. According to the WHO (World Health Organization) data published in April 2011, oral cancer deaths in Malaysia reached 1587 or 1.55% of total deaths (Zainal Ariffin & Nor Saleha, 2011). The incidence of oral cancer is predominant among the Indian ethnic group where mouth and tongue cancers were among the 10 most common cancer among both male and female. The incidence of oral cancer is highest in Indian females where the ASR

was 10.2/100,000 female populations. Of those cases reported with staging, only 35.4% of the cases were diagnosed at stage I and II. When detected early, oral cancer is almost always cured. But unfortunately, so many people still present it at such a late stage (Zainal Ariffin & Nor Saleha, 2011).

2.2.4 Etiology of Oral Cancer

The etiology of oral cancer is multifactorial. Among the risk factors that influence the development of oral cancer, smoking and alcohol are the most important factors. In India and other parts of Southeast Asia, betel quid is traditionally consumed. Viral infections such as human papillomavirus affecting at least 50% of oropharyngeal cancers, particularly those affecting the tonsils and base of the tongue (Gillison et al., 2000). Another risk factor may be genetic susceptibility that predisposes some individuals to the development of oral cancer (Jefferies & Foulkes, 2001). Chronic irritation of the mucosal edges of teeth, ill-fitting dentures or chronic infections can also act as a cancer promoter. Dietary and nutritional factors may also have some participation in the development of oral cancer (Gillison et al., 2000).

Smoking is the main risk factor associated with the development of premalignant lesions and oral cancer, especially of OSCC (Hirota et al., 2008). Chronic exposure to smoking carcinogens causes genetic changes in epithelial cells of the oral mucosa of the upper digestive tract. The accumulation of genetic changes leads to genomic instability, the development of epithelial dysplasia and eventually to invasive carcinoma. Parallel to the direct effect of the components of smoking, it can also induce proliferative activity through the activation of EGFR and its downstream mechanisms including MAPK and ERK1 and PKC alpha. This activates cyclin D1, which leads to increase the proliferative activity and increase the frequency of mutations so that cells are more vulnerable to

permanent genetic changes which in turn can lead to genomic instability and invasive cancer (Hirota et al., 2008).

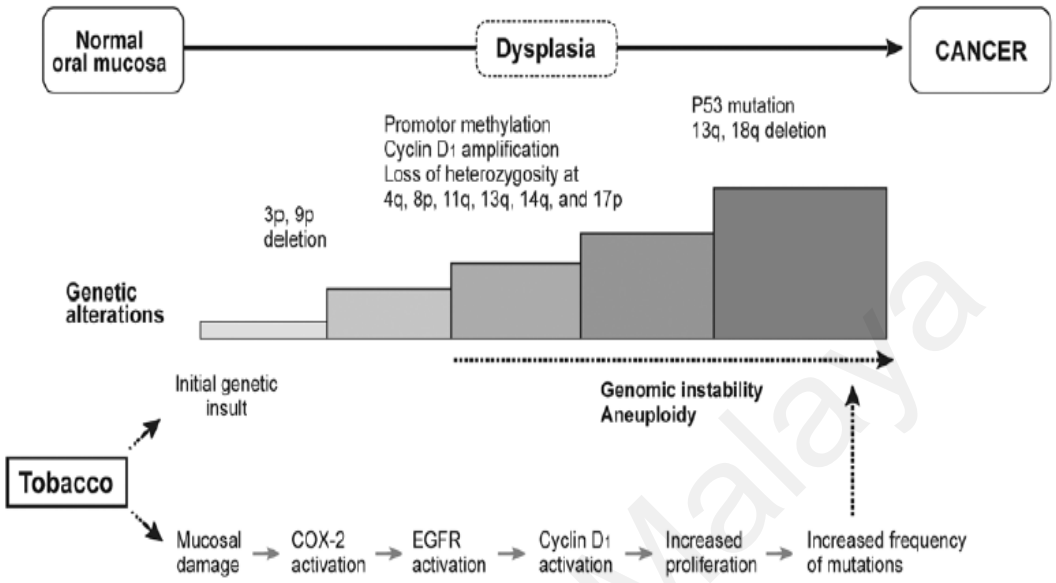


Figure 2.3: Genetic Progression in oral carcinogenesis. Transformation of normal epithelium to dysplasia and invasive carcinoma by the action of carcinogens such as smoking (Choi & Myers, 2008).

The most carcinogenic components of smoking are N-nitroso-nor-nicotine, aromatic hydrocarbons such as benzopyrene and polonium, being harmful locally and favoring the absorption of carcinogenic substances (Rodu & Jansson, 2004). It is estimated that between 85 and 90% of patients with upper aerodigestive tract cancer are explained by exposure to smoking and the risk is proportional to the intensity of exposure (Blot et al., 1988). In addition, a synergistic effect was observed between smoking and alcohol in the pathogenesis of oral cavity cancer (Hashibe et al., 2009; Znaor et al., 2003). Smoking increases the burden of the following acetaldehyde to alcohol consumption which in turn increases activation of pro-carcinogens in snuff (Seitz et al., 2009). It is difficult to distinguish the separate effects of these two agents, as consumers of alcohol tend to smoke and vice versa. Smoking inverted (with the fire toward the palate), common in parts of India, South America, and the Philippines, it strongly associated with lesions on the palate

that can lead to a high risk of developing oral cancer. There is a synergistic effect between hyperemia and direct action of smoking carcinogens (Stich et al., 1992).

Alcohol consumption has been identified as one of the major risk factors for oral cancer and the entire upper aerodigestive tract. Alcohol produces a fivefold increased risk of cancer for heavy drinkers, and there is a strong dose-response relationship (Pelucchi et al., 2008)). Although the exact mechanisms by which chronic alcohol ingestion stimulates carcinogenesis are not fully elucidated, experimental animal studies support the concept that ethanol is not a carcinogen, but under certain experimental conditions acts as a co-carcinogen and/or tumor promoter. Ethanol metabolism by the enzyme alcohol dehydrogenase leads to the generation of acetaldehyde and free radicals (Boccia et al., 2009). These highly reactive compounds to cellular components and possibly rapidly bind to DNA. Acetaldehyde decreases DNA repair mechanisms and cytosine methylation in DNA. It also damages the function of glutathione, an important role in the detoxification of carcinogens peptide. Another mechanism by which alcohol stimulates carcinogenesis is by inducing CYP2E1 cytochrome P450 enzyme, which leads to activation of several procarcinogens present in alcoholic beverages, which enhance the carcinogenic effect of smoke snuff and carcinogens in the diet (Pöschl & Seitz, 2004). Alcohol also acts as a solvent, allowing the absorption of other carcinogens.

Alcohol consumption also seems to be associated with tissue damage and secondary compensatory hyper-regeneration. So, the association between oral cancer and the use of oral rinses with high alcohol content has questioned (La Vecchia, 2009; McCullough & Farah, 2008). based on the assumption that the permanency of alcohol in contact with the oral mucosa for longer than ingesting an alcoholic beverage might suggest a possible adverse effect from a local mechanism. However, it has not been able to confirm a causal relationship between the use of mouthwashes and the development of oral cancer, but on

the other hand, some authors believe that it is not justified the use of alcohol in mouthwashes (Carter et al., 2004).

There is also a relationship between oral cancer and consumption betel nut (Areca), which is a custom in India and other parts of Southeast Asia (Cogliano et al., 2004). IARC long said that betel nut was carcinogenic to humans, and has subsequently been confirmed (Thomas & Kearsley, 1993; Zhang & Reichart, 2007). The constituents of the betel vary in different cultures but usually consists of betel nut, you obtained piper betel plant (inflorescence, leaf or root) and slaked lime (either as a powder or paste). In many countries also contains snuff and spices, betel chewing so often associated with the consumption of snuff. Similar chewing habits, such as khat, also may be involved in oral cancer in some communities (Fasanmade et al., 2007; Sawair et al., 2007). Arecoline, main alkaloid of the betel nut, could block tumor suppressor genes by hypermethylation, inhibiting DNA repair (Tsai et al., 2008).

Participation of Human Papillomavirus in the oral and pharyngeal cancer was first proposed by Syrjänen et al. in 1983, and subsequently supported by several authors (Syrjänen et al., 1983; Zur Hausen, 2002) on the basis of the following tests: 1) the well-established Epithelial Tropisms HPV, 2) the morphological similarities between the oropharynx and genital epithelia, 3) the ability to immortalize human oral keratinocytes in vitro, and 4) firmly established the etiologic role of HPV in cervical cancer human neck. This association is established for high-risk HPV, including HPV -16 and HPV-18. Some authors reported that HPV-16 is associated with approximately 50% of carcinomas of the oropharynx. These HPV-related tumors have a lower rate of mutations in the p53 gene, little relation to smoking, alcohol consumption and a better prognosis. This also justifies the increased incidence of OSCC and its appearance in young people, the most

common risk factors are not being present. HPV 16 oncoproteins encoded by genes E6 and E7, cause genomic instability in cells infected and inactivate suppressor genes.

Another aspect to be considered is the genetic susceptibility to cancer of the oral cavity. The appearance of tumors of the oral cavity in young patients and in non-smoking patients suggests the role of a genetic component. Some patients with oral cancer have decreased the ability to repair damage to DNA by carcinogens. Villaret et al. (2000) used the technique of DNA microarrays and identified that certain genes such as keratin 17 and 19, laminin-5, connexin-26, and VEGF were upregulated in the biopsied areas of oral squamous cell carcinomas (Villaret et al., 2000).

The development of oral squamous cell cancer is a multistep process which requires the accumulation of multiple genetic alterations, influenced by individual genetic predisposition and exposure to environmental carcinogens. Many genetic alterations that can be found in oral cancer gains or amplification or loss of heterozygosity (LOH) or deletions in certain chromosomes may appear. Some alterations appear early and carry a prognostic value for patients with premalignant lesions or carcinomas in early stages. Chromosome 9 appears to be one of the earliest and sensitive cancer development targets, having documented allelic losses in the 9p21 region in most of the premalignant oral lesions (Jiang, 2001) and developing carcinoma (Bockmühl et al., 1998). 9p21 region contains genes encoding inhibitors of cyclin-dependent kinases p16 and 14, which act as important regulators of cell proliferation. This alteration is associated with the transition from normal to hyperplasia/hyperkeratosis and occurs before the development of histologic atypia (Mao et al., 1996). Moreover, some regions of chromosome 3 are common carriers of chromosomal aberrations in oral cancer. Another genetic alteration that occurs in a more advanced stage of progression of squamous cell carcinoma is the

LDH 17p. This aberration causes mutation of p53 tumor suppressor gene and is associated with progression to dysplasia (Boyle et al., 1993).

2.2.5 Diagnosis of Oral Cancer

Early detection in asymptomatic stages ensures not only increased survival rates but also an improvement in the quality of life accordingly less aggressive and mutilating treatments (Sciubba, 2001). However, at the time of diagnosis of oral cancer, 36% of patients present with localized disease, 43% have a disease with regional involvement, 9% have distant metastases, and for the remaining 12%, the stage of the disease cannot be identified (Silverman Jr, 2007). Although the oral cavity is often accessible and examined the area, the fact that this type of cancer is usually painless can be a cause of late diagnosis. After the clinical diagnosis, a biopsy of intraoral lesions must perform to make a pathological study and a definitive diagnosis (Sciubba, 2001). Other non-invasive techniques such as oral cytology by scraping or staining lesions with toluidine blue (Jr et al., 1984). should be considered complementary aa biopsy but should never replace. Imaging techniques (CT, MRI, and PET) are currently very standardized and are fundamental for determining tumor extension element, the involvement of regional lymph nodes and the presence of distant metastases. Recently, the genetic study of molecular markers has become more common since detect abnormalities before they become visible clinically (Garcia del Muro et al., 2005). Oral squamous cell carcinoma has been linked to different types of cell and tissue markers, such as PCNA and p53, which can provide additional information to that provided by clinical examination and histopathology information.

The nuclear proliferation nuclear antigen (PCNA) clone PC10, is indicated for the identification of proliferating cells in normal tissues and in some forms of neoplasia (Hall et al., 1990). PCNA is an essential protein for cell cycle progression, which is expressed

in the G1 phase (last 5%) and the first 35% of the S phase of the cell cycle. It is a component of the machinery of DNA replication, acting as an auxiliary protein of DNA polymerase required for DNA synthesis. The absence or low levels of functional PCNA can induce the cells to apoptosis (Kelman, 1997). P53 (clone DO-7) is a nuclear phosphoprotein with a molecular mass of 53 kDa. The p53 wild-type protein is present in normal cells but has a very short half-life and thus, it presents only in very small quantities (Vojtěšek et al., 1992), generally below the detection level of the immunocytochemical methods. Somatic mutation of the p53 gene is a frequent event in the development of human neoplasia and since proteins p53 mutant-type are usually much more stable than wild-type, mutant p53 protein accumulates in large amounts (Vojtěšek et al., 1992).

2.2.6 Treatment of oral cancer

In the 1890s, the x-ray and ionizing radiation were discovered, allowing the use of radiotherapy in cancer treatment. In 1940 it was observed that nitrogen mustards, used as weapons of war, led to non-Hodgkin's lymphoma regression chemically react with DNA. Around the same time, the action of antifolates was also discovered and the result was the emergence of Cancer Chemotherapy National Service Center, National Cancer Institute (NCI) in the United States in 1955. In 1975, a combination of cyclophosphamide, methotrexate, and fluorouracil (CMF) shown effective in adjuvant treatment for breast cancer, this discovery followed by the approval of Cisplatin in the treatment of ovarian cancer and other solid tumors. The main antineoplastic agents can be divided into the following categories: alkylating agents (cyclophosphamide, Cisplatin) antimetabolites (methotrexate, 5-fluorouracil), cytotoxic antibiotics (doxorubicin, bleomycin) and vegetable products (vinca alkaloids, taxanes, etoposide, camptothecin) (Fennell & Rudd, 2004).

2.3 Oral Cancer Animal Models

Previous research has acknowledged the paucity of reliable biomarkers to identify those lesion of oral potentially malignant disorders that may proceed to the greatest risk of malignant progression has been acknowledged (Kanojia & Vaidya, 2006). Partly, this implies that availability of animal models that can be used for studying how the mechanisms of OSCC are formed and for testing substances that may halt/reverse these processes is still restricted (Czerninski et al., 2009). Moreover, whereas the molecular analysis of human biopsy samples is regarded the ideal, it is difficult to find tissue from each of the multiple stages involved in the evolution of a particular lesion (i.e. hyperplasia, various grades of dysplasia and carcinoma in-situ). On the other hand, tissue derived from animal models allows reproducing each stage of precancerous development, and its availability assists in carrying out a histological and molecular analysis (Herzig & Christofori, 2002).

It is rare to find such spontaneous cases of OSCC in animals (Thurman et al., 1997). Yet, there are several methods, including chemical induction, xeno-transplantation, and transgenesis which have been used for inducing development of OSCC in animal models (Mognetti et al., 2006).

2.4 Natural plant products

Nature has been the source of medicinal products for millennia. With the improvement of methods of isolation, identification, and synthesis, during the last century, many drugs have been developed from natural sources, especially herbal species (Cragg et al., 2009). It is difficult to date the beginning of drug use in the treatment of cancer, as herbs and other preparations were used for this purpose since ancient times (Baguley, 2002). Hartwell, in his review in 1982, listed over 3000 plants used in the treatment of cancer (Cragg et al., 2009). As a result, over 60% of anticancer agents used today are derived

from natural products, including plants, microorganisms, and marine organisms (Cragg & Newman, 2005). The anticancer agents derived from natural products that mostly used, belong to four main classes, including synthetic or semisynthetic derivatives: Vinca alkaloids of vinblastine and vincristine epipodophyllotoxins of *Podophyllum peltatum* L., taxanes from *Taxus brevifolia* Nutt and camptothecins of *Camptotheca acuminata* Decne (Balunas et al., 2006). In general, these substances act in events related to DNA and cell division, preventing the formation of the mitotic spindle and its polymerization (vinblastine, vincristine, taxanes), inhibiting the action of topoisomerases and then the DNA replication process (camptothecin, etoposide) (Fennell & Rudd, 2004). Today, other classes of natural products such as bacteria, already in clinical trials phase, such as epothilone B from *Sorangium cellulosum*, romidepsin from *Chromobacterium violaceum* and dibenzodiazepine ECO-4601from *Micromonospora sp* (Gordaliza, 2007).

2.4.1 Goniothalamin

Goniothalamin (GTN) is a molecule (Fig. 2.5) derived from a natural source with potent activity antiproliferative. It is a styryl lactone found in Annonacea family members in plants of the genus goniothalamus sp (Table 2.2 and Fig. 2.6). This molecule has been isolated in 1967 by first from the bark of Cryptocarya caloneura (Hlubucek & Robertson, 1967). In 1972, it was reported that this molecule was present as a constituent of the shell G. andersonii , G. macrophyllus and G. malayanus (Jewers et al., 1972). In other styryl lactones, the GTN can be found in goniothalamus species such as allolactose and cardiopetalolactona (Wiart, 2007).

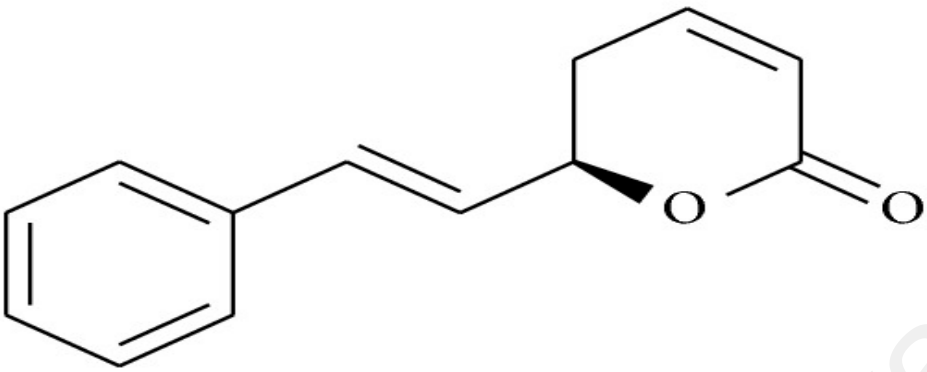


Figure 2.3: Goniothalamin chemical structure

Figure 2.5: Goniothalamus *macrophyllus* (Blume) Hook plant

Table 2.2: Goniothalamus species with their common names, part used, and their traditional uses

Species	Vernacular name	Part used	Traditional uses
G. macrophyllus Hook.f andThoms	Selada, SelayarHitam Gajah Beranak, Penawar Hitam (Malaya) Ki Cantung (Indonesia), Limpanas Putih (Brunei), Chin Dok Diao (Thailand)	Leaves	The leaves of G. macrophyllus Hook.f and Thoms. are used to allay fever.
G. macrophyllus Hook.f and Thoms; G.macrophyllus (Bl.)	Selada, SelayarHitam Gajah Beranak, Penawar Hitam (Malaya) Ki Cantung (Indonesia), Limpanas Putih (Brunei), Chin Dok Diao (Thailand)	Roots	Decoction of the roots are given as a postpartum remedy and for abortion. Root decoction is given for anti-aging purposes. Mixed with Eurycoma longifolia, it is used as a male tonic. Different parts of Goniothalamus macrophyllus are used by the Temuan in Peninsular Malaysia to treat various ailments such as body pains, rheumatism, and skin complaints. The decoction of the root is used to eliminate excessive gas in body. The decoction is used as a lotion to treat body pains and rheumatism Pounded leaves and bark are used for skin complaints.

GTN has δ-lactones α, β-unsaturated in its chemical structure. Studies have shown that molecules with the δ-lactones α, β-unsaturated ring act as inhibitors of protein phosphatases (Buck et al., 2003; Ghatge et al., 2006; Lewy et al., 2002; Theobald et al., 2013), have antibacterial activity (Mosaddik & Haque, 2003), antifungal (Martins et al., 2009), immunosuppressant (de Fátima et al., 2008) and anticancer (de Fátima et al., 2005; de Fátima et al., 2008; Lewy et al., 2002). It is believed that this structure plays a key role in the biological activities presented for these molecules because of their potential to act as a Michael acceptor in the presence functional protein groups (Barcelos et al., 2014; Boucard et al., 2007). Structural changes made in the GTN molecule showed that the biological activity depends on the Michael acceptor of existence in its structure (Kasaplar et al., 2010)).

2.4.1.1 GTN as a promising anticancer agent

Previous studies have reported that GTN has many bioactive properties such as antibacterial (Mosaddik & Haque, 2003), antifungal (Martins et al., 2009), larvicide (Senthil-Nathan et al., 2008), trypanocidal (De Fátima et al., 2006) and antimalarial (Mohd Ridzuan et al., 2006). However, several in vitro studies have reported the cytotoxicity of GTN against a variety of cell lines with no cytotoxicity on normal cells.

2.4.1.2 In vitro Studies of GTN

GTN has been reported as an antiproliferative and antitumor agent against various tumor cell lines (Chen et al., 2005; Inayat-Hussain et al., 2003; Rajab et al., 2005; Wattanapiromsakul et al., 2005). Some authors suggest a possible selectivity of GTN against tumor lines with little activity against normal cells, which it has made a great interest in this molecule in the last two decades (Azimahtol Hawariah et al., 1998; Orlikova et al., 2013; Wattanapiromsakul et al., 2005).

The selective antiproliferative activity of GTN was first identified by Azimahtol Hawariah et al, (1998) using methylene blue staining technique. GTN showed a strong antiproliferative activity against MCF7 line in the concentration of 6.30×10^{-7} M. At the same concentration, however, there was no inhibition of proliferation of normal human liver cells, CCL 13, and normal kidney cells bovine MDBK (Azimahtol Hawariah et al., 1998).

Wattanapiromsakul et al. (2005) reported that selective cytotoxicity of GTN was demonstrated by the sulforhodamine B assay (SRB). All IC_{50} values of the tumor cell lines of colon cancer (LS-174T), breast cancer (MCF-7) and lung carcinoma (COR-L23) were lower than those for normal cell lines (ST3). The IC_{50} for colon cancer cells (LS174T), breast cancer cells (MCF7) and lung cancer cells (CORL23) were 0.51 ± 0.02 µg/m, 0.95 ± 0.02 µg/ml and 3.51 ± 0.03 µg/ml, respectably. As for normal lines, the IC_{50} for mouse skin fibroblasts (ST3) was 26.73 ± 1.92 µg/ml and human fibroblasts (HF) 11.99 ± 0.15 µg/ml (Wattanapiromsakul et al., 2005).

Tian et al. (2006) investigated the effects of GTN and two more styryl lactones on three hepatocyte cell lines, HepG2, drug-resistant HepG2 (HepG2R) and primary cultured of normal mice hepatocyte. In the MTT assay, a drastic reduction in cell proliferation was observed in a concentration-dependent manner with IC_{50} values of 8.83 µM for HepG2, 8 µM for HepG2R and 23.33 µM for the normal mouse hepatocyte line, which suggests a more significant cytotoxicity against the cancerous cells (Tian et al., 2006).

Orlikova et al., (2013) investigated the cytotoxic effects of GTN on K562 chronic myeloid leukemia and Jurkat T acute leukemia, and also on peripheral blood mononuclear cells (PBMC). K562 cells showed a 70% reduction in cell viability when exposed to the concentration of 20 µM GTN and Jurkat T cells showed 50%reduction in cell viability.

As for healthy cells (PBMC), GTN did not present a cytotoxic effect (Orlikova et al., 2013).

Failures in the apoptotic pathway consider one of the biggest obstacles to success in cancer treatment. Therefore, the modulation of apoptosis is one of the main mechanisms of interest in the development of new compounds and therapeutic strategies for the treatment of cancer (Reed, 2002). Several previous studies suggest that the induction of apoptosis by GTN is the main antiproliferative mechanism (Alabsi et al., 2012; Alabsi et al., 2013; Barcelos et al., 2014; Chan et al., 2006; Chen et al., 2005; Chiu et al., 2011; Inayat-Hussain et al., 2003; Inayat-Hussain et al., 1999; Kuo et al., 2011; Orlikova et al., 2013; Petsophonsakul et al., 2013; Reed, 2002; Semprebon et al., 2014; Tian et al., 2006; Yen et al., 2014).

Some studies report that GNT-induced apoptosis occurs through the intrinsic pathway of apoptosis. Inayat-Hussain et al. (2003) reported that human HL-60 promyelocytic cells that exposed to GTN (50 μM) using the analysis of phosphatidylserine exposure by flow cytometry resulted in the induction of cell death by apoptosis. The intrinsic pathway of apoptosis was activated with loss of mitochondrial membrane potential, activation of the initiator caspase-9 and effector caspases-3 and 7. Treatment with the Z-VAD.FMK caspase inhibitor did not lead to complete inhibition of phosphatidylserine, suggesting that the apoptosis observed in HL-60 cells may also have occurred through caspase-independent mechanisms (Inayat-Hussain et al., 2003).

Another study by Chen et al. (2005) revealed that MDA-MB-231 human breast cancer cells that exposed to GTN had an increase in the sub-G1 population and characteristic morphological changes of apoptosis, such as chromatin condensation and nuclear fragmentation. A time-dependent increase of cytochrome c in the cytosol and a

mitochondrial efflux of this protein was observed. The treatment also led to the activation of effector-3 caspase and poly (ADP-ribose) polymerase cleavage (PARP), a DNA repair enzyme that is a substrate of caspase-3. Treatment with the Z-VAD.FMK caspase inhibitor attenuated the induction of apoptosis (Chen et al., 2005).

De Fatima et al. (2008) reported a reduction in TNFR1 cell surface death receptor expression levels after treatment of 786-O cells with S-GTN (IC_{50}: 4 nM) and R-GTN (6.4 μM). Exposure to R-GTN reduced the expression levels of the anti-apoptotic protein Bcl-2 and led to an increase in the cleavage of PARP (Poly (ADP-ribose) polymerase), which suggest that apoptosis was activated (de Fátima et al., 2008).

Chan et al., (2010) studied the molecular mechanisms of GTN-induced apoptosis on coronary artery smooth muscle cells CASMCs and they found that GTN induces apoptosis in a concentration-dependent manner. GTN induced DNA damage associated with an increase in P53 level (Chan et al., 2010). However, this increase in P53 level was independent of NAD(P)H: quinone oxidoreductase 1 and Mdm-2 expression. They also observed that caspase-2 and -9 were activated but not caspase-8 which led to downstream of caspase-3 cleavage. In their study, they confirmed the role of mitochondria in the apoptosis induced in CASMCs the role of oxidative stress in GTN mechanism of treatment (Inayat-Hussain et al., 2010).

Kuo et al. (2011) studied the apoptotic mechanisms of GTN on the hepatocellular carcinoma cells Hep-3B (TP53-negative) and Sk-Hep1 (TP53-positive). They reported that GTN triggered apoptosis via TP53-dependent and -independent pathways. In TP53-positive SK-Hep1 cells, GTN induced TP53 transcription-dependent and -independent apoptosis. Moreover, GTN induced apoptosis in a dose-dependent manner in both cells. In SK-Hep1 cells, GTN treatment induced cleavage of the Casp8, Casp9, Casp3 and

PARP proteins. In the Hep-3B cells, there was only the cleavage of CASP8 and CASP3. In both cell lines, the changes in the mitochondrial membrane potential and the release of mitochondrial cytochrome c to the cytosol confirmed the participation of the intrinsic pathway in apoptosis induced by (Kuo et al., 2011).

PetsophonsakuL et al. (2013) reported that treatment of HL-60 and U937 cells with concentrations of IC_{10}, $IC_{20,}$ and IC_{50} of GTN led to a dose-dependent increase of the sub Gl population and exposure of phosphatidylserine by both cell lines. The intrinsic pathway of apoptosis was activated, with a reduction in mitochondrial membrane potential, increase in Smac/Diablo expression and activation of the initiator-9 caspase. The activation of initiator-8 caspase was also observed, suggesting that the extrinsic pathway also participates in GTN-induced apoptosis (Petsophonsakul et al., 2013).

A study by Alabsi et al. (2013) investigated the effect of GTN against cytotoxicity effect against cervical cancer cells (Hela) and demonstrated that GTN induced cell deathcytotoxicity via apoptosis. The IC_{50} value of GTN was 3.2±0.72 μg/ml. Evidence such as chromatin condensation and nuclear fragmentation, DNA damage, increased sub-G1, and also the increase in caspase-9 levels, with no change in levels of caspase-3, 7 and 8, confirmed that GTN induces apoptosis in a time-dependent manner (Alabsi et al., 2013).

Barcelos et al. (2014) evaluated the effect of GTN against a highly metastatic human pancreatic cancer cell line PANC-1 and revealed that GTN caused pancreatic cancer cell cycle arrest and exhibited cell death by apoptosis and, as evidenced by the increase in the ratio of Bax proteins(Pro-apoptotic) / Bcl-2 (anti-apoptotic), reduction in procaspase-3 levels and increased PARP cleavage (Barcelos et al., 2014).

The IAP family (Inhibitor of Apoptosis Protein) is a class of regulatory proteins that play some important roles in the cell as inhibition of apoptosis and promotion of cell cycle progression (Rumble and Duckett, 2008). Researchers studied the effect of S-GNT (a synthetic enantiomer of GTN) on a human non-small cell lung cancer NCI-H460 cells reported that the cytotoxicity of-GTN is due to its ability to induce cell death via apoptosis. S-GTN inhibited the expression levels of the BIRC5 gene, which encodes the survivin protein, belonging to the IAP family (Semprebon et al., 2014).

In response to cellular stress conditions, physiological levels of autophagy promote cell survival. It is a process of encompassing the cytosol that occurs in a random and indiscriminate way. Some major stages lead to autophagy. Initially, the phagophore formation occurs, where the ULK/Atg1 complex activates the class III phosphatidylinositol 3 kinase complex, which recruits new proteins to form an isolation membrane. In the next stage, the conjugation of the Atg5-Atg12 proteins occurs and the conversion of LC3-I to its phosphatidylethanolamine-conjugated form (LC3-II). Then, the mature autophagosome membrane fuses with a lysosome to form an autolysosome in order to degrade the cytoplasmic material that is in the vesicle (Glick et al., 2010; Levine & Kroemer, 2008). De Fatima et al. (2008) reported that S-GTN (IC 50: 4 nM) stimulates the processing of LC3-I in LC3-II, suggesting the participation of the autophagy process in autophagy process in inhibiting the cellular proliferation of 786-O renal adenocarcinoma cells (de Fátima et al., 2008).

Oxidative stress is the state in which oxidation exceeds antioxidant defense systems. Some atoms and molecules have unpaired electrons and are called free radicals. These unpaired electrons tend to form pairs with other electrons, and therefore, these molecules are highly reactive. Some examples of reactive oxygen species (ROS) are superoxide, hydrogen peroxide, hydroxyl radicals, nitric oxide, among others. ROS is capable of

causing damage to proteins, DNA, RNA, and lipids (Ye et al., 2014). The hepatocellular carcinoma cells, SK-Hep1 and Hep-3B when treated with GTN, the treatment resulted in the induced intracellular formation of reactive oxygen species (ROS) (Kuo et al., 2011). Inayat-Hussain et al., (2010) also suggested that the ability of GTN to induce DNA damage is due to the occurrence of oxidative stress. In their study, when Jurkat T cells were treated with 50 μM GTN for 2 h, a significant increase in the production of ROS (reactive oxygen species) and loss of GSH was observed. After that, DNA damage was also analyzed through the Comet Assay, and consistent with the loss of GSH and increased ROS production, the results showed a significant increase in damage to the genetic material (Inayat-Hussain et al., 2010).

Chan et al., (2010) reported an increase in peroxide levels, but not in superoxide anions in CASMC cells. The authors suggest that GTN induces a reduction in oxygen consumption by cells, causing a reduction in ATP levels with a concomitant increase in peroxide levels. The level of reduced GSH decreased in the cells exposed to GTN (Chan et al., 2010).

The tripeptide glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine) is the main modulator of the redox state of the cells, which protects them from oxidative stress generated by free radicals (Townsend and Tapiero, 2003; Mari et al., 2009). Pretreatment with exogenous GSH led to the reduction of GTN cytotoxicity (1-100 μM), even at high concentrations, against PANC-1 cells and an increase in intracellular ROS levels after the treatment with GTN was also observed (Barcelos et al., 2014). Chen et al. (2005) reported that the increase in ROS levels and reduction in intracellular levels of intracellular free thiol content was also observed for MDA-MB-231 cells. In their study, the treatment of cells with NAC and GSH reduced the ability of GTN to induce apoptosis, suggesting that cytotoxicity is due to its pro-oxidant properties (Chen et al., 2005).

DNA damage can disrupt genomic integrity and, therefore, molecules with genotoxic potential that cause extensive damage to the DNA, are not amenable to repair, induce apoptosis and thus block tumor growth (Riedl & Shi, 2004). Some authors attribute the cytotoxic effect of GTN to its genotoxicity (Inayat-Hussain et al., 2009; Kuo et al., 2011; Rajab et al., 2005; Semprebon et al., 2014). Exposure of CHO ovarian cells to GTN (10 and 5 μM) led to the induction of damage to DNA. The main types of aberrations found were gaps in chromatids and in whole chromosomes, breaks and exchanges, endoreduplication and chromosomal rings (Umar-Tsafe et al., 2004). Rajab et al. (2005) evaluated the genotoxicity of GTN at concentrations of IC_{10} and IC_{25} for the leukemic cell lines HL-60 (IC_{50}: 4.5 μg/mL) and CEM-SS (IC_{50}: 2.4 μg/mL) through the Assay of the comet with two hours of treatment. In both treatments, there was the induction of genotoxicity and GNT was more cytotoxic and genotoxic for the CEM-SS line than for HL-60. Semperbon et al. (2014)studied the activity of S-GTN against NCI-H460 lung cancer cells and the reported that induction of DNA damage was observed at all concentrations of the tested (2.5, 12.5 and 25 μM) in a concentration-dependent manner (Semprebon et al., 2014).

2.4.1.3 *In vivo* Studies of GTN

Tumors have been recognized as organs due to their complexity. Tumor biology is influenced by the surrounding microenvironment and by several cell types that contribute to tumor development and maintenance (Hanahan & Weinberg, 2011). Thus, *in vivo*studies are essential for understanding the new mechanism of action anticancer drugs. Although several studies have reported the potent activity antiproliferative the GTN against several tumor cell lines *in vitro*, little is known on the antitumor activity of this molecule *in vivo*.

The antitumor activity of the racemic GTN (R-GTN|) and synthesized GTN (S-GTN) was evaluated in solid Ehrlich tumor. The doses of 30, 100 and 300 mg/kg of the racemic GTN inhibited 48.5 ± 9.4, 40.9 ± 9.5, and 66.7 ± 15.9, respectively, of tumor growth compared to the control group. The authors also evaluated the antitumor activity of each of the enantiomers and it was observed that the maximum inhibition of tumor growth was 53.7 ± 20.9% on the 10^{th} day after administration of the 3^{rd} dose in the group treated with the synthetic enantiomer (S-GTN) and 37.4 ± 10.8% in the group treated with the natural enantiomer (R-GTN). There was no evidence of toxicity or weight loss for the groups treated with the R-GTNor S-GTNwas noticed. However, the group treated with Doxorubicin (3 mg/kg) showed weight loss at the end of the experiment (Vendramini-Costa et al., 2010).

2.5 Cell Death

The process of cell death is fundamental for the normal development and maintenance of the multicellularity of the organism. An abnormal pattern of death caused by loss or gain of the functionality of genes associated with cell death is correlated with the appearance of different pathological conditions, such as neurodegenerative disorders, lymphoproliferative diseases, and cancer (Kissil et al., 1997). Cell death can be triggered by multiple causes: loss of function, mechanical damage, infection by microorganisms or viruses, the action of toxic chemical agents or lack of nutrients. Cell death according to classical criteria can be divided into a death that goes by regulated mechanisms, such as apoptosis, and unregulated necrosis (Degterev & Yuan, 2008).

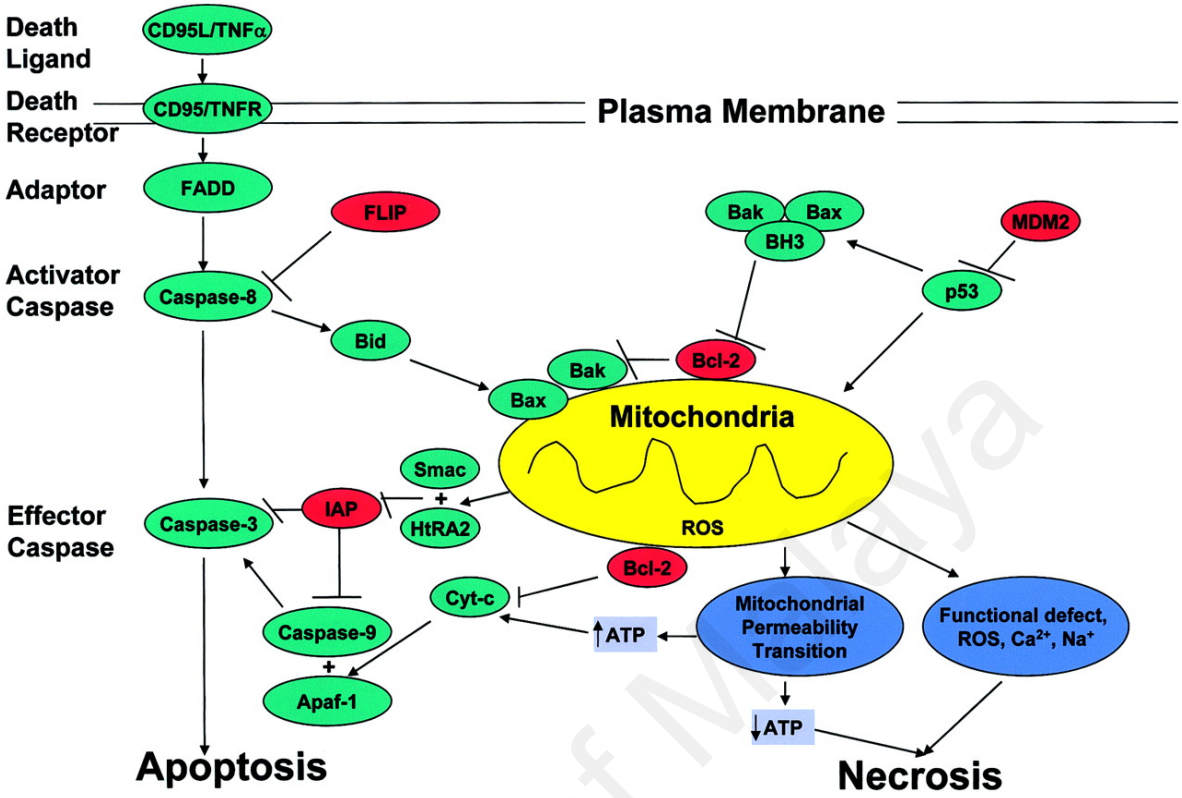


Figure 2.6: Apoptosis and necrosis (Bhatia, 2004)

2.5.1 Apoptosis

Apoptosis, programmed cell death, is a phenomenon where the cell is stimulated to trigger mechanisms that culminate in its own death. It is a way where the initial stimulus induces molecular events cascade complex that results in the activation of a group of cysteine proteases called "caspases" and other key proteins, with consequent cell suicide (Elmore, 2007). In 1972, Kerr et al. described the morphological changes that occur and suggested the name "Apoptosis" for this type of cell death. This process begins with the shrinkage of the cell and the nucleus, as well as chromatin condensation in well-defined masses attached to the nuclear membrane (Kerr et al., 1972). Later, the nucleus fragments (karyorrhexis) followed by the invagination of the plasma membrane, and fragmentation of the cell into apoptotic bodies, which are delimited by membrane, cytoplasmic

organelles compound and compacted with or without nuclear fragments. These apoptotic bodies are rapidly engulfed by macrophages or adjacent parenchymal cells (Kerr et al., 1972).

This process is critical in embryonic development to remodeling of organs, so it is essential in organogenesis process (Jin & El-Deiry, 2005). In the adult organism this process is involved in various physiological events, such as sloughing of the intestinal lining, death of neutrophils, tissue renewal, ensuring tissue homeostasis and also acting as a defense mechanism of body, being instrumental in the removal of cells that have undergone some damage, preventing pathological processes such as cancer (Reed, 2002). Deregulation of the apoptotic mechanism is associated with various diseases. According to Melnikova and Golden (2004), Inhibition of apoptosis and increased cell survival is associated with cancer; autoimmune diseases such as systemic lupus erythematosus, inflammatory diseases such as asthma and rheumatoid arthritis and viral infections. Since excessive apoptosis with consequent increase of cell death is associated with AIDS, neurodegenerative diseases such as Alzheimer's, Huntington's disease, multiple sclerosis and Parkinson's disease, hematological diseases such as aplastic anemia, lymphocytocytopenia and myelodysplastic syndrome, tissue damage such as myocardial infarction and other diseases such as alopecia and muscular dystrophy (Melnikova & Golden, 2004).

During apoptosis, an enforcement machinery is triggered by various extra or intracellular signals that result in an intense cleaving of cellular proteins by activated caspases. They are synthesized in the cell as inactive zymogens, the procaspases, which are activated by cleavage of the aspartic acid residues by other caspases or self-activation. Once activated, these caspases cleave and activate other procaspases and other key proteins (Elmore, 2007). Functionally, apoptotic caspases are divided into two groups:

primers (2, 8, 9 and 10) which activate effector caspases (3, 6 and 7), when effector caspases activated, they directly degrade various substrates including regulatory and structural proteins in the nucleus, cytoplasm, and cytoskeleton, leading to the execution of cell death by apoptosis (Zimmermann et al., 2001). Activation of procaspases can be triggered by activation of cell surface death receptors (extrinsic pathway) or through the mitochondrial pathway, which occurs when the cell is stressed or damaged (intrinsic pathway) (Jin & El-Deiry, 2005). These pathways converge to a common path, the execution apoptosis, which begins with the cleavage of caspase3 and result in some changes of the morphological cellular structure as DNA fragmentation, cytoskeletal degradation, and nuclear proteins, protein crosslinking, formation of apoptotic bodies, expression of ligands for recognition of phagocytic cells and finally, phagocytosis (Elmore, 2007).

2.5.2 Mechanisms of Apoptosis

Apoptosis is a sophisticated and highly intricate mechanism of the organism involving an energy-dependent cascade of molecular functions (Elmore, 2007). Apoptosis occurs via various internal or external stimuli and these signals are controlled by two distinct pathways; intrinsic and extrinsic (Chowdhury et al., 2006; Li et al., 2012). The pathways are linked, where the molecules within one are influential upon the other (Igney & Krammer, 2002).

2.5.2.1 The extrinsic pathway

The extrinsic pathway is initiated when an extracellular ligand binds to death receptor on the membrane surface. The sequence of events that occurs on the extrinsic pathway is well characterized by the FasL/PRSA model. The FasL death binder joins its death receptor fasL and the formation of this complex recruit some cytosolic factors as FADD and procaspase-8 forming a signaling complex inducer of death. The formation of

signaling complex inducer of death will then trigger autocatalytic activation of procaspase-8, once activated will cleave and activate procaspase-3 activator, inducing apoptosis (Riedl & Shi, 2004).

2.5.2.2 The intrinsic signaling pathway

The intrinsic signaling pathway initiates apoptosis through stimuli diversified and not involving receptors, such as DNA damage, deprivation growth factors, heat, hypoxia, cytotoxic drugs, radiation and oxidative stress, which produce intracellular signals that act directly on targets within the cell. The intrinsic pathway normally begins in the mitochondria (Jin & El-Deiry, 2005). These stimuli cause changes in the mitochondrial membrane, causing a great increase in their permeability, which is accompanied by leakage of proapoptotic proteins in the intermembrane space of mitochondria to the cytosol (Riedl & Shi, 2004). The cytochrome c released into the cytosol will bind to the cytosolic protein Apaf1, causing a conformational change that will facilitate the binding of ATP/dATP. This complex, called "apoptosome", will connect to procaspase-9 and promote your cleavage giving rise to the initiator caspase-9 active, which will be responsible for the activation of caspases activator 3 and 7, resulting in induction of apoptosis (Huerta et al., 2007). SMAC/DIABLO and Omi/HtrA2are recruited to promote apoptosis inhibition of Inhibitors of Apoptosis Proteins (IAPs) in order to increase the activity of caspases. The apoptosis-inducing factor (AIF) and endonuclease G are responsible for DNA fragmentation (Huerta et al., 2007). Due to the fundamental importance of apoptosis for homeostasis of the organism, this process is strictly regulated. Some of the proteins involved in this regulation may be good targets for cancer therapy since they are altered in most tumors, such as the Bcl-2 family and the family of IAPs.

Bcl-2 family are regulators of apoptosis and a family of proteins that regulate apoptosis by actively participating in this process. These proteins are divided into two classes, those

inhibiting (antiapoptotic) and those promoting (proapoptotic) apoptosis (Petros et al., 2004). Functionally, the stoichiometry between proteins anti and proapoptotic and the interaction between them determines whether the cell will or will not answer different apoptosis-inducing stimuli (Levine & Kroemer, 2008; Willis et al., 2003). The Bcl-2 family proteins are divided into three subfamilies according to the number of homology domains of Bcl-2 (Bcl-2 homology): antiapoptotic Bcl-2, BclxL, Bclw, Mcl1, BclA1 and B with four homology to Bcl-2 domains (BH1, BH2, BH3 and BH4), which protect the cell from apoptosis and proapoptotic protiens are Bax, Bak and Bok, with three regions of conserved Bcl-2 homology (BH1, BH2 and BH3) that induce apoptosis; and the proapoptotic BH3only proteins, Bik, Bad, Bid, Bim, Bmf, HRK, and Noxa Puma, with only one region of homology to Bcl-2 (BH3) which trigger or sensitized to apoptosis (Adams & Cory, 2007; Borner, 2003; Ghibelli & Diederich, 2010). Apoptosis induced by certain stress requires the participation of both groups of proapoptotic proteins, both of BH3only proteins, which act as damage sensors activating Bax and/or antagonists of antiapoptotic proteins. Proapoptotic proteins Bax subfamily, which when activated acts permeabilizing the outer mitochondrial membrane (Adams & Cory, 2007; Ghibelli & Diederich, 2010). As a result, cytochrome c and other effector proteins are released into the cytosol causing the activation of the proteolytic cascade and cell death induction apoptosis (Lessene et al., 2008). Different stimuli leading to activation of different proapoptotic proteins BH3only. The proapoptotic protein Bax is the most studied of the members of proapoptotic subfamily. This protein is found in the form of monomers of 21 kDa. They have cytoplasmic or perimitocondrial location. Normally, the activation of Bax during apoptosis does not require an increase in the transcription of this gene (Lessene et al., 2008). In response to an apoptotic stimulus, proapoptotic proteins BH3 only promote the translocation of Bax into the outer mitochondrial membrane (Willis et al., 2003). Two models have been proposed to explain the release of the effector

molecules of mitochondria occurs. The first model suggests that Bax form multimers, a nonspecific mega canal, which induces the decrease in mitochondrial membrane potential and swelling of mitochondria, causing the rupture of the outer mitochondrial membrane and release of cytochrome c into the cytosol. The second model suggests that multimers Bax interact directly with specific channels like DVCA (voltage-dependent anionic channel) causing conformational changes that make them permeable to cytochrome c (Er et al., 2006). Moreover, the formation of homodimers by antiapoptotic proteins Bcl-2 family, can prevent the increase in mitochondrial permeability and prevent forming pores in the mitochondrial membrane (Antignani & Youle, 2006).

TP53, the human tumor suppressor gene P53 is located on the short arm chromosome 17 P13.1 (Bertheau et al., 2008). The expression patterns of this gene are quite complex, with more than 10 different isoforms of proteins produced by splicing and alternative translation (Hollstein & Hainaut, 2010). The p53 protein, encoded by the gene P53 is a transcription factor, considered the "guardian of the genome" capable of regulating the expression of various genes with a variety of functions including cell cycle arrest, apoptosis, changes in metabolism, among others (Green & Kroemer, 2009).

This protein is involved in several pathways in response to stress that prevents the growth and survival of potentially malignant cells. In normal cellular conditions, p53 levels are kept very low and this happens because p53 interacts with a Mdm2 protein, the primary regulator of p53 levels, which acts as a ubiquitin ligase that binds it to be destroyed by proteasomes. In cellular stress situations, such as genotoxic damage, oncogenic activation, deprivation of nutrients and hypoxia. The protein kinase phosphorylates p53 and reduces its connection with Mdm2 which in turn reduces degradation and increases its levels in the cell (Gottlieb & Vousden, 2010; Hollstein & Hainaut, 2010; Meek, 2009). The increase in p53 levels in the cell will trigger the

transcriptional transactivation of its target genes such as components of the machinery of repair and replication, cell cycle regulators, apoptosis regulators, etc. (Bouchet et al., 2006; Vousden & Prives, 2009). Furthermore, p53 can also inhibit transcription of Bcl-2, and this is fundamental in the process of cell death, in which the increase of Bax in mitochondria and the low levels of antiapoptotic Bcl-2, will release the cytochrome c into the cytosol (Hemann & Lowe, 2006).

The p53 choose between cell cycle arrest and apoptosis according to nature and extent of the inducing signal, and according to the cell type and the tissue (Bouchet et al., 2006). One possible explanation of the reason for the choice is that genes involved in cell cycle arrest have a high affinity for p53 in its promoters, while lower affinity sites are present in the promoter of genes related to apoptosis. Thus, increased levels of p53 may trigger apoptosis after reaching a certain limit in their levels (Bertheau et al., 2008; Chen et al., 1996). As p53 helps the body to pass cellular stress safely when mutated or missing, the necessary cellular responses will not occur and the damaged cells escape apoptosis and continue dividing. In about 50% of human cancers, gene TP53 is found mutated, and in most cases there are increased inhibitors, reducing their activators or inactivating their targets downstream (Green & Kroemer, 2009). Therefore, p53 is identified as an important target for cancer therapy, since its activation causes tumor cytotoxicity. Among the various studies involving therapeutic use of p53, one great progress has been achieved with the use of gene therapy for delivery of a copy functional p53 pathway oncolytic adenovirus vector or adenovirus ONYX015; small molecules which has also been used to inhibit Mdm2; among several approaches which aim directly to modulate p53 conformation and induce normal operation (Chen et al., 2010; Lee et al., 2008). The strain used in this study (NCIH460) expressed mRNA levels of p53 comparable to those presented by normal lung tissue cells (Takahashi et al., 1989).

2.5.3 Necrosis

Necrosis is another type of cell death in a multicellular organism, derived from the Greek word “nekros”, for a corpse. It is also functional in multiple physiological processes, such as inner ear development (Zong & Thompson, 2006) and the tissue renewal of small and large intestines (H. BARKLA, 1999; Murdoch et al., 1999), follicular maturation during oogenesis and loss of interdigital cells in the mouse embryo (Chautan et al., 1999; Murdoch et al., 1999). Taking into account common histology, distinguishing apoptosis from necrosis is a complex issue. Cell deaths and its associated mechanisms can happen simultaneously, depending on factors such as intensity and the period of stimulus, the amount of ATP depletion and also the accessibility to caspases (Zeiss, 2003). Necrosis can be initiated by external factors, such as mechanical trauma, damage to blood vessels, ischemia, thermal effects, viruses, bacteria, protozoa, and toxins. It can also be caused by internal factors, such as components of the immune system, activated natural killers and peritoneal macrophages. Moreover, insufficient secretion of cytokines, nitric oxide (NO) and reactive oxygen species (ROS) may also cause necrotic cell death (Proskuryakov et al., 2003; Raffray, 1997). Although apoptosis and necrosis have been described according to their respective distinguishable morphological features, they may involve in a number of common signaling and execution mechanisms (Leist et al., 1999).

2.5.4 Cell Cycle

Cell cycle is defined as the sequence of processes by which a cell duplicates its contents and divides into two. The two main phases of the cell cycle are mitosis (M) and interphase (Vermeulen et al., 2003). Interphases include G1, S and G2 phases. In the G1 phase (growth phase), bioactive amino acids are generally synthesized for the production of proteins and enzymes. The numbers of organelles increased while the cell grows in

size. This phase is followed by the S phase, whereby DNA synthesis subsequently occurs. The next phase of the interphase is G2. It is characterized by the biosynthesis of proteins, such as microtubules, which are essential in the segregation of chromosomes during the M phase (Tyson et al., 2003). During interphase cells transcribed genes, synthesized proteins and grow in size.

2.5.4.1 Cell Cycle Control

There are a few identifiable cell cycle control mechanisms; a cascade of protein phosphorylations, transmitting a cell from one stage to another, followed by a series of checkpoints that check for the completeness of important functions while delaying progression, all in the quest to make sure that the cell is not in any way duplicating DNAs that are damaged (Morgan, 1995). The initial type of control involves a family of protein kinase complexes, including cyclin-dependent kinases (CDKs) and their activating partners, cyclins. Regulatory phosphorylation and dephosphorylation helps alter the activity of CDK-cyclin complexes, which helps define a well-delineated transition between the cell cycle phases. Another type of cell cycle regulation is known as checkpoint control (Collins et al., 1997). At critical transitions (G1/S, intra-S-phase, G2/M or mitotic spindle), the eukaryotic cell cycle progression help mark the pathways and confirm the successful and effective completion of upstream events before it moves on to the next stage. These regulatory pathways are defined as cell cycle checkpoints (Grasso et al., 2012; Hartwell & Weinert, 1989). Checkpoint signaling may result in activation of pathways, leading to the apoptosis if cellular damage remains unrepaired (Pietenpol & Stewart, 2002).

2.5.4.2 Cell Cycle and Cancer

Cell cycle is the main regulator in cell proliferation and growth, while cancer is a disease of inappropriate cell proliferation (Collins et al., 1997). In other words, deregulation of cell cycle will push cancer cells into uncontrolled proliferation. Aberrations in cell cycle progression occurs in the majority of human malignancies (Zhang et al., 2006). In the development of tumors, cell cycle deregulation provides cancer cells with an infinite life span, since differentiation can be aborted, cellular senescence can be prevented and the cells can remain insensitive to growth inhibitory signals (Cangi et al., 2000). Many of the anticancer agents from natural products and chemotherapy agents exert their respective effects by interrupting or arresting cell cycle progression at specific phases (Shapiro & Harper, 1999). Drugs such as methotrexate and mercaptopurine act at the S phase, preventing DNA synthesis (Kanemitsu et al., 2009; Tsurusawa et al., 1990).

2.6 Metastasis

Generally, OSCC is characterized by a high rate of recurrence and metastasis to the regional lymph nodes. Nodal metastasis is considered to be a significant prognostic indicator (Noguti et al., 2012).

Metastatic cancer cells must undergo certain processes, such as interacting with the local microenvironment, migration, invasion, resistance to apoptosis and induction of angiogenesis (Geiger & Peeper, 2009). Epithelial tissues are the genesis of most solid tumours and are capable of forming relatively rigid sheets of cells. They are separated from the stroma using a basement membrane and are arranged within lateral belts of cell-cell adhesion complexes. During the development from a tumour in situ to an invasive carcinoma, epithelial tumour cells are released from their adjacent cells and break the basement membrane barrier. Epithelial-mesenchymal transition (EMT) has been

suggested to be the fundamental factor in this process (Christofori, 2006) and is characteristic of cancer metastasis. It is also the process that helps epithelial cell to lose interactions with the basement membrane due to it possessing the mesenchymal cell phenotype, made up of improved migratory ability, invasiveness and resistance to apoptosis (Singh & Settleman, 2010). Cell migration is imperative towards multiple physiologic, as well as pathologic processes, including normal development, angiogenesis, inflammatory responses, wound repair and also tumour invasion. Lymph node metastatic tumors occur in 40% of OSCC patients (Noguti et al., 2012). Moreover, up to 25% of OSCC cases have evidence of distant metastasis (Woolgar, 2006).

2.7 Chemical carcinogenesis induction in animal models.

Since the 1950s of the last century, researchers have carried out experiments on chemical Carcinogenesis protocols in the field of cancer research (Levy & Ring, 1950). The use of chemical carcinogenesis in models of carcinogenesis in animals is mainly attributed to the speedy identification of the chemical components of tobacco and alcohol which are the causes of most human SCC of the oral cavity (Mognetti et al., 2006). There are also various rodent models which have been employed *in vivo* for the purpose of evaluating cancer. They depend on the use of chemicals, transplant and genetic methods (Mognetti et al., 2006).

2.7.1 4-Nitroquinoline-1-Oxide (4NQO)

The 4NQO carcinogen is have been extensively studied by researchers (Table 2.3). It is defined as a synthetic derivative of quinoline soluble in water which is, sensitive to high temperature and light (Wilkey et al., 2009). The administration of this carcinogenic agent to rodents in water simulates leads to damaging DNA. And simulates tongue carcinogenesis in rodents (Minicucci et al., 2009). The mechanism of action of 4NQO takes place by generating reactive oxygen species (ROS) and nitrogen (RNS) such as

superoxide radicals, hydrogen peroxide and nitric oxide inducing an intracellular oxidative stress (Nunoshiba & Demple, 1993). ROS/RNS directly and indirectly damage macromolecules, including DNA, joining predominantly of guanine residues, which cause adducts (Kanojia & Vaidya, 2006). The lesions that caused by 4NQO represents the lesions that caused by other carcinogens existing in the smoking, which is an important risk factor for OC. Researchers have carried out studies comparing between human and hamster's oral cancer in relation to the carcinogen activation agents, oxidative stress, cell proliferation, apoptosis, invasion, and angiogenesis. Such studies aimed at validating models of chemical carcinogenesis in animals. They obtained results are indicative of the aberrant expression of multiple molecules in major signaling pathways in oral cancer. Such results also emphasize the importance of these models as a tool to monitor oral oncogenesis (Nagini et al., 2009). Regarding the method of application of 4NQO, previous research gained best results especially when dissolving carcinogen in the drinking water of animals (Lu et al., 2006; Tang et al., 2004). Researchers introduced the first study models for oral carcinogenesis by using polycyclic aromatic hydrocarbons induce oncogenesis. DMBA (dimethylbenzanthracene), the methylcholanthrene, benzopyrene and nitroquinoline oxide (4NQO) are important examples of polycyclic aromatic hydrocarbons. 4NQO plays an important role in inducing a strong intracellular oxidative stress while generating reactive oxygen species (ROS) such as superoxide radicals and hydrogen peroxide, which seems to be the reason behind inducing mutations in nitrogenous bases of DNA, and genetic deletions and chromosomal aberrations conversions, as well as breaks and chromatin changes (Kanojia & Vaidya, 2006). Ribeiro et al., (2004) reported that the DNA damage sites, including incomplete repair significantly increased in just 4 weeks of use of 4NQO, which is concurrent with this activity (Ribeiro et al., 2004). In addition, according to Nishimura et al., (1999) and Okazaki et al., (2002), there was an increasing expression of Bcl-2 before occurrence of

histological changes in tissue when using the carcinogen (Kanojia & Vaidya, 2006; Nishimura, 1999; Okazaki et al., 2002). From the reviewed literature, it is apparent that the initial application of nitroquinoline oxide (4NQO) in the oral cavity initially was traced to 1965 when Fujino et al., used the carcinogen diluted to 0.25% in benzene, which was applied topically (Tanaka et al., 2002; Tang et al., 2004). This study suggested a variety of methods for applying 4NQO in experimental studies on carcinogenesis. However, the carcinogen which had been employed by dilution benzene was substituted by preparing 4NQO diluted to 0.5% propylene glycol and applied three times a week using a brush (Wallenius & Lekholm, 1973). The same method of application was used by Henriques et al., (2011) in his study where experimental groups received the application of 4NQO diluted to 0.5% propylene glycol, with a brush on the left side edge of the tongue. The most important routes of oral administration of carcinogens is through liquid solutions (Okazaki et al., 2002; Ribeiro et al., 2005; Vered et al., 2003).

A study by Noguti et al., (2012), the researchers focused on the application of the carcinogen in the water of the animals for a minimum period of 4 weeks, 12 and maximum of 20 weeks (Noguti et al., 2012). It was found that from the 12th week of application of 4NQO there were the first changes only that occurred in the form of hyperplasia, hyperkeratosis, and dysplasia to moderate forms. However, from the 20th week, the researchers observed dysplasia that was moderate to severe, and squamous cell carcinoma, particularly of the latter type. Whereas concentration of 4NQO appears to have an influence on and to modify the amount and the time of onset of lesions, the application method needs to be considered. Henriques et al., (2011) carried out a study in mice by applying 4NQO topically in the left lateral border of the tongue. It was found that two months after applying 4NQO, the mild dysplasias became moderate and appeared more in the form of marked changes only from the third and fourth month, being present

in addition to moderate dysplasia, carcinoma in situ, and superficial invasive carcinoma, respectively. In repeating the 4NQO application, Mognetti et al., (2006) reported production of squamous cell carcinoma in mice tongue (Mognetti et al., 2006). These histopathological changes which were also seen in the lingual epithelium during the process are hyperplasia, mild, moderate and severe atypia prior to forming invasive carcinoma (Tanaka et al., 2002). Other few studies attempted to address the impact of nitroquinoline oxide on gingival tissues. For instance, Wallenius and Lekholm (1973) reported the injury to three lower gum among the thirteen rats treated with 4NQO for seven months. In other places, there were higher rates of lesions, and 100% of lesions on the palate and 75% of tongue lesions (Wallenius & Lekholm, 1973). In another study by Ohne et al., (1981), it was found that there was squamous cell carcinoma induction after 7 months of treatment in all treated mice. Moreover, the results indicated that there were tongue injuries in 18 rats, 15 in the hard palate, 4 soft palates, 2 in the jugal mucosa, while 3 rats displayed lesions on the gums, and 2 of them showed ulcerated lesions (Ohne et al., 1981).

Table 2. 3: 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., 2014)
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)

CHAPTER 3: THE ANTICANCER EFFECT OF GONIOTHALAMIN ON ORAL CANCER CELLS *IN VITRO*

3.1 Introduction

Oral cancer, a subtype of head and neck cancer is any cancerous tissue growth located in the oral cavity (Werning, 2007). There are several types of oral cancers, but around 90% are squamous cell carcinomas. Approximately, 263,900 cases of OSCC and 128,000 OSCC-related deaths are projected annually around the world (Jemal et al., 2011). Current modes of treatment for oral cancer include adjuvant chemotherapy, radiotherapy and surgical ablation (Jemal et al., 2011). A number of undesired side effects sometimes occur during cancer chemotherapy. The use of natural products or compounds, such as plant-derived products is one of the promising approaches in cancer chemotherapy as it may reduce adverse side effects (Cragg & Newman, 2005).

Goniothalamin, locally known as “gajahberanak”, “penawarhi- tam” or “monsoi” is a natural occurring styryl-lactone compound isolated from the roots, stems and leaves of Goniothalamus macrophyllus (Al-Qubaisi et al., 2011). In Malaysia, the decoctions of G. macrophyllus are used for the treatment of colds, fever, malaria, and swellings. A recent study on GTN documented a wide spectrum of therapeutic properties including anti-microbial, anti-cancer and apoptosis-inducing in mammalian cancerous cell lines (Chen et al., 2005). Goniothalamin was reported to exert potent cytotoxicity against various cancer cell lines. This included colon gastric (HGC-27), kidney (768-0), breast carcinomas (MCF-7, T47D, MDA- MB-231) and leukemia (HL-60, Jurkat, and CEM-22) (Inayat-Hussain et al., 2010; Rajab et al., 2005). In addition, GTN showed lower toxicity to normal liver Chang cell line as compared to doxorubicin (a chemotherapy drug) (Al-Qubaisi et al., 2011). Besides, GTN was able to induce apoptosis in cervical cells (HeLa), oral cancer cells (Ca9-22), vascular smooth muscle cells (VSMCs) and coronary artery

smooth muscle (CASMCs) as well as arrested cell cycle in liver (HepG2) cells (Al-Qubaisi et al., 2011; Alabsi et al., 2013; Chan et al., 2006; Yen et al., 2012). Therefore, this study was carried out to investigate the cytotoxicity properties and the mechanism of apoptosis inductions of GTN in oral squamous cell carcinoma (H400) cells.

3.2 Materials and methods

3.2.1 Goniothalamin

Goniothalamin (GTN) was kindly obtained from Professor Dr. Abdul Manaf Ali, Faculty of Bioresource and Food Industry, University Sultan Zainal Abidin, Terengganu, Malaysia.

3.2.2 Cell culture

H400 Human Oral Squamous Cell Carcinoma, isolated from the alveolar process of the oral cavity, and the normal human oral fibroblasts (NHOF)were obtained from Prof. Ian Charles Paterson, Department of Oral Biology and Biomedical Sciences, Faculty of Dentistry, University of Malaya. The cells were grown in a volume of 5 mL in a 25 cm^2 flask, or 10 mL in a 75 cm^2 flask (Corning, USA). The growth and morphology of the cells were regularly monitored and the culture medium was renewed 2-3 times weekly. The cells were harvested using 0.25% trypsin-EDTA (Nacalai Tesque, Kyoto, Japan) when they reach 70-80% confluency in culture flasks. All cell culturing procedure was conducted under sterile conditions in a class-II biological safety cabinet to prevent the onset or proliferation of contaminations.

3.2.2.1 Cell Passaging

Cell passaging or splitting is a technique that enables an individual to keep cells alive and growing under cultured conditions for extended periods of time. In the current study, the old growth media was discarded, the adherent cells were rinsed with sterile phosphate

buffer saline (without Ca_{2+}or Mg_{2+}) to remove serum or other proteins that might inhibit the action of trypsin and the cells were trypsinized with 0.25% trypsin-EDTA. The detached cells were re-suspended in a serum-containing growth medium (to neutralize the trypsin) and the suspension was gently aspirated a few times to avoid cell clumps and then added to a 15 mL conical centrifuge tube. The cells were spun at low speed for 3-4 min to pellet the suspended cells. The supernatant was discarded and the cell pellet was mixed with 1 mL of medium.

3.2.2.2 Cell Counting

Vital stain trypan blue (10 μL) was thoroughly mixed with the cell suspension (10 μL) and carefully loaded on a LunaTM cell counting slide. The number of total, live and dead cells and the percentage of viability were then obtained by LunaTM automated cell counter machine (Logos Biosystems, USA).

3.2.2.3 Cryopreservation of the Cells

Cryopreservation of the cells was performed to store the cells in liquid nitrogen for future use. The freezing medium consists of 90% pure foetal bovine serum (FBS) and 10% Dimethyl sulphoxide (Vivantis, USA). Generally, the cell suspension was spun at 1000 rpm for 10 min at 4ºC. Cell pellet was re-suspended in a freezing medium and transferred to a cryogenic vial (Nunclon TM, Denmark) at 2×10^6 cells/vial. The vials were tightly capped, properly labelled and placed in a freezing container, which was then stored at -80ºC overnight prior to being transferred into a liquid nitrogen tank for long-term storage.

3.2.2.4 Thawing of Frozen Cells

Cryopreserved cells from liquid nitrogen were thawed when required for the experiments. Basically, the frozen cells were thawed by rapid agitation in a 37ºC water

bath (Memmert, Germany) and transferred to a 15 mL conical centrifuge tube containing warm growth medium. Following the centrifugation at 1000 rpm for 10 min, the supernatant was discarded and the cell pellet was re-suspended with fresh medium and transferred to a 25 cm^2 culture flask (Corning, USA). The flasks were then incubated at 37ºC in a humidified atmosphere of 5% CO_2.

3.2.3 MTT cytotoxicity assay

Cytotoxicity of GTN was determined through Microculture Tetrazolium Test (MTT) Assay as previously described (Mosmann, 1983). H400 and NHOF were cultured in 96 well-plates at a density of 1×10^5 cells per well and incubated for 24 h until they attached. The cells were treated with varying concentrations of GTN and incubated for 72 h. After 72 h a volume of 20 µl of MTT (5 mg/ml) was added per well then incubated at 37 ºC in a 5% CO2 atmosphere for 4 h. The supernatant was removed and 100 ml of DMSO was added. Finally, the absorbance was measured at 570 nm and 630 nm as references on Tecan Infinite M200 Pro ELISA microplate reader (Männedorf, Switzerland). In order to investigate whether the cytotoxicity of GTN was specific towards cancer cells, the cytotoxic effect on Normal Human Oral Fibroblast (NHOF) cells was determined using MTT assay. Selectivity Index (SI) was defined as the ratio of cytotoxicity (IC_{50}) on normal cells to cancer cells (IC_{50} on NHOF/IC_{50} on H400 cells). SI value greater than 3 was considered specific towards cancer cells (Mahavorasirikul et al.,2010).

3.2.4 Mitochondrial membrane potential

Mitochondrial membrane potential was determined using JC- 10 mitochondrial membrane potential assay kit (ABCAM, USA) according to manufacturer's instruction. Briefly, about 90 µl of H400 cells (1×10^5 cells/ml) were seeded in a 96 well black color plate and incubated at 5% CO2, 37 ºC for 24 h. Treatment with GTN for 24 h was carried out by adding 10 µl of 10 times concentrated IC_{50} of GTN. Untreated cells were used as

negative control. Fluorescence intensity at excitation/emission 490 nm/525 nm for green color and 490 nm/590 nm for red color were measured using Tecan Infinite M200 ELISA plate reader (Männedorf, Switzerland).

3.2.5 Cytochrome c detection

The intracellular level of cytochrome c in GTN-treated H400 cells was determined by cytochrome c ELISA Kit (Invitrogen, USA) according to the manufacturer's instructions. Briefly, 1×10^5 cell/ml of cells were seeded and treated with GTN at a concentration of IC_{50} for 24 h. Untreated cells were used as negative control. After 24 h of treatment, cells were harvested for proteins extraction purpose. The concentration of Cytochrome c standard used was 0–5 ng/ml. Absorbance reading at 450 nm for samples and standard was measured using Tecan Infinite M200 Pro ELISA plate reader (Männedorf, Switzerland). The concentration of cytochrome c in H400 treated cells was determined using the cytochrome c standard curve and expressed as ng per ml.

3.2.6 Caspases activity

Caspase-3/7, caspase-8, and caspase-9 were performed using Caspase-Glo 3/7, 8 and 9 Assay kits from Promega according to to manufacturer protocol. Briefly, H400 cells were seeded in a 96 well white plate and kept under 5% CO_2 at 37 C for 24 h. Cells were then treated with GTN at a concentration of IC_{50} and IC_{25} for 24 h. Untreated cells were used as negative control. Caspase-Glo buffer was thawed and equilibrated to room temperature prior to use. This buffer was then used to dissolve lyophilized Caspase-Glo substrate and the mixture was vortexed to obtain a homogenous solution. For caspase-Glo 8 and 9, the MG132 inhibitor was added. Prior to the addition of Caspase-Glo reagent, culture plate was equilibrated to room temperature. Then, about 100 µl of the reagent was added to each well and placed on an orbital shaker for 30 s. The plate was then further

incubated at room temperature for 2 h in dark. The luminescence signal was then measured using Tecan Infinite M200 Pro ELISA plate reader (Männedorf, Switzerland).

3.2.7 Involvement of Human Nuclear factor-kappa B (NF-kb) assay

Quantification of NF-kb was carried out using Human Nuclear factor-kappa B (NF-kb) ELISA kit (Cusabio, China) according to manufacturer's instruction. Briefly, 1×10^5 cell/ml of H400 cells were seeded and treated with GTN at a concentration of IC_{50} for 72 h. untreated cells were regarded as the negative control. After 72 h of treatment, GTN-treated H400 cells were harvested and lysed through three freezes–thaw cycle to obtain the cell lysate. The concentration of NF-kb standard used was 0–5 ng/ ml. Absorbance reading for measurement wavelength and refer- ence wavelength at 450 nm and 540 nm, respectively was carried out using Tecan Infinite M200 Pro ELISA plate reader (Männedorf, Switzerland). The concentration of NF-kb in treated-H400 cells was determined using NF-kb standard curve and expressed as ng per ml.

3.3 Statistical analysis

Data were expressed as mean ± SEM in triplicates. Statistical analysis was performed using t-test (SPSS version 17). A p-value < 0.05 was considered statistically significant.

3.4 Result

3.4.1 MTT cytotoxicity assay

Cytotoxic effect of GTN on H400 oral cancer cells were evaluated using MTT assay (Table 3.1). As shown in Table3.1 percentage of H400 cell viability decreased with an increased in the concentration of GTN. The IC_{50} value of GTN (concentration causing the death of 50% of H400 oral cancer cells) was 0.3 µg/ml after 72 h of treatment. In order to investigate whether the cytotoxicity of GTN was specific towards H400 cells, the cytotoxic effect of GTN was screened on normal human oral fibroblast (NHOF) cells and

Selectivity Index (SI) was calculated. Table 3.1 showed the response of NHOF cells towards GTN. An IC_{50} value of 22.27 µg/ml and SI value of 12.37 were recorded, suggesting that the cytotoxicity of GTN was selective towards H400 oral cancer cells (SI >3). Among the tested cell lines, maximum growth inhibition was observed in H400 (0.3 µg/ml) and thus this cell line was selected for further study.

Table 3.1: Cytotoxic effects of GTN on the viability of oral cancer cell lines (H400) and normal human oral fibroblast (NHOF) cells at 72h treatment. Values were presented as mean±SEM from three independent experiments.

Cell lines	**IC_{50}**	**SI**
H400	0.3 ± 0.01 µg/ml	12.37
NHOF	22.27 ± 2 µg/ml	

3.4.2 Mitochondrial membrane potential

Mitochondrial permeability transition is an important step in the induction of cellular apoptosis. Loss of mitochondrial potential (depolarization) is a classical evident of apoptosis. Fig. 3.1 showed an increase in the ratio of green/red relative fluorescence unit for H400 cells treated with GTN (1.18±0.02 RLU) as compared to untreated cells (0.44± 0.27 RLU) upon 24 h of treatment. These indicated that GTN induced depolarization of mitochondrial membrane potential in H400 cells during apoptosis.

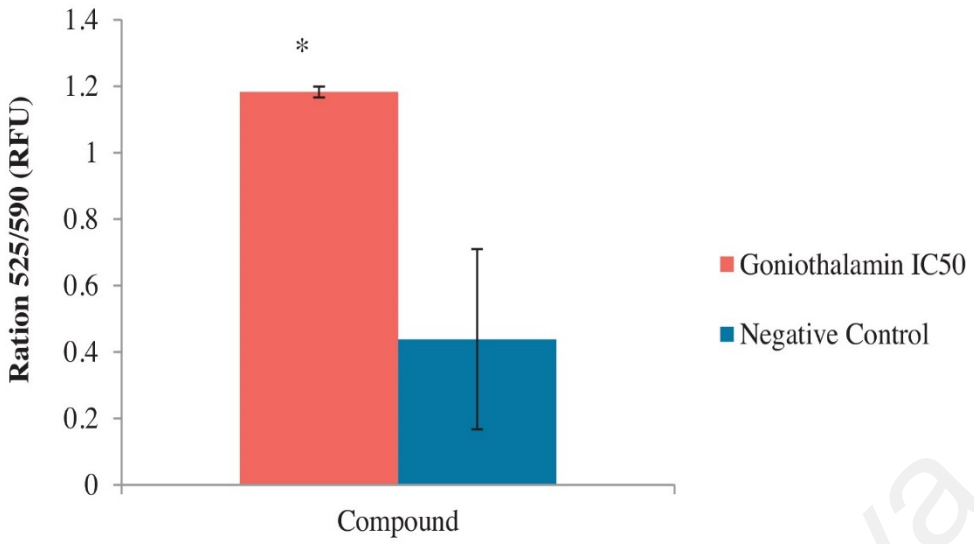


Figure 3.1: Changes in mitochondrial membrane potential in H400 cells treated with GTN at a concentration of IC_{50} for 24 h. Untreated cells were used as negative control. Data were expressed as mean ±SEM (n = 3). *Significant differences as compared to negative control ($p < 0.05$) when statistical analysis using t-test was performed.

3.4.3 Cytochrome c detection

The release of cytochrome c from the inner membrane of the mitochondria into the cytosol is an indicator of mitochondrially dependent apoptosis. In the present study, there was a slight increment of cytochrome c in GTN-treated H400 cells (99.05±0.45 ng/ml) compared to untreated cells (98.92±1.51 ng/ ml). However, this increment was not statically significant. This result is in agreement with the suggestion that GTN induced depolarization of MMP which lead to the translocation of cytochrome c into the cytosol (Fig. 3.2).

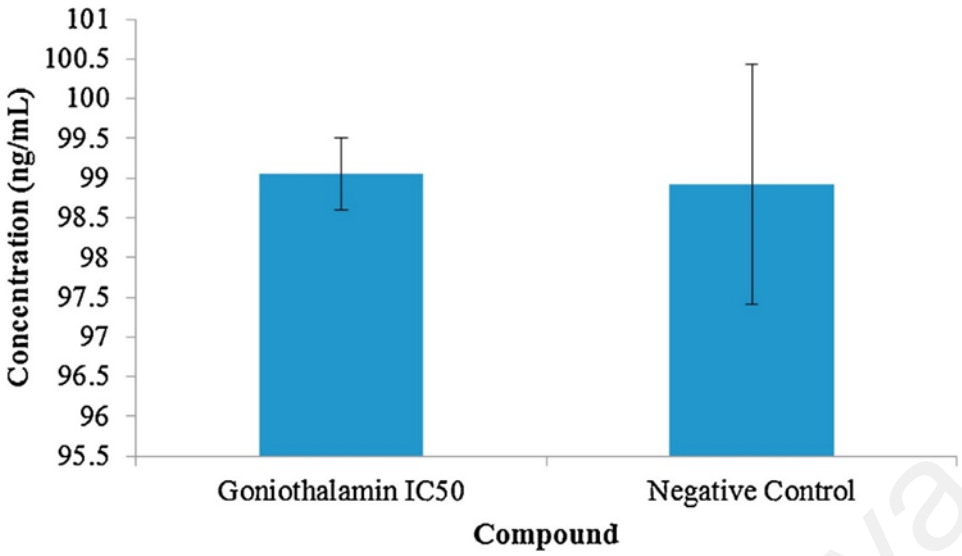


Figure 3.2: Effects of GTN on intracellular cytochrome c in H400 cells. Cells were exposed for 24 h at a concentration of IC_{50}. Untreated cells were used as negative control. Data were expressed as mean ±SEM (n = 3) using t-test statistical analysis.

3.4.4 Caspases activities

The caspase activation was determined using Caspase-Glo reagent and the amount of luminescence produced was calculated as Relative Luminescence Unit, RLU. Results demonstrated that GTN-treated H400 cells showed a significant increment of caspases activities compared to the negative control in a concentration-dependent manner at IC_{50} and IC_{25}. However, the amounts of RLU were different between these 3 caspases. Fig. 3.3 showed that caspase 3/7 showed the highest amount of RLU followed by caspase 9 and the least is Caspase 8. The higher amount of RLU of caspase 9 compared to caspase 8 showed that the induction of apoptosis in H400 cells by GTN was mainly through the intrinsic pathway. The higher amount of RLU of caspase 9 compared to caspase 8 indicates an activation of the extrinsic pathway but not as much as the intrinsic pathway.

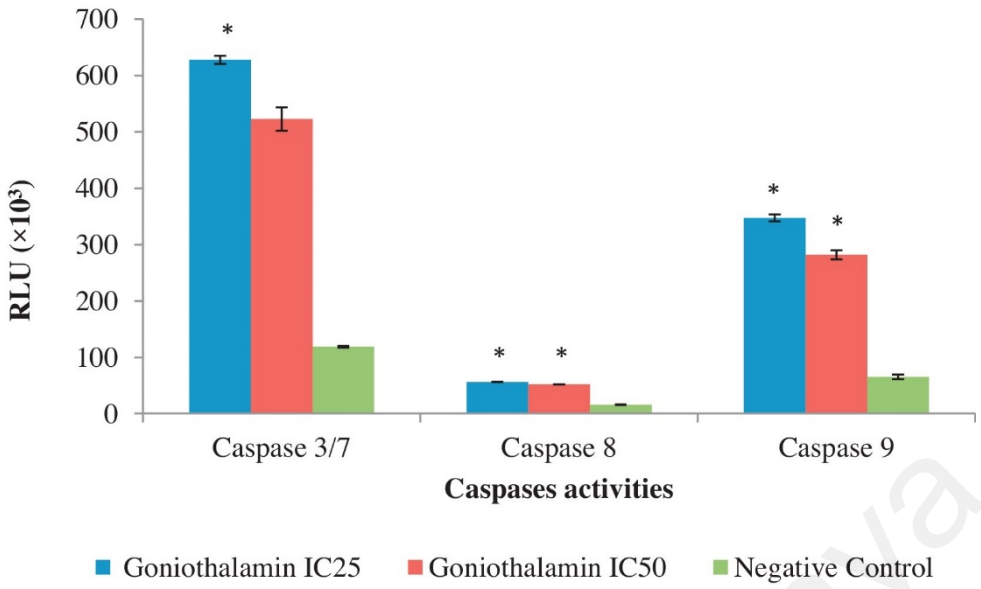


Figure 3.3: Relative luminescence expression of Caspase-3/7, 8 and 9 in H400 cells treated with GTN at concentration of IC_{50} and IC25 for 24 h. Untreated cells were used as negative control. Data was expressed as mean ± SEM (n = 3). *Significant differences as compared to negative control ($p < 0.05$) when t- test statistical analysis was performed.

3.4.5 Involvement of Human Nuclear factor-kappa B (NF-kb) assay

The blockage of apoptosis and cell proliferation is closely associated with the activation of NF-kb. Hence, we next examined the effects of GTN against NF-kb activation in H400 cells. Results demonstrated that H400 cells treated with GTN at a concentration of IC_{50} showed decreased in NF-kb concentration (17.20±0.70 ng/ml) compared to untreated cells (19.68±2.06 ng/ml). This indicated that GTN exerted an inhibitory effect against the activation of NF-kb in H400 cells that halt the translocation of NF-kb from the cytoplasm to nucleus. In other words, GTN induced apoptosis was associated with the inhibition of the NF-kb (Fig. 3.4).

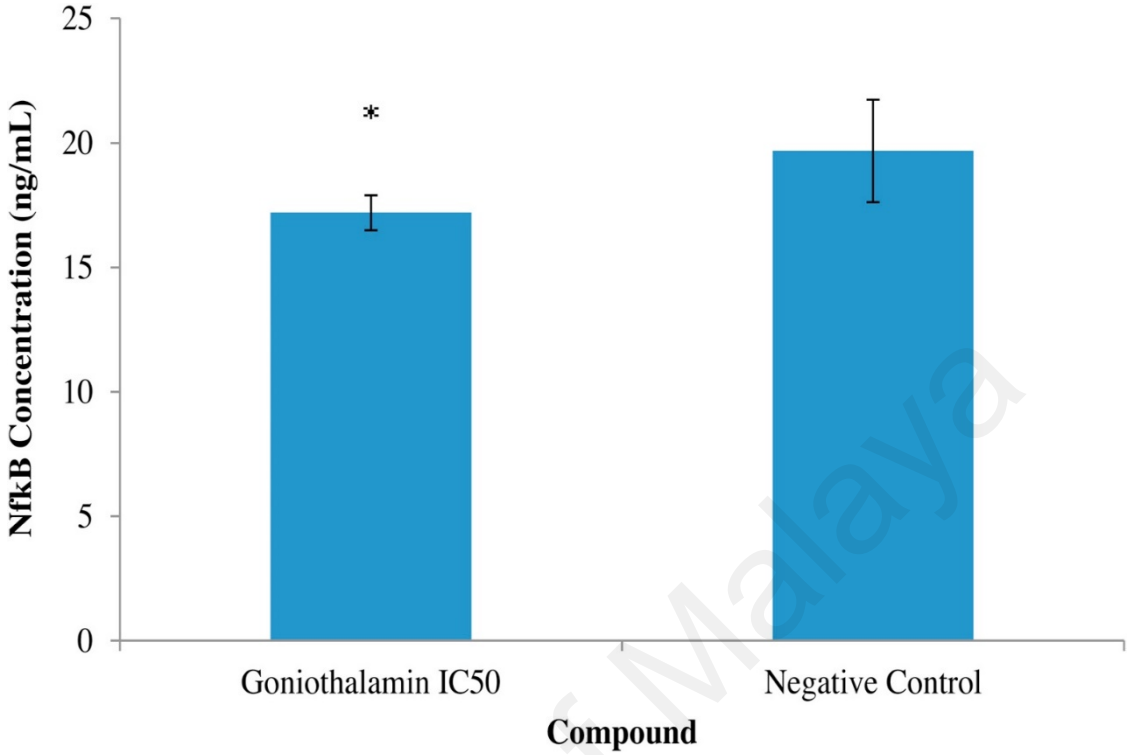


Figure 3.4: Effect of GTN at concentration of IC_{50} on inhibition of NF-kb in H400 cells after treatment for 72 h. Untreated cells were regarded as negative control. Data were expressed as mean ±SEM. *Significant differences as compared to negative control ($p < 0.05$) when t-test statistical analysis was performed.

3.5 Discussion

The previous study on GTN reported a promising anticancer property with potent cytotoxic effects towards various cancer cell lines. In this study, the cytotoxic effect of GTN on the viability of H400 oral cancer cells was determined through MTT assay. MTT assay is a colorimetric assay measuring the activity of dehydrogenase enzymes from living cells that reduces the yellow MTT into purple formazan (Mosmann, 1983). MTT assay is used to determine the IC_{50}, concentration of GTN required for 50% growth inhibition in oral cancer cells in vitro. In the present study, results suggested that GTN exhibited potent cytotoxic effects on H400 cells with an IC_{50} value of 1.8 µg/ml. The cytotoxic effects exerted by GTN on H400 cells were in concentration-dependent manners following 72 h of treatment. This finding was supported by previous studies

done by other researchers which reported that GTN was cytotoxically active on not only oral cancer cell (Ca9-22) but also several others type of cancer cells including human breast cancer, vascular smooth muscle cells (VSMCs), Jurkat leukemia cells, HL-60 leukemia cells, Chinese hamster ovary (CHO) and hepatoblastoma HepG2 cells (Al-Qubaisi et al., 2011; Azimahtol Hawariah et al., 1998; Chan et al., 2006; Chen et al., 2005; Inayat-Hussain et al., 2003; Inayat-Hussain et al., 1999; Mosmann, 1983; Umar-Tsafe et al., 2004; Yen et al., 2012). An ideal anticancer drug should incapacitate cancer cells without causing excessive damage to normal cells (Taraphdar et al., 2001). Compounds with a selectivity index (SI) greater than 3 were considered to have high selectivity towards cancer cells (Mahavorasirikul et al., 2010). In the present study, a selectivity index (SI) greater than 3 was recorded, suggesting that the cytotoxicity of goniothalmin was selective towards H400 oral cancer cells.

Apoptosis can be an extrinsic pathway mediated via activation of death receptors or by an intrinsic mitochondria-mediated pathway (Jiang et al., 2013). Mitochondrial plays an important role in the regulation of apoptosis (Simon et al., 2000). Fluorescent probes are a tool frequently used to assess the mitochondrial function by monitoring the mitochondrial membrane potential (Dym) (Perry et al., 2011). In the present study used JC–10 was used as a ratio metric probe which was capable of entering selectively into mitochondria and changed reversibly its color from green to orange as the membrane potential increase. This measurement is independent of variation in mitochondrial mass or volume (Griffiths, 2000; Nicholls, 2006). We demonstrated that GTN induced depolarization of mitochondrial membrane potential (MMP) in H400 cells. This is in agreement with the earlier study conducted by Yen *et al.* (2012) which reported that GTN significantly decreases MMP in Ca9-22 oral cancer cells. Besides, depolarization of

MMP was also documented in leukemia cells and coronary artery smooth muscle cells (Chan et al., 2010; Inayat-Hussain et al., 2010).

Changes in MMP cause the translocation of cytochrome c from mitochondrial into cytosol which in turn activates a full caspase cascade activity. We next examined the intracellular level of cytochrome c using ELISA. In agreement with the depolarization of MMP, GTN-treated H400 cells showed a slight increase of cytochrome c as compared to untreated H400 cells. However, this increase of cytochrome c was not statistically significant. Our data support the previous study which documented the release of cytochrome c upon treatment with GTN in human breast cancer cell (MDA-MB-231) as well as coronary artery smooth muscle cells (Chan et al., 2010; Chen et al., 2005).

The induction of apoptosis is associated with the activation of caspase ((Thornberry & Lazebnik, 1998). The released of cytochrome c trigger the activation of caspase 9 through the formation of the apoptosome. Subsequent activation of the initiator caspase 9 causes the cleavage of effectors caspase 3/7 which then activates DNase and cause DNA fragmentation in the nucleus (Kang & Reynolds, 2009). To confirm whether the cytochrome c released plays its role in further activated downstream mitochondrial-mediated apoptosis pathway, caspases 3/7, 8 and 9 activities were determined. Results demonstrated a significant increment in initiator caspase 9 and executioner 3/7 activities in dose-depending manners, indicating the activation of caspase cascade activity with the release of cytochrome c into the cytosol of GTN- treated H400 cells. Besides, higher activity of caspase 9 compared to caspase 8 imply that caspase 8 plays an insignificant role as an initiator caspase in activating executioner caspase 3/7 in GTN- treated H400 cells. The previous study on GTN- treated HeLa cells and CASMCs (Coronary Artery Smooth Muscle Cells) reported the sequential activation of caspase 9 but not caspase 8 leading to the downstream caspase 3 cleavage (Alabsi et al., 2013; Chan et al., 2010).

Besides, GTN induced apoptosis in HL-60 and Jurkat cells via mitochondrial pathway (Inayat-Hussain et al., 2010). NF-kb plays a vital role in regulating cellular growth and apoptotic cell death (Barkett & Gilmore, 1999). Chemotherapy agent with the NF-kb inhibition properties could enhance the therapeutic efficacies. Our result demonstrated that goniothalmin inhibited the activation of NF-kb in H400 oral cancer cells upon treatment. The previous study reported similar results in Jurkat and K562 leukemia cells, in which GTN prevented TNF-a-induced NF-kb activity (Orlikova et al., 2013).

In the present study, GTN was dissolved using 0.09% Dimethyl Sulfoxide (DMSO). DMSO considers safe solvent at ≤ 0.1% and has been used to dissolve GTN previously (Mi et al., 2016; Vendramini-Costa et al., 2010).

3.6 Conclusion

In conclusion, this study clearly demonstrates for the first time that GTN exhibited selective cytotoxic effects and induced apoptosis in H400 oral cancer cells through mitochondrial-mediated pathway associated with the depolarization of MMP, releasing cytochrome c into the cytosol and further activated caspases 3/7 and 9. Hence, it is worth to highlight that GTN has the potential to be developed as an anticancer agent for oral cancer.

CHAPTER 4: HISTOLOGICAL, BIOCHEMICAL AND HAEMATOLOGICAL EFFECT OF GTN COMPOUND ON SELECTIVE INTERNAL ORGANS OF RATS

4.1 Introduction

Toxicity studies are important in determining the toxicity of a given substance using a selected animal model in the prediction of adverse effects, the extrapolation of these effects in humans and in the determination of the safety level of the substance used (Katzung et al., 2004).

Medicinal plants have been used from prehistoric times to the present. People used the natural elements to cure both human and animal diseases. This knowledge was transmitted from generation to generation and was perfected with experience and gained wide acceptance in recent times. The high cost of orthodox medicine which poorer people might not afford and the impression that natural plant medicines had fewer side effects could be the reason for this trend (Cohen-Kohler, 2007; Osujih, 1993). The World Health Organization (WHO) estimated that about 70-80% of the population has used traditional medicine in some way or another in many developed countries (Organization, 2008). Similarly, there is also an increasing interest in herbal medicines among people in developed countries (Aschwanden, 2001). However, safety and efficacy of herbal medicines are still not sufficiently explored (Organization, 2008). Herbal medicine has been used with an assumed impression that it is naturally safe and has fewer side effects (Chan, 2009; Larrey, 1994). In order to determine any toxicity, it is important to study these natural products for a possible cause of poisoning in consumers.

The use of herbal medicines attracts criticism largely due to the lack of scientific assessment of their toxicity. some of these herbal medicines have been found to be toxic (Ernst, 2005; Yeung et al., 2008). For this reason, toxicity should be evaluated on those

herbal medicines for which some medicinal properties have already been confirmed (Saad et al., 2006). One such widely used and studied plant derivatives is GTN which is a bioactive styryl-pyrone isolated from Goniothalamus species within Annonaceae family. It is widely distributed throughout Asia and is used as a medicinal plant (Al-Qubaisi et al., 2011; Chen et al., 2005; de Fátima et al., 2005; Wiart, 2007). Several studies reported the cytotoxicity of GTN against many cancer cell lines (Alabsi et al., 2012; Inayat-Hussain et al., 2010; Rajab et al., 2005). In comparison with other anticancer agents, GTN showed cytotoxicity cancer cell lines with no toxicity against normal cells (Lin & Pihie, 2003). Al-Qubaisi et al. (2011) compared GTN toxicity with the chemotherapeutic drug doxorubicin, against normal Chang liver cells. A much lower toxicity of GTN on those cells was reported. However, there is insufficient data on acute and sub-acute toxicity of this compound on animals is available. Hence, this study aims to investigate the histological, biochemical and hematological changes associated with GTN administration on selected internal organs of rats.

4.2 Materials and Methods

4.2.1 Goniothalamin

Goniothalamin (GTN) was kindly obtained from Dr. Abdul Manaf Ali, Faculty of Bioresource and Food Industry, University Sultan Zainal Abidin, Terengganu, Malaysia. The GTN compound was dissolved in phosphate-buffered saline and 1% DMSO and stored in the fridge at 4 ºC.

4.2.2 Animals

Male Sprague Dawley rats of 6 to 8 weeks old and an average weight of 200 - 230 g were purchased from Animal Experimental Unit (AEU) Faculty of Medicine (FOM), University of Malaya, and used as the experimental animals. The rats were housed in plastic cages, three rats per cage, and maintained in the (AEU). They were maintained

under standard conditions of temperature 25°C ± 2°C and relative humidity 70% ± 5% and 12 hours light-dark cycle. Feeding and water intake of the animals were done with standard rat pellets and tap water ad libitum throughout the experiments. In general, animal handling, from the beginning to the end of the study, was ethically done according to the agreed guidelines for the Animal Ethics Committee for Animal Experimentation, FOM, University of Malaya, Malaysia (Ethics No. 2016-190607/DENT/R/A).

4.2.3 Acute Toxicity Study

The experiment protocol was designed according to a previous study published elsewhere (Ahmed et al., 2012). Animals were kept for 5 days to allow for acclimatization to the laboratory conditions before the commencement of the study. The rats were divided randomly into 6 groups with 7 rats in each group. The assessment of the acute toxicity was conducted using intraperitoneal administration of GTN at doses of 100, 200, 300, 400 and 500 mg/kg, respectively. A control group was simultaneously administered a vehicle solution containing PBS and 1% of DMSO and all groups of animals were observed for 4 hours after administration of the dose and then for 24 hours. During the experiment, all observations were systematically recorded and maintained as an individual record. At the end of the time of the experiment, the deceased animal was counted in each group and LD_{50} (dose that kills 50% of animals) was calculated (420 mg/kg) according to the previous study published elsewhere (Miller & Tainter, 1937).

4.2.4 Sub-acute Toxicity Study

The rats in this part of the study were divided into two groups with 7 rats in each group. One group served as the experimental group while the other group served as the control. Rats in the experimental group were injected with 42 mg/kg (1/10 of LD_{50}) of GTN compound while rats in the control group were injected with vehicle solution (PBS and 1% of DMSO) daily for 14 days (Ahmed et al., 2012). The rats were observed

continuously for 4 hours and then daily for a period of 14 days. The body weights of animals were calculated and recorded shortly before the tested substance was administered and at the end of the experiment. The food consumption and water intake were recorded daily.

For both acute and subacute toxicity experiments, the following parameters of general toxicity signs such as effects on locomotion, behaviour (agitation, decreased activity, and somnolence) breathing, salivation, lacrimation, cyanosis, and death were evaluated (Iwamoto et al., 2015; LITCHFIELD & Wilcoxon, 1949).

4.2.5 Hematological and Biochemical Evaluation

At the end of both acute and sub-acute toxicity experiments, all the rats were generally anesthetized using an 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 samples were collected via *cardiac puncture* using a disposable syringe for haematological and biochemical evaluations.

4.2.6 Histopathological Evaluation

An autopsy was carried out in all rats. The selected organs (kidney, liver, lung, heart, spleen, and brain) were harvested and preserved in 10% buffered formalin until subjected to histopathological evaluation.

4.2.6.1 Tissue Preparation

(a) ***Fixation of the samples***

The rat organs were placed in a container containing 50 ml of neutral buffered formalin in 10% natural buffer formalin (NBF) and fixed for 24 hours. The ratio of tissue to fixative volume was 1:20.

(b) ***Grossing of the samples***

The NBF fixed samples were grossed according to the previous study published elsewhere (Parkinson et al., 2011).

(c) ***Processing of the samples***

Samples were proceed using a Leica TP 1020 tissue processor. This is an automatic machine where the specimen (organs) 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.

(d) ***Sectioning of the samples***

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

(e) ***Staining of the samples***

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 followed by 95 %, 70 % alcohol bath for 3 minutes and then in running water bath for 3 minutes at room temperature. Sections were stained with Harris' hematoxylin working solution for 5 minutes. Then, it was washed in slow running tap water until the excess blue color was washed 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 then 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 then placed in xylene I, II and III bath for 3 minutes and finally, the stained tissue sections were mounted and a coverslipped with DPX mounting medium and checked under the microscope. Hematoxylin and eosin stained slides were stored for microscopic examination.

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.

4.3 Statistical analysis

Results were expressed as a mean ± standard deviation. The differences between groups of acute and sub-acute toxicity tests were determined by analysis of variance (one-way ANOVA) and independent *t*-test. Differences were considered significant at $p < 0.05$.

4.4 Result

4.4.1 Acute toxicity

4.4.1.1 Clinical observation of acute toxicity

Among the animals treated with 300 mg/kg of GTN, the following sings were observed; eye secretion within one hour of treatment, reduce in mobility with no respond to stimuli and the appearance of tremors after 2 hours of treatment. All animals recovered after 4 hours of the treatment with no mortality. In animals treated with 400 mg/kg of GTN, 2 rats suffered from tremors, breathing difficulty and died within 4 hours of the

experiments. The remaining rats showed eye secretion after one hour of treatment, reduce mobility with no response to stimuli, and the appearance of tremors was observed within two hours of treatment. Four hours later, the 5 rats recovered. The animals treated with the dose of 500 mg/kg showed many signs of toxicity during the first hour of the treatment in which tremors, breathing difficulty, Straub tail, opisthotonos (head and neck arched back) and scattered seizures were observed. All the treated animals died within 4 hours.

4.4.1.2 Hematological and Biochemical Evaluation

The hematological profile of control and treated groups are summarized in Table 4.1. The results concluded that all hematological parameters such as hemoglobin (Hb) and total white blood cell count were within the normal range in both control and GTN-treated groups with no significant changes were observed between different doses of GTN-treated groups and the control group. Similarly, as shown in Table 4.2, there were no significant alterations observed between all biochemical parameters tested in the current study and different doses of GTN-treated groups and the control group.

Table 4. 1: Haematological evaluation for acute toxicity

INDEXES	Control	GTN doses mg/kg					*P* value
		100	200	300	400	500	
HGB (g/dL)	16.52±1.22	15.46±1.12	15.62±1.31	15.12±2.21	16.13±1.46	15.11±2.08	0.525
WBC (10^9/L)	6.33±0.32	6.11±0.52	6.61±0.58	5.99±1.14	6.47±053	5.71±1.21	1.000
Neutrophils	18.22±0.23	18.41±0.31	17.17±2.35	17.22±3.15	17.30±0.50	18.33±1.21	0.506
Lymphocyte	78.21±2.26	80.16±5.31	76.86±2.17	80.66±3.11	79.23±1.11	76.57±1.55	0.060
Monocytes	0.89±0.11	1.00±0.10	0.87±0.12	0.87±0.21	0.85±0.12	0.88±0.10	0.346
Eosinophils	3.23±3.68	3.13±2.79	3.41±3.21	3.22±2.31	3.11±2.45	3.51±2.55	1.000
Basophisl	0.01±0.21	0.01±0.11	0.01±0.02	0.01±0.07	0.01±0.10	0.01±0.06	1.000

Values are Mean±SEM, $*P$ values < 0.05 were considered significant using one-way ANOVA.

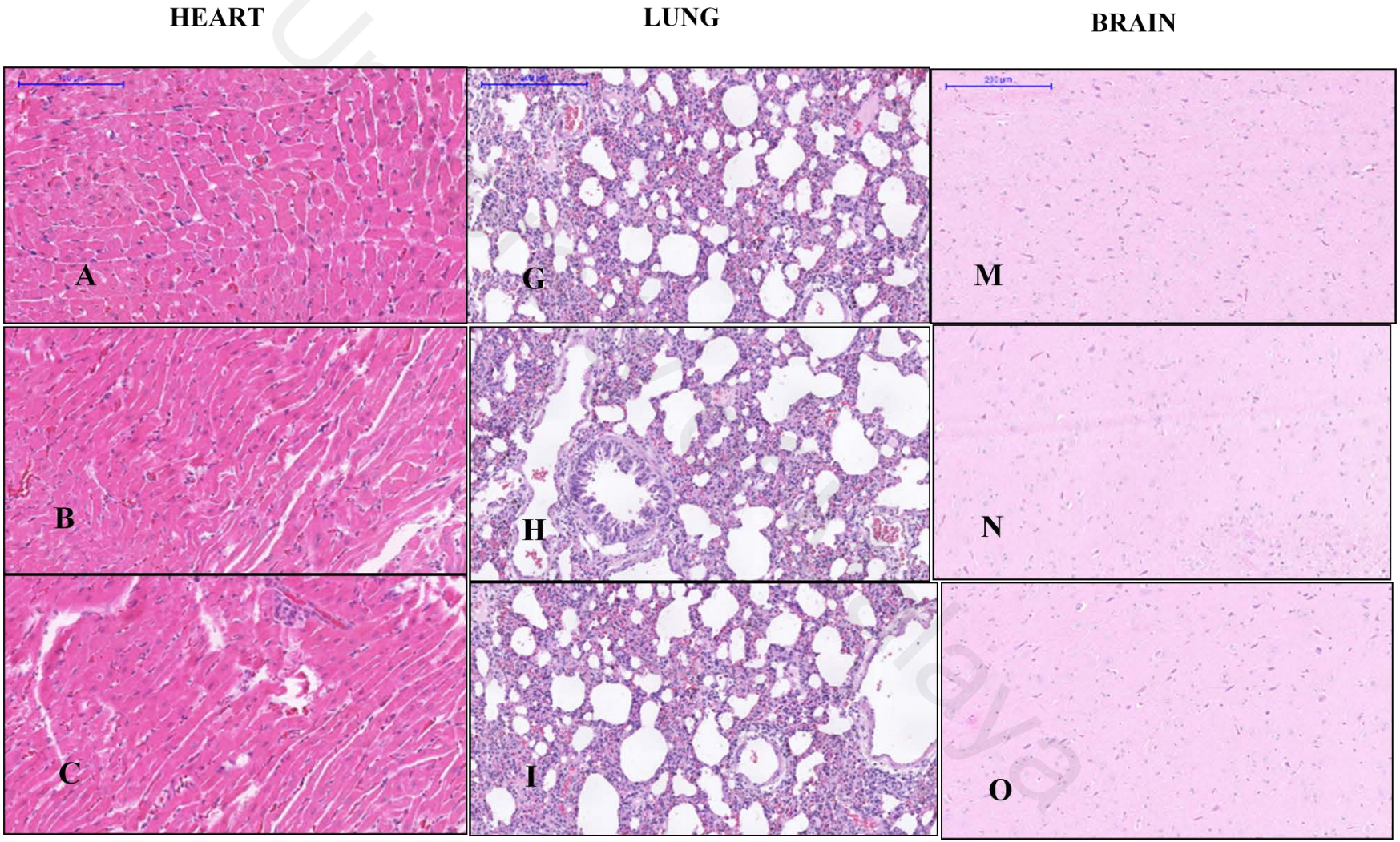
Table 4. 1: Biochemical evaluation for acute toxicity

Indexes	GTN doses mg/kg						*P* value
	Control	100	200	300	400	500	
Creat(μmol/l)	25.22±1.13	25.13±1.11	24.00±2.15	24.52±2.14	25.00±1.22	26.24±1.14	0.174
Urea(mmol/l)	5.95±0.33	5.60±0.15	5.87±0.13	5.90±0.21	5.91±0.32	5.85±0.15	0.089
Albumin (g/l)	35.9 ± 3.2	34.6 ± 3.7	34.2 ± 7.8	34.3 ± 3.2	33.5 ± 5.3	33.2± 4.3	0.928
Globulin (g/l)	23.2 ± 2.8	21.5 ± 1.8	22.3 ± 3.1	22.6 ± 2.9	21.2 ± 4.2	21.3 ± 3.9	0.815
T.bil (μmol/l)	2.0 ± 0.8	2.1 ± 0.7	2.0 ± 0.5	1.9 ± 0.4	1.9 ± 0.7	2.1 ± 0.9	0.988
ALP(U/l)	432 ± 11	424 ± 29	431 ± 26	422 ± 13	420 ± 14	435 ± 17	0.637
ALT(U/l)	118 ± 31	119 ± 11	116 ± 21	120 ± 10	117 ± 18	115 ± 12	0.996
AST(U/l)	153 ± 56	155 ± 32	153 ± 21	152 ± 42	155 ± 23	152 ± 32	1.000

Values are Mean±SEM, $*P$ values < 0.05 were considered significant using one-way ANOVA

4.4.1.3 Histopathological Evaluation

As shown in Figure 4.1 and Figure 4.2, microscopic examination of the tissue of all GTN treated animal showed that all rat organs (kidney, liver, heart, spleen, brain, and lung) for the five groups were normal. The histological and cellular structure of all organs showed unnoticeable differences. In liver, the cellular structure of hepatocytes, sinusoids, and central vein were similar to those in control group. In the heart, the cellular structure of cardiac muscle cell and connective tissue were normal. In the lung, the cellular structure of bronchioli, alveoli, alveolar duct and blood vessel was normal. Similarly, no changes were observed in the spleen and brain of the rats following the GTN administration compared to the normal control group.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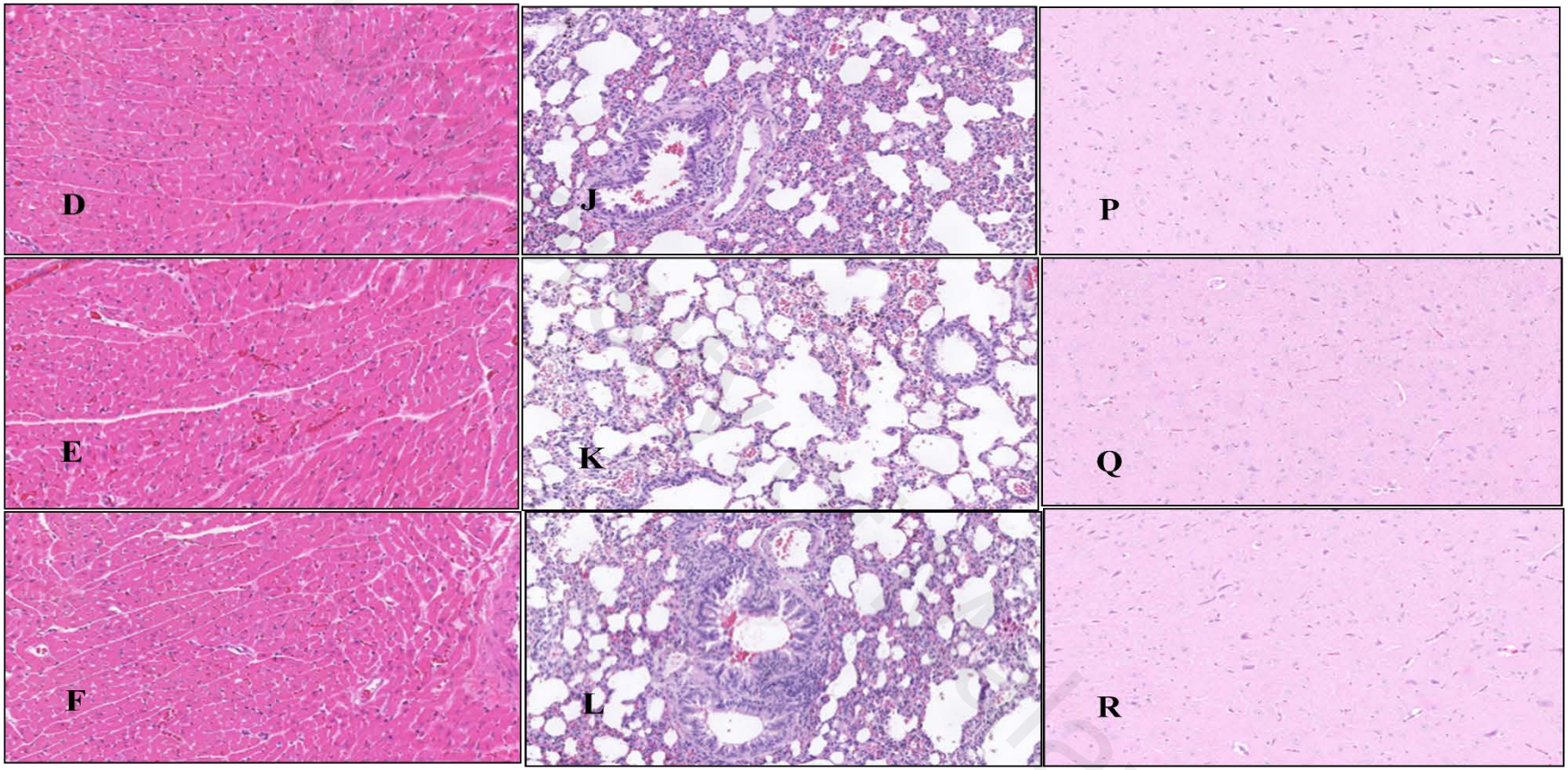

Figure 4. 1: Photomicrographs of selected rat organs for the acute toxicity study of control and GTN-treated groups (A) normal heart (B to F) GTN-treated heart using 100, 200, 300, 400 and 500 mg/kg of GTN, respectively. (G) Normal lung from control. (H to L) GTN-treated lung using 100, 200, 300, 400, 500 mg/kg of GTN respectively. (M) Normal brain. (N to R) GTN-treated using 100, 200, 300, 400, 500 mg/kg of GTN respectively. (Original magnification: 100×. Stain: H&E).

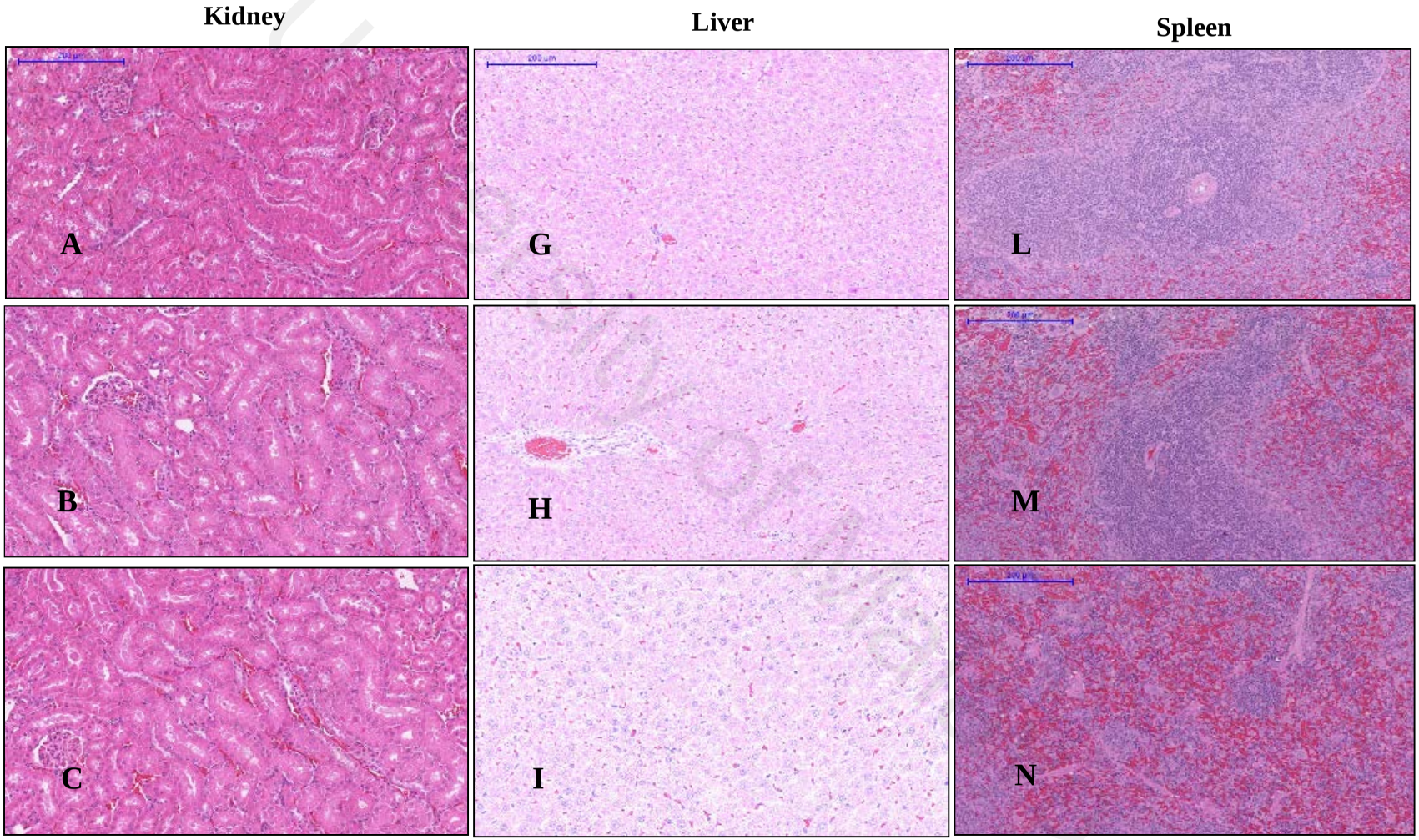
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Figure continued

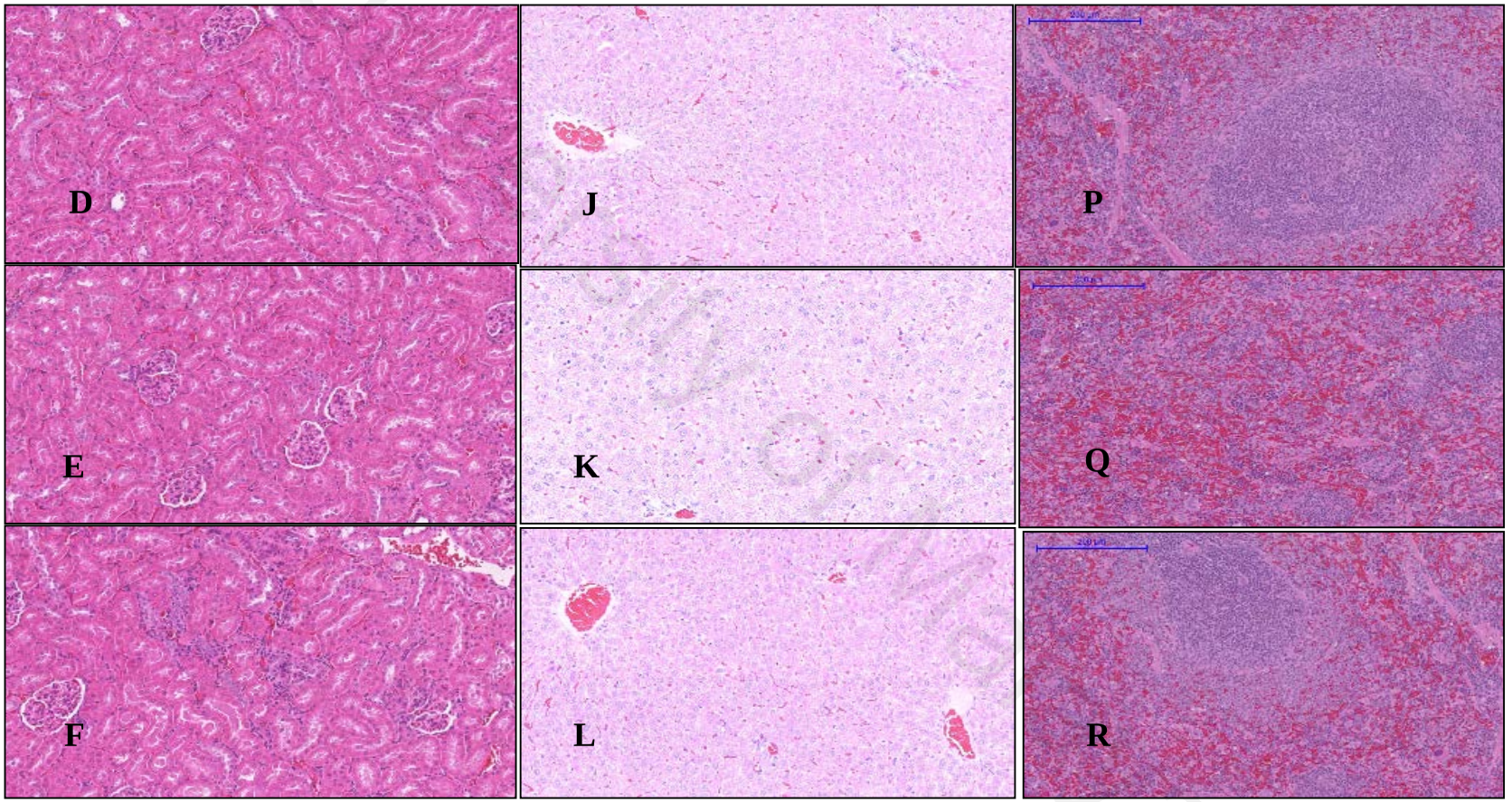


Figure 4. 2: Photomicrographs of selected rat organs of the control and GTN-treated groups for the acute toxicity. (A) Normal kidney (B, to F) GTN-treated kidney using 100, 200, 300, 400, 500 mg/kg of GTN respectively. (G) Normal liver. (H to L) GTN-treated liver using 100, 200, 300, 400, 500 mg/kg of GTN respectively. (M) Normal spleen. (N to R) GTN-treated spleen using 100, 200, 300, 400, 500 mg/kg of GTN respectively. (Original magnification: 100×. Stain: H&E

4.4.2 Sub-Acute Toxicity

4.4.2.1 Clinical Observation

No deaths were observed after 14 days of treatment with 42 mg/kg GTN. The changes in animal body weights (Table 4.3) and organs weights (Table 4.6) in 14 days GTN treated rats were normal comparing to control group. There were no significant ($p>0.05$) differences in the weight of heart, spleen, and kidney GTN-treated and control rats. The macroscopic examinations of the organs of rats treated with GTN 42 mg/kg (1/10 of LD_{50}) did not show any changes in color compared with control group.

4.4.2.2 Body weight of sub-acute toxicity

As seen in Table No. No statically significant changes were obtained between the body weight of the GTN-treated group and the control group.

Table 4. 2: Rats body weight for sub-acute toxicity

Groups	0 day	Week 1	Week 2
Control	250.25±4.13	267.19±5.11	288.22±4.19
GTN treated	245.8±8.11	263.8±6.13	284.8±9.08
P value	-	0.283	0.383

Values are Mean±SEM, $*P$ values < 0.05 were considered significant using independent *t* test

4.4.2.3 Food Consumption and Water Intake of Rats

As shown in Table 4.4, there is no significant changes in animal food consumption were achieved between the GTN-treated group and the control group. As for the water intake, as shown in Table 4.5 it is clear that there is no significant differences between the GTN-treated group of rats and the control group.

Table 4. 3: Food consumption of rats

Time period	Control	GTN 42 mg/kg	P value
Week 1	73.34±3.23	75.32±1.52	0.168
Week 2	82.62±1.34	80.12±1.42	0.065

Values are Mean±SEM, ∗*P* values < 0.05 were considered significant using independent t test

Table 4. 4: Water intake of rats

Time period	Control	GTN 42 mg/kg	*P* value
Week 1	92.54±4.13	91.22±1.02	0.428
Week 2	93.14±2.12	92.31±2.11	0.477

Values are Mean±SEM, ∗*P* values < 0.05 were considered significant using independent t test

4.4.2.4 The weight of Selected Organs

As shown in Table. 4.6, the weight of the selected organs of the GTN-treated group were within the normal range and no significant differences were observed between the GTN treated group and the control group of rats.

Table 4. 5: Weight of selected organs

Organs	GTN 42	Control	*P* value
Kidney	0.87±0.10	0.85±0.02	0.613
Liver	3.55±0.12	3.62±0.11	0.278
Brain	2.25±0.33	2.26±0.12	0.941
Lung	0.45±0.04	0.46±0.02	0.565
Heart	0.37±0.01	0.38±0.02	0.260
Spleen	0.28±0.01	0.27±0.02	0.260

Values are Mean±SEM, ∗*P* values < 0.05 were considered significant using independent t-test

4.4.2.5 The Haematological and Biochemical Evaluation

The hematological measurements of the GTN-treated group were similar when compared to the control group as shown in Table 4.7. Similarly, the biochemical

parameters assessed for the GTN-treated and control group showed unnoticeable alteration (Table 4.8).

Table 4. 6: Haematological evaluation of GTN-treated sub-acute toxicity

Parameters	Control	GTN 42 mg/kg	*P* value
HGB (g/dL)	155.21±0.76	154.11±1.30	0.850
WBC (10^9/L)	6.57±0.21	6.73±0.14	0.119
Neutrophil (%)	9.13±1.23	10.10±2.11	0.314
Lymphocyte %	77.22±26.44	79.39±21.22	0.868
Monocyte (%)	2.21±0.21	2.33±0.14	0.232
Eosinophil (%)	0.89±0.90	0.91±0.80	0.966
Basophil (%)	0.01±0.00	0.01±0.15	1.000

Values are Mean±SEM, $*P$ values < 0.05 were considered significant using independent t test

Table 4. 7: Biochemical evaluation for sub-acute toxicity

Parameters	Unit	GTN 42 mg/kg	Control	P value
Creatinine	μmol/l	26.24±1.14	25.22±1.13	0.119
Urea	mmol/l	5.85±0.15	5.95±0.33	0.480
Albumin	g/l	33.2± 4.3	35.9 ± 3.2	0.207
Globulin	g/l	21.3 ± 3.9	23.2 ± 2.8	0.316
Total bilirubin	μmol/l	2.1 ± 0.9	2.0 ± 0.8	0.830
ALP	U/l	14.2 ± 0.5	13.9 ± 1.1	0.524
ALT	U/l	115 ± 12	118 ± 31	0.815
AST	U/l	100 ± 7.1	99.5 ± 5.6	0.886

Values are Mean±SEM, $*P$ values < 0.05 were considered significant using independent t test

4.4.2.6 Histopathological Evaluation

A histopathological study was carried out to confirm the biochemical findings as shown in Table 4.8 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 GTN-treated and control groups for sub-acute toxicity (Figure 4.3) did not reveal any gross pathological lesions.

The photomicrographs of the liver and kidney of the control and GTN-treated groups showed normal morphological architecture. Under microscopic examination, the liver of GTN-treated animals showed normal cellular architecture and binucleation and was without any distortions similar to the control group. Furthermore, signs of injury, necrosis, congestion, fatty acid accumulation, or hemorrhagic regions around the central vein or sinusoids of the liver were not observed. The hepatocytes arranged in cords and were 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. As for the kidneys, histologically there was no morphological change for the GTN-treated group. 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 groups. In addition, there was no interstitial and intraglomerular congestion or tubular atrophies. All the nephron cells were normal and showed clearly visible nucleoli with no degeneration, bleeding, necrosis or infiltration with lymphocytes. In both the control and GTN-treated rats, the heart showed normal cardiac muscle fibers and lungs showed a normal alveolar structure with no treatment-related inflammatory response. Similarly, normal structure and histology of the spleen also observed in all the rats. Thus, the histopathological evaluations of the selected organs did not reveal any morphological abnormalities that could be attributed to the administration of GTN compound to the rats.

Normal **GTN-Treated**

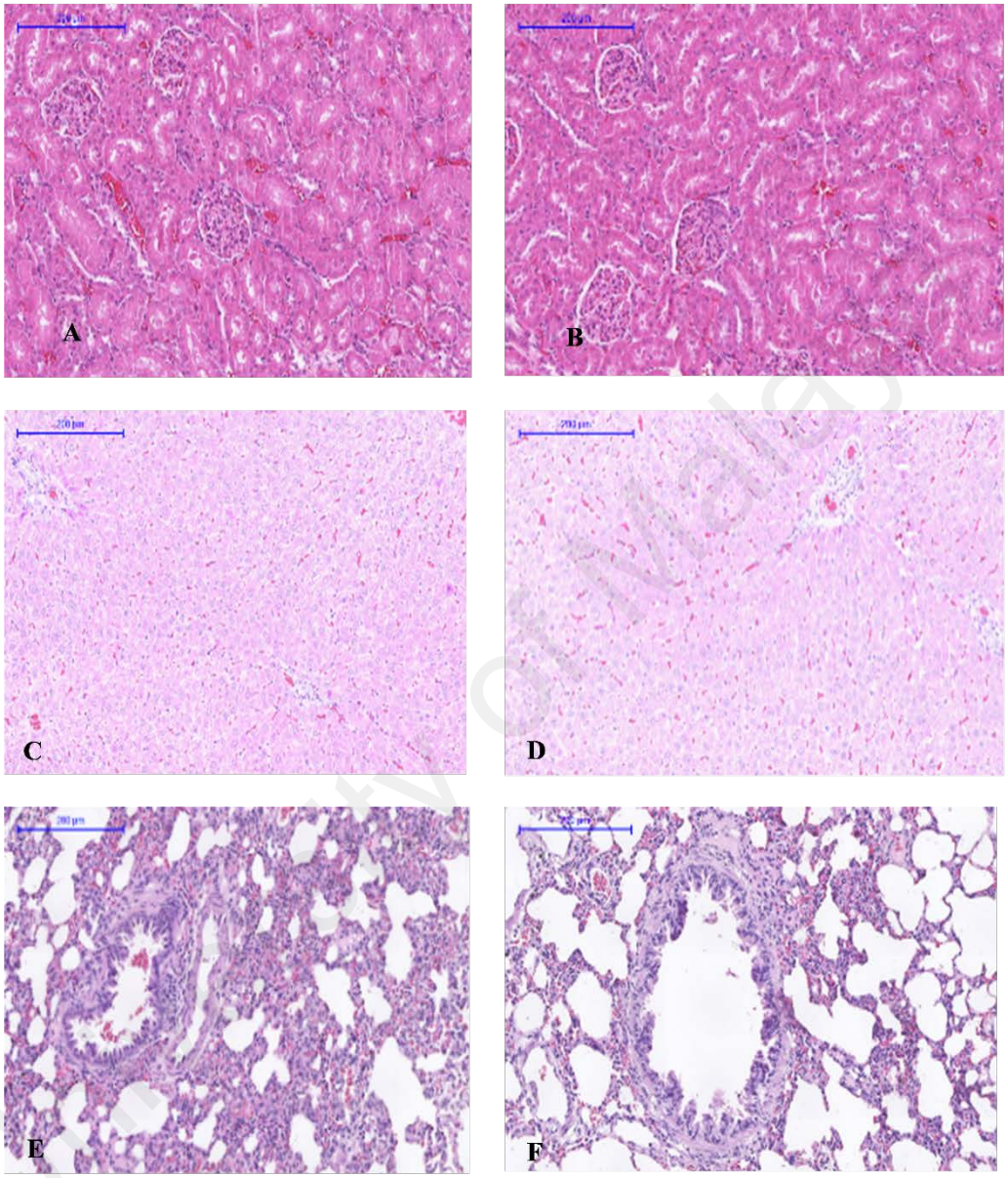


Figure 4. 3 continued

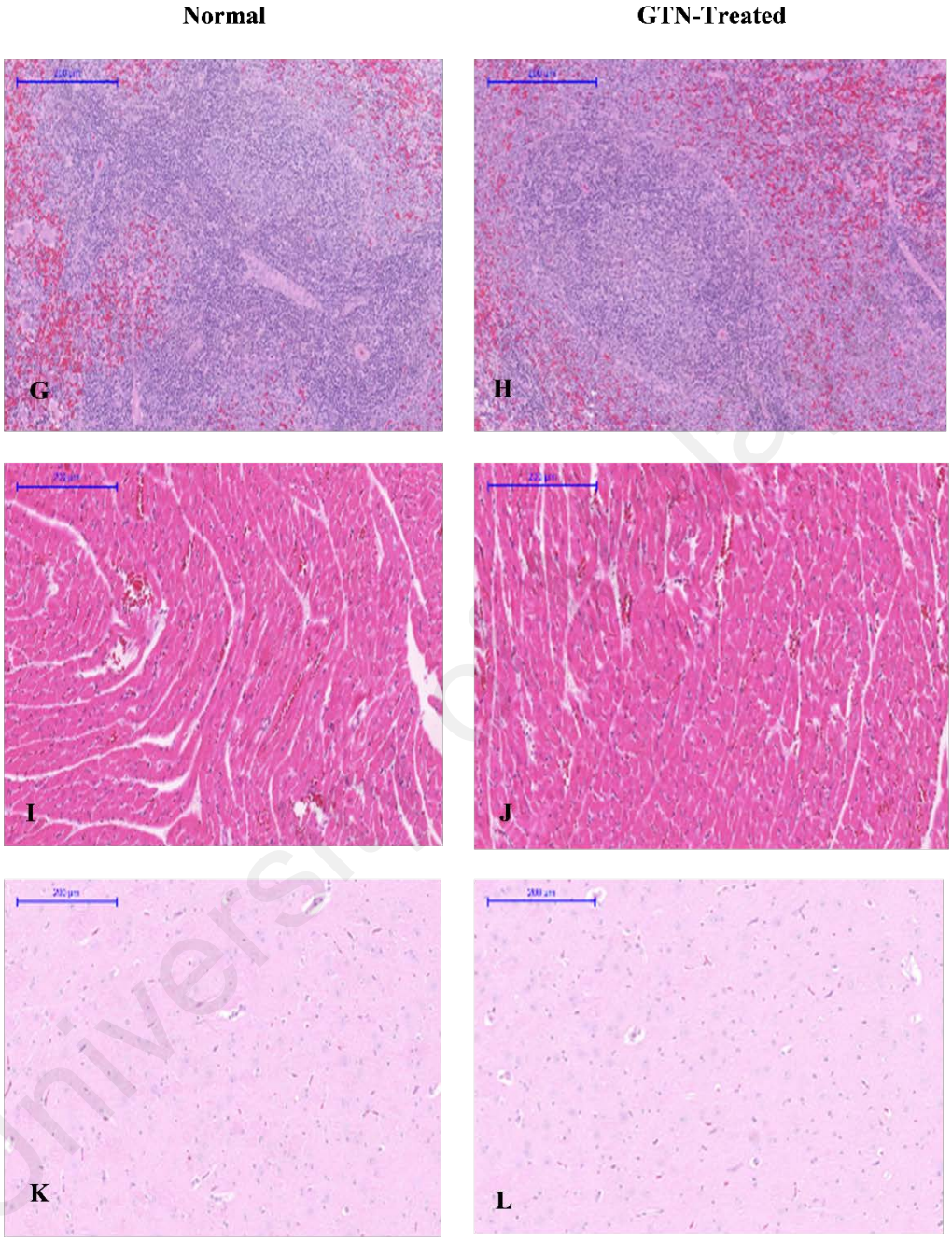


Figure 4.3: Photomicrographs of selected rat organs for sub-acute toxicity of the control group and the GTN-treated groups. (A)Normal kidney (B)| Kidney treated with 42 mg/kg of GTN. (C) Normal liver. (D) Liver treated with 42 mg/kg of GTN. (E) Normal lung. (F) Lung treated with 42 mg/kg of GTN. (G) Normal spleen. (H) Spleen treated with 42 mg/kg of GTN. (I) Normal heart. (J) Heart treated with 42 mg/kg of GTN. (K) Normal brain. (L) Brain treated with 42 mg/kg of GTN. (Original magnification: 100×. Stain: H&E).

4.5 DISCUSSION

GTN active compound is a natural compound derived from a plant called *goniothalamus macrophyllus* distributed in the rainforest of Peninsula Malaysia, Sabah, and Sarawak and also in Southeast Asian countries. In the current study, a toxicological study was conducted to evaluate the effect of GTN on selected internal organs of rat models using hematological, biochemical and histological parameters.

Due to the lack of previous toxicity studies on GTN, comparing the present study findings to other studies in the literature is limited. In 1999, Mosaddik and Haque conducted a study at University of Rajshahi, Bangladesh to evaluate the toxicity of GTN isolated from *Bryonopsis laciniosaLinn*. In their study, GTN was administered intraperitoneally (300 µg/day) into a group of four rats for 14 days. The results were compared to a control group of four rats receiving only water. The body weight, hematological, biochemical and histological parameters of GTN-treated rats were compared with the control group. They found that GTN had no toxic effects on rats. However, as the dose used in their study was very low, it is difficult to compare the present study findings with their result (MOSADDIK & Haque, 1999). The present study findings showed that GTN has a higher safety range with a remarkably high safe upper limit in comparison with already clinically established anticancer agents such as Cisplatin, tamoxifen, doxorubicin, taxol and 5-Fluorouracil(5-FU) (Chow et al., 2010; Lin & Pihie, 2003; Magherini et al., 2010; MOSADDIK & Haque, 1999; Yan et al., 2010).

Similar to tamoxifen or taxol drugs, GTN showed cytotoxicity against ovarian cancer cell lines (Caov-3) but is toxicity against normal kidney cells (MDBK) such as was found in the case of tamoxifen or taxol agents (Lin & Pihie, 2003). Another study compared

GTN toxicity with the chemotherapeutic drug doxorubicin against normal Chang liver cells, a much lower toxicity of GTN on those cells was reported (Al-Qubaisi et al., 2011).

With regard to body weight, the present study findings do not show any statistically significant differences in groups of rats treated with different doses of GTN when compared to the control group. Comparing these records with previous studies the values are within the normal range (Lillie et al., 1996; Said & Abiola, 2014). Body weight is an important indicator as it is involved in a series of organic changes at different stages of life. Significant variation in the rat behavior suggests an alteration in the general state of the animal (Poole & Leslie, 1989). Other anticancer agents such as Cisplatin may reduce the body weight of rats. Atasayar *et al.* (2009) reported that a single dose of 7.5 mg/kg of Cisplatin injected to rats, significantly decreased the rat's body weight (Atasayar et al., 2009).

In toxicity studies, hematological parameters are evaluated to determine the toxic effect of drugs and or plant extracts. These parameters are used to determine abnormalities in the metabolic processes of the animal, and to obtain further evidence about the reactions in the body to injury or lesion, deprivation, and drug-related stress (Debelo et al., 2015).

In the current study findings of both acute and sub-acute toxicity, no significant alterations were observed in different doses of GTN-treated rat groups compared to the control group, and all of the hematological parameters values were within the normal range (Poole & Leslie, 1989; Said & Abiola, 2014). Therefore, it is assumed that at the tested doses of GTN it does not cause any alteration of biological importance in hematological parameters. In contrast to the present study result, Wood and Hrushesky (1995) had treated rats with 2 mg/kg of Cisplatin for 4 weeks and observed that the

hemoglobin value was only 5.9 g/dl. The white blood cells and platelets values were also reduced to the lower normal range (Wood & Hrushesky, 1995).

Serum determination of specific enzymes and metabolites is an indirect way of assessing possible organic damage an animal. There are established normal ranges of universal markers for the detection of organic damage (Debelo et al., 2015; Petterino & Argentino-Storino, 2006). The liver and kidneys are important organs responsible for the metabolism, detoxification, storage and excretion of xenobiotics and their metabolites, and are susceptible to damage caused by external substances (Seif, 2016; Zbinden, 1991). The liver is a complex organ, composed of several cell types and responsible for various functions. Therefore, it can be damaged by different pathways and there is no single biochemical marker that serves as a universal test of liver damage (Solter, 2005). Once the hepatic cell membrane is damaged, the cytosol enzymes are released into the blood. Possible toxicity of liver can be measured by evaluating the transaminases aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase. These are biochemical markers commonly used in clinical practice and are considered good indicators of hepatic function. AST and ALT are the serum enzymes that have been shown to be the most effective in clinical practice, as sensitive indicators of hepatocellular injury (Nicholson & Wilson, 1991).

AST also exists in many organs apart from the liver, including the heart and other muscles. Its release is not specific for acute liver diseases. It is also found in acute myocardial infarction and diseases affecting skeletal muscle (Mythili & Malathi, 2015). In contrast, ALT is primarily found in the liver (Oh & Hustead, 2011). Both transaminases are intracellular enzymes and their appearance in blood is indicative of cellular damage. About 80% of AST of hepatocytes (both rat and human) is found in mitochondria, while ALT is located in the cytosol (Oh & Hustead, 2011).

In the present study, no statistically significant variations were observed in the serum concentrations of AST and ALT in both acute and sub-acute toxicity studies of different doses of GTN-treated animal groups. This indicates that GTN active compound does not result in toxic effects on the liver. However, in the case of acute toxicity, since all animals were sacrificed after the end of the experiments, the time interval of testing may not have been enough for these markers to be detectable. To compare the present study findings on Cisplatin to the previous study by Palipoch and Punsawad (2013), investigated oxidative stress involved in Cisplatin-induced liver and kidney injury pathogenesis. They treated rats with a single dose of Cisplatin at 10, 25 and 50 mg/kg for 24, 48, 72, 96 and after 120 hours and they found that ALT and AST were significantly increased in the rat's serum (Palipoch & Punsawad, 2013).

The current study findings showed that GTN does not produce toxic effects on serum alkaline phosphatase (ALP) concentration in both acute and sub-acute GTN-treated rat groups since there were no statistically significant differences in comparison to the control. ALP activity is present in several tissues, including liver, bone, kidney, intestine, and placenta. The exact role differs from tissue to tissue. The majority of serum ALP is associated with liver and bone ALP (Sharma et al., 2014). Hepatic and bone isoforms of ALP behave differently in varying temperatures, allowing for its identification in clinical diagnosis. It has been reported that ALP is rapidly elevated (values can exceed ten times the upper limit) when bile flow is impaired, or expansive lesions of different types have developed (de Barros Pereira, 2014). In the absence of bone disease, elevated ALP levels often reflect altered biliary tract function. Increase in the catalytic concentration of ALP in plasma occurs because of increased synthesis of the enzyme by the cells, in response to cholestasis (McPherson & Pincus, 2017). In contrast, Al-Malki and Sayed (2014) reported that 12 mg/kg of Cisplatin administered intraperitoneally into rats resulted in a

significant increase of the hepatic levels of ALT, ALP, AST, γGGT, total bilirubin and LDH (Al-Malki & Sayed, 2014). Taking the above into account, the GTN compound tested in the current study did not cause any changes in the serum ALP levels, which may suggest that GTN does not exert toxic effects on the biliary tract.

To evaluate the renal function of the rats in the present study, urea and creatinine levels were measured. Serum urea concentrations in this study showed no statistically significant difference in both acute and sub-acute studies of all doses of GTN compared to the control group, and all values are considered within the reference ranges. Similar findings were observed in the serum creatinine concentration. The kidney has three main functions: elimination of toxic substances produced during metabolism or directly digested, regulation of internal liquid medium, and production of hormones. Any of these functions can be used to assess renal status (Oh & Hustead, 2011). When the kidneys fail, acutely or chronically, the end product of nitrogen metabolism builds up, increasing non-protein nitrogen levels. This is reflected in elevated urea nitrogen in the blood and serum creatinine (Oh & Hustead, 2011). Renal function in blood can be evaluated through the determination of urea, which is the end product of protein metabolism, formed in the liver from ammonia and later eliminated by the kidney (Hosten, 1990) or through the determination of creatinine, which is a product of spontaneous non-enzymatic cleavage of phosphocreatine in the muscle, being excreted unchanged through the kidney. In most species this occurs only by filtration, plasma creatinine levels being taken as a measure of glomerular filtration rate. However, some extrarenal factors such as massive myonecrosis or prolonged strenuous exercise may temporarily affect the level of creatinine (Hosten, 1990). Plasma creatinine concentration is a poor sensitive indicator of renal function, and may not be elevated until the flow rate of glomerular filtrate has fallen below 50% of normal. (Oh & Hustead, 2011).

The liver is the main metabolizing and detoxing organ for drugs, and therefore, is a common target of xenobiotic damage. Foreign compounds that are biotransformed in the liver predominantly by metabolizing enzymes include cytochrome P450 microsomal enzymes, mixed-function monooxygenases, glutathione-stranspheres, sulfotransferases and UDP-glucuronosyltransferases. They may be induced by drugs (Cederbaum, 2015). Many apparently safe drugs may occasionally produce severe liver-related adverse reactions (Cederbaum, 2015). In fact, drug-related liver-related adverse reactions are the most common cause of withdrawal of drugs from the market (Stickel et al., 2005). Adverse drug reactions in the liver can be difficult to diagnose because drugs that induce liver damage can mimic almost any type of hepato-biliary disease. In addition, it is almost impossible to differentiate histologically between liver damage caused by drugs and that produced spontaneously by a disease, since drug-induced damage includes virtually all types of known acute and chronic liver damage (Cederbaum, 2015).

The current study results of both acute and sub-acute experiments show that there are no significant differences between the relative weights of the liver of rats treated with GTN compared to those in the control group. In the microscopic evaluation of the liver sections of the animals treated and untreated with GTN, no lesions were observed. This indicates that GTN is not toxic to the liver of rats.

Regarding kidneys, the histopathological evaluation within the present study of the acute and sub-acute experiments, does not reveal any significant differences between the GTN-treated groups and the control group. Findings obtained in the current study show that GTN active compound does not produce toxic effects at the renal level. Similarly, the relative weights of the rest of the studied rats' internal organs were also in the normal range and without apparent significant differences. Microscopic analysis of these organs does not show any histological alteration.

The histological findings of the current study of acute and sub-acute toxicity, demonstrate that GTN shows no renal tubular or tubular necrosis. Comparing this finding to other anticancer agents, GTN showed high renal tolerance. For instance, many previous studies reported that Cisplatin is nephrotoxic at the lower end doses of the therapeutic range for clinical use. Acute tubular necrosis, cystic tubular dilatation, tubular regeneration and renal tissue inflammation were reported when Cisplatin was administered into rats at a dose 7.5 mg/kg of body weight (Bunel et al., 2017; Choie et al., 1981; Dobyan et al., 1980; Miller et al., 2010; Nonclercq et al., 1989; Ozkok & Edelstein, 2014; Uehara et al., 2005; Ward & Fauvie, 1976). Other researchers administered a low dose of Cisplatin (0.4 mg/kg) into rats daily for 8 weeks and it resulted in an irreversible kidney injury of acute tubular necrosis, a severe atrophy of glomerulus and marked dilation of proximal convoluted tubules (Ravindra et al., 2010). Recent chemotherapeutic agents are often associated with nephrotoxicity complications leading to minimizing the therapeutic doses and thus to limit the antitumor effect of those agents (Katsuda et al., 2010; Remesh, 2017). Moreover, in a recent study, Ribeiro et al. (2017), investigated the effect of obesity on the nephrotoxic effects of 20 mg/ kg of body weight of Cisplatin in mice. They found that Cisplatin causes acute renal injury especially to obese mice (Ribeiro et al., 2017). The possible explanation for undetectable renal toxicity of GTN could be due to its anti-inflammatory activity. Many previous studies reported the anti-inflammatory activity of GTN. After being isolated in 1972, many subsequent studies have reported different GTN properties such as: anti-inflammatory, gastroprotective, cytotoxicity against cancer cell lines, and apoptosis induction (Al-Qubaisi et al., 2011; Alabsi et al., 2012; Inayat-Hussain et al., 1999; Jewers et al., 1972; Kido et al., 2017; Orlikova et al., 2013; Vendramini-Costa et al., 2016; Vendramini-Costa et al., 2014).

Regarding the liver, although hepatotoxicity is not considered as much a dose-limiting factor as nephrotoxicity, previous studies reported that high doses of Cisplatin also induce hepatotoxicity (Liu et al., 1998; Martins et al., 2008). Taking the above into account, GTN may be considered as a promising chemotherapeutic agent.

4.6 CONCLUSIONS

1. Peritoneal administration of GTN into Sprague Dawley rats produced no changes in the levels of hematological parameters.

2. Peritoneal administration of GTN into Sprague Dawley rats produced no changes in the levels of biochemical markers of hepatic and renal function.

3. Peritoneal administration of GTN at a dose of 42 mg/kg for 14 days to Sprague Dawley rats produced no histological alterations in kidney, liver, spleen, heart, brain, and lung of Sprague Dawley rats.

CHAPTER 5: THE ANTICANCER EFFECT OF GONIOTHALAMIN ACTIVE COMPOUND ON 4NQO-INDUCED ORAL CANCER IN RATS

5.1 Introduction

Cancer is one of the most common causes of death in developed and developing countries. So far, over one hundred types of cancer have been recognized and cancer classified according to the type of affected tissue or cells (Shield et al., 2017; Siegel et al., 2017).

Oral cancer along with Oro-pharyngeal cancer is considered the third common cancer in the developing countries and the sixth worldwide. It was reported that there are up to 400,000 new cases recorded every year (Ferlay et al., 2010; Ogbureke & Bingham, 2012; Shield et al., 2017; Siegel et al., 2017). In Malaysia, according to the World Health Organization (WHO), oral cancer causes 1.55% of the total deaths in Malaysia (Zainal Ariffin & Nor Saleha, 2011). It was reported that oral cancer is the eleventh most common cancer in Malaysia with about 32,000 new cases in 2008 increased to 37,000 new cases in 2012. In 2007, the National Cancer Registry (NCR) revealed that oral cancer ranks the second (13.2%) of the top five leading cancers in Malaysia (Cheong et al., 2017).

Various animal models such as rats, mouse, and hamster have been involved in oral cancer research and are utilized to study and evaluate many natural and synthetic anticancer agents and their chemopreventive potential (Suzuki et al., 2005). There are several carcinogenic agents like coal tar, 20 methylcholanthrene (20 MC), 9,10-dimethyl-1,2-benzanthracene (DMBA) and 4-nitroquinoline-1-oxide (4NQO) have been used in experimental oral carcinogenesis. However, 4NQO is the preferred carcinogen in the development of experimental oral carcinogenesis. 4NQO is a water-soluble carcinogen, which encourages tumors development mainly in the oral cavity. It produces all the stages

of oral carcinogenesis like hyperplasia, dysplasia, severe dysplasia, carcinoma-in-situ and squamous cell carcinoma. In addition, there is evidence suggest that similar histological, as well as molecular changes, are observed in the human system (Kanojia & Vaidya, 2006).

There is a great improvement in chemotherapy, radiotherapy and surgical therapy of cancer, however, treatment using these techniques still are not sufficiently effective to reduce the 5-year survival rate and the high incidence of oral cancer (Moongkarndi et al., 2004). Recent cancer studies focus on finding new anticancer agents isolated from medicinal plants that can be used as anticancer or preventive agents.

Yet, various anticancer agents have been identified, however, the side effects and toxicity are still intolerated. Radical removal of cancer and the surrounding tissues resulting in a large tissue defect. Moreover, radiation therapy of squamous cell carcinoma of oral and pharyngeal regions results in side effects such as oral mucositis and xerostomia (Silverman Jr, 2007). It is also reported that there is no medication that can treat or inhibit oral mucositis on its own and the strategies that can reduce oral mucositis still are uncertain. There is evidence that describes a treatment with a proven efficiency and is superior to the other treatments for this condition but that evidence are still not enough (Rodriguez-Caballero et al., 2012). Hence, new anticancer agents, that can preserve tissues and organs with low toxicity and side effects, are needed.

Regarding Goniothalamin (GTN), a styryl lactone compound isolated from plants of the Goniothalamus genus plants that distributed in many parts of Malaysia and other Southeast Asian countries (Al-Qubaisi et al., 2011; Chen et al., 2005; Lin & Pihie, 2003). Previously, several in vitro studies reported that demonstrated selective toxicity against different cancer cell lines (Al-Qubaisi et al., 2011; Sam et al., 1987). Moreover, in vivo

study carried out by Vendramini-Costa et al. (2010), GTN showed anti-proliferative features in an Ehrlich solid tumor model mice (Vendramini-Costa et al., 2010).

GTN has demonstrated cytotoxic activity and induces apoptosis in various cancer cell lines; such as liver, breast, kidney, ovarian and leukemic cell lines including cervical (HeLa), gastric (HGC-27), kidney (768- 0), breast carcinomas (MCF-7, T47D and MDA-MB-231) and leukemia (HL-60, Jurkat and CEM-SS) (Alabsi et al., 2012; Inayat-Hussain et al., 2010; Rajab et al., 2005). As compared with tamoxifen or taxol treated cells, GTN has shown cytotoxicity against ovarian cancer cell lines (Caov-3) and there was no cell death in normal kidney cells (MDBK) as the case with tamoxifen or taxol (Lin & Pihie, 2003). Moreover, lower toxicity to normal liver Chang cell line has been reported compared with the chemotherapeutic drug doxorubicin (Al-Qubaisi et al., 2011).

Vendramini-Costa et al. (2010) has reported GTN anti-inflammatory and anti-proliferative activity and confirmed its potential as a therapeutic agent (Vendramini-Costa et al., 2010). Cytotoxicity of GTN in human leukemia (HL-60 and Jurkat), human breast carcinoma (MDA-MB-231) and cervical (HeLa) occurs via apoptosis after treated with GTN (Alabsi et al., 2012; Chen et al., 2005; Inayat-Hussain et al., 2010). GTN induces apoptosis in which oxidative stress and mitochondria-mediated pathway involves in its mechanism of action (Chan et al., 2010; Inayat-Hussain et al., 1999). Moreover, GTN induces DNA damage, generation of reactive oxygen species (ROS), loss of mitochondria membrane potential, the release of cytochrome c and activation of caspases (Chen et al., 2005; Inayat-Hussain et al., 2003; Rajab et al., 2005). However, data from previous studies is still insufficient and less convincing because most are done in vitro and not in vivo using an animal model or human body. Moreover, there has been no in vivo study conducted to investigate the anticancer activity of GTN on oral cancer. Hence, in vivo studies using various experimental cancer animal models are needed to explore the

anticancer features of GTN. Within this context, the present study was conducted to investigate the anticancer activity of GTN on 4NQO-induced oral cancer rat model.

5.2 Materials and Methods

5.2.1 Goniothalamin

Goniothalamin (GTN) was kindly obtained from Dr. Abdul Manaf Ali, Faculty of Bioresource and Food Industry, University Sultan Zainal Abidin, Terengganu, Malaysia.

5.2.2 Cisplatin Preparation

Cisplatin 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/m2 once every 3 weeks parallel with radiotherapy is a commonly recommended schedule for head and neck SCC treatment. Treatment protocol schedule was achieved by administration of Cisplatin in week 1, 4 and 7 (Cisplatin 100 mg/m2) over a period of 2 to 3 days every 3 weeks treatment (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.

5.2.3 Animal

A number of 56 male Sprague-Dawley rats weighing 200 ± 20 grams were used for the present study. The animals were housed in standard cages in a temperature-controlled room (24±2°C) with a 12-hours light/12-hours dark cycle during the experiments. The animals were provided a standard rat pellet diet ad libitum. Drinking water containing the 4NQO was prepared by dissolving the carcinogen in distilled water three times per week.

It was given in light-opaque bottles because the 4NQO is a synthetic carcinogen derivative of quinoline, soluble in water and sensitive to high temperature and light.

5.2.4 Experimental Design

Experimental procedures were conducted according to the approval of the Institutional Animal Care and Use Committee (IACUC), University Malaya (Ref. Number: 2016-190607/DENT/R/A).

The animals were randomly divided into eight groups (7 animals in each group). The normal group (Group 1) served as a normal group and the animals received normal water while group 2 served as untreated group (4NQO-induced oral cancer group) and the animals was given 4-nitroquinoline 1-oxide (4NQO) (Sigma Aldrich) at the concentration of 20 ppm in drinking water for 12 weeks and then the animals were taken off 4NQO and immediately exposed to normal drinking water without any treatment. The treatment groups; 3, 4, 5, and 6 received 4NQO at the concentration of 20 ppm in their drinking water for 12 weeks and then the animals were taken off 4NQO and the animals were exposed to normal water. At the end of the twelve weeks, the treatment groups were administered GTN intraperitoneally 3 times weekly at 25, 50, 100, 150 mg/kg for 10 weeks. Group 7 received 3 mg/kg Cisplatin only while group 8 received a combination of 3 mg/kg Cisplatin and 50 mg/kg of GTN. All the animals were weighed before and after the experiment; weight gain was calculated for each one of the animals. At the end of the experiment, 22 weeks, the animals were euthanized under the anaesthetic condition, as mentioned in the methodology of Chapter 4. All rats 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 was fixed in 10% buffered formalin, routinely processed, embedded in paraffin wax and subjected to histological processing. The other half was stored in RNA later solution (Qiagen) and then transferred and stored at -80°C to be used in molecular analyses study.

5.2.5 Estimation of Tumour Volume

The dissected tongues were investigated to evaluate the size of the tumors. The tumor volume (size) was measured according to the formula ($\pi/6 \times$ width $\times$ length $\times$ height) as reported in studies previously published (Tomayko & Reynolds, 1989; Wali et al., 2012).

5.2.6 Histological Evaluations

Similarly, as mentioned in Chapter 4 for methodology, formalin-fixed, paraffin-embedded (FFPE) tissue samples were trimmed into 5 µm thick tissue sections. Afterward, the tissue sections were stained by the hematoxylin-eosin staining method. Histological evaluations were performed with light microscopy and a pathologist qualified the samples blindly. Then the tongue tissue sections were analyzed and graded as normal, hyperplasia, dysplasia and squamous cell carcinoma per animal as described by previous studies (El-Rouby, 2011; Lindenblatt et al., 2012).

5.2.7 Gene Expression Evaluation

5.2.7.1 RNA Extraction

RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) was used to extract a total RNA from the frozen tongue tissue samples according to manufacturer´s protocol. Total RNA was extracted from 4 groups; the untreated group (4NQO oral cancer-induced) (Group 2), GTN-treated (50 mg/kg) (Group 3), Cisplatin group (Group 7) and GTN-Cisplatin combination group (Group 8). Briefly, the frozen tissue sections were incubated in 350 µl Buffer RLT, which contained 3.5 µl beta mercaptoethanol and the mixture was homogenized with 1 ml syringe. Then, one volume of 70% ethanol was added into 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 second at 10,000 rpm. The flow-through solution was discarded. After that, an amount of 350 µl of Buffer RW1 was added into the RNeasy MinElute spin column assembly with 2 ml

collection tubes and centrifuged for 15 seconds at 10,000 rpm in order to wash the spin column. The flow-through solution and collection tubes was discarded. RNeasy MinElute spin column was placed with a new 2 ml collection tube and 500 µl Buffer RPE was added to the spin column and centrifuged for 15 seconds at 10,000 rpm in order to wash the spin column membrane. Total of 500 µl of 80% ethanol was added into the RNeasy MinElute spin column and centrifuged for 15 seconds at 10,000 rpm to wash the spin column membrane. And then, the flow through solution and collection tube was discarded. RNeasy MinElute spin column was placed with a new 2 ml collection tube. The lid of the spin column was opened during centrifugation at full speed for 5 minutes. Similarly, the flow through solution and collection tube was discarded. After that, the RNeasy MinElute spin column was placed in a new 1.5 ml collection tube. Total of 32 µl of RNase free water was directly added into the centre of the spin column membrane and centrifuged for 3 minutes at full speed to elute the RNA. To determine the quantity and purity of the extracted RNA, the absorbance at 260 nm (A260) and 280 nm (A280) was measured using a spectrophotometer (Nanodrop ND-2000, Thermo Scientific, MA, USA). A260/A280 ratio between 1.8 and 2.1 indicate high quality RNA, while A260 determines the yield of total 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.

5.2.7.2 First Strand cDNA Synthesis

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

The first step in cDNA synthesis was the genomic DNA elimination step. This was done by addition of genomic DNA elimination mixture 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.

a) Genomic DNA Elimination Mixture:

For each RNA sample, solutions were combined in a sterile PCR tube as shown in Table 5.1.

Table 5.1: Combined solution for RNA sample

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 to the above mixture (Table 5.1 and Table 5.2), 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.

b) Cocktail:

Table 5.2: Reverse Transcription 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)	2µl
RNase free H_2O	3 µl
Final Volume	10 µl

5.2.7.3 Real-time qPCR

A total of 675 µl of master mix was prepared by mixing 25.5 µl of 1 µg of cDNA synthesized from each sample with 337.5 µl RT2 SYBR Green/ROX qPCR Master Mix (Qiagen) and water. Then, amount of 25 µl of the aliquot mixture was loaded onto each well of a pre-designed 96-well RT2 Profiler PCR Array (Qiagen) customized for 4 samples. Each well from the Array plate was pre-hybridized with primers pair for a gene member that listed in Table 5.3. A housekeeping gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control.

Real-time PCR was performed by ABI 7500 HT 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 analyzed using the GeneGlobe Data Analysis Center on QIAGEN's website (http://www.qiagen.com/my/shop/genes-and-pathways/data-analysis-center-overview-page/).

Table 5.3: Selected genes

Symbol	Description
bax	BCL2-Associated X Protein
bcI-2	B-cell CLL/lymphoma 2
casp3	Caspase 3
tp53	Tumor protein p53
***ptgs2* (*cox-2*)**	Prostaglandin-endoperoxide e synthase 2
ccnd1	Cyclin D1
egfr	Epidermal growth factor receptor
gapdh	Glyceraldehyde-3-phosphate dehydrogenase

5.2.8 Immunohistochemical Evaluation

Immunohistochemical evaluation was conducted in the present study to determine the effect of GTN on the expression of tumor markers; cyclind1, ki67, cox2, bcl-2, p53, β-catenin and E-cadherin in the tongue tissue sections of Sprague Dawley rats. Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated, and then the antibodies were applied according to the manufacture's protocol.

Formalin-fixed paraffin-embedded (FFPE) blocks were sent to the Pathology Lab, Department of Pathology, Faculty of Medicine at 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 cyclind1 (clone SP4, Thermo Fisher Scientific). Automated IHC staining was performed according to routine guidelines of Department of Pathology Faculty of Medicine, University of Malaya.

5.2.8.1 Digital image analysis of Immunohistochemical Expression

The verification for the immunohistochemical reaction of cyclind1, ki67, bcl-2, p53, β-catenin, and E-cadherin in three samples from each group was done by light

microscope. Then, immunostained sections were imaged using a digital slide scanner (3D HISTECH Ltd, Hungary). A captured image for each sample was taken per five fields using the magnification 400×. The immunoreactivity for each tumor 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 color selection and automatic statistical color detection model that presented in ImageJ's IHC Toolbox. Threshold settings that successfully quantified staining in a positive staining specimen were then duplicated in every image for comparison purpose.

5.2.9 Statistical analysis

Statistical analysis of area percent of stain detection data was performed by one-way ANOVA test. A *p* value less than 0.05 was taken to be significant.

5.3 Result

5.3.1 Effect of GTN on body weight of 4NQO-Induced Oral Cancer rats

As shown in Table 5.4, in a comparison to the normal control group, a statistically significant decrease in mean body weight was seen in untreated cancer at the end of the experiment (22 weeks) comparing to the normal group (p=0.0002 using posthoc test). For both Cisplatin (group 7) and GTN 25 mg/kg group (group 3), a statistically significant decrease in body weight was observed, (p=0.0035. p=0.0013, respectively using posthoc test). No significant differences were shown between the normal control group (group 1) and the groups of GTN at 50, 100 and 150 mg/kg (group 4, 5 and 6) or the GTN-Cisplatin combination group (group 8).

Table 5.4: Effect of GTN on body weight of 4NQO-Induced Oral Cancer rats

Group (n=7)	(G 1) Normal	(G 2) Untreated Cancer (4NQO)	Treatment Groups						*P* value
			GTN dose mg/kg				(G7) Cisplatin	(G8) GTN + Cisplatin	
			(G3) 25	(G4) 50	(G5) 100	(G6) 150			
Body weight	520± 45	407±33	421± 40	467± 23	469± 45	469± 34	428± 44	499± 60	0.000

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

5.3.2 Effect of GTN on Haematological and Biochemical Parameters of 4NQO-Induced Oral Cancer in rats

The mean values (±standard deviation) of hematological parameters for the all groups are presented in Table 5.5. At the end of the experiments (22 weeks), no significant differences were found between GTN-treated (group 3, 4, 5 and 6) and untreated group (group 2) (4NQO-induced cancer) with respect to lymphocyte and basophil. In a comparison to the normal control group (group 1), a significant reduction in the levels of haemoglobin (Hb) and eosinophil were presented in Cisplatin-treated rats (group 7)(Tukey HSD posthoc test p=0.000 and p=0.023, respectively), which returned to near normal level in rats that were treated with GTN (group 3, 4, 5 and 6). 4NQO administration did not affect the levels of Hb and eosinophil as the induced cancer showed no significant difference compared to the normal control group (group 1). The level of WBC was decreased in the induced cancer (group 2) and Cisplatin (group 7) groups. However, a significant difference was obtained only in the untreated cancer group (group 2) (Tukey HSD posthoc test p=0.000). The value of monocyte was significantly decreased in the untreated cancer groups (group 2) (Tukey HSD posthoc test p=0.000). However, GTN compound treatment (group 3, 4, 5 and 6) returned the value of monocyte to near normal value (Table 5.5).

Table 5.5: Effect of GTN on hematological parameters of 4NQO-induced oral cancer in rats

Groups (n=7)	Hematological Parameters						
	Hb	**WBC**	**N**	**L**	**M**	**E**	**B**
(G 1) Normal rats	15.52±1.11	6.77±1.11	1.06±0.07	9.05±0.66	0.34±0.05	0.15±0.01	0.02±0.01
(G 2) 4NQO	14.11±2.10	3.21±1.26	1.20±0.06	8.43±0.43	0.17±0.03*	0.15±0.04	0.03±0.01
(G 3) GTN 25	14.52±1.34	5.11±1.21	1.05±0.16	8.54±0.76	0.32±0.09	0.14±0.02	0.02±0.01
(G 4) GTN 50	14.67±1.13	6.41±1.12	0.97±0.06	8.57±0.46	0.31±0.02	0.14±0.03	0.02±0.01
(G 5) GTN 100	14.74±1.13	5.91±1.14	1.10±0.26	8.54±0.32	0.33±0.06	0.15±0.03	0.02±0.01
(G 6) GTN 150	14.34±1.16	6.43±2.45	1.01±0.07	8.43±0.38	0.28±0.03	0.14±0.03	0.02±0.01
(G 7) Cisplatin	11.34±1.11*	4.34±1.24*	1.13±0.16	9.13±0.21	0.26±0.05	0.10±0.01*	0.02±0.01
(G 8) GTN+Cisplatin	14.90±1.45	6.45±1.32	1.02±0.09	8.52±0.76	0.31±0.04	0.14±0.03	0.02±0.01
	0.000	0.000	0.060	0.094	0.000	0.023	0.533

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

The mean values (±standard deviation) of biochemical parameters for the all groups are presented in Table 5.6. Significant changes were found in all biochemical parameters studied treated and both untreated cancer and Cisplatin groups using Tukey HSD posthoc test.

Table 5.6: Effect of GTN on biochemical parameters of 4NQO-induced oral cancer in rats

Group (n=7)	Biochemical Parameters					
	Urea	Creatinine	Albumin	ALP	ALT	AST
(G 1) Normal rats	2.11±1.11	4.43±1.23	1.88±1.46	14.24±1.26	110±5.95	121±10.21
(G 2) 4NQO	7.21±2.10*	15.21±4.56*	0.18±1.26*	34.33±19.37*	165±7.36*	168±14.68*
(G 3) GTN 25	2.82±1.12	5.22±1.13	0.75±1.36	12.13±3.36	109±1.59	137±10.02
(G 4) GTN 50	2.70±1.15	4.71±1.55	1.77±1.16	13.67±1.35	115±3.22	122±12.43
(G 5) GTN 100	2.64±1.14	5.86±1.24	1.35±1.36	13.41±1.22	121±4.26	111±10.13
(G 6) GTN 150	3.20±1.16	8.43±2.45	1.71±1.47	13.34±3.58	113±6.93	128±10.43
(G 7) Cisplatin	8.11±3.11*	16.74±4.44*	0.20±1.16*	31.23±19.24*	173±7.35*	147±10.01*
(G 8) GTN+Cisplatin	2.70±1.23	4.65±1.22	1.96±1.21	13.23±3.35	112±3.54	126±2.11
***P* value**	0.000	0.000	0.045	0.000	0.000	0.000

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

5.3.3 Effect of GTN compound on Tongue Tumour Size in 4NQO-induced oral cancer in rats

Administration of GTN is beginning at the 12th week of the experiment and was continued for the next 10 consecutive weeks. GTN-treated groups (group 3, 4, 5 and 6) showed a significant decrease in the tongue tumor size induced by 4NQO (Table 5.7) and posthoc test showed p values of 0.0016, 0.0029, 0.0061 and 0.0012 respectively. Induced cancer developed large tumors in the oral cavity, mostly seen in the posterior region of the tongue. A one-way ANOVA presented that the administration of GTN to the rats at doses of 50, 100 and 150 mg/kg and in combination with Cisplatin as well as Cisplatin

alone, developed smaller tumors (tumor volume) than those seen in the induced cancer (Table 5.7). The combination group of GTN-Cisplatin (G8) showed no significant relation compared to other treatment groups (G3, G4, G5, G6, G7), and posthoc test showed p values of 0.999, 0.065, 0.999, 0.996, 0.978 respectively.

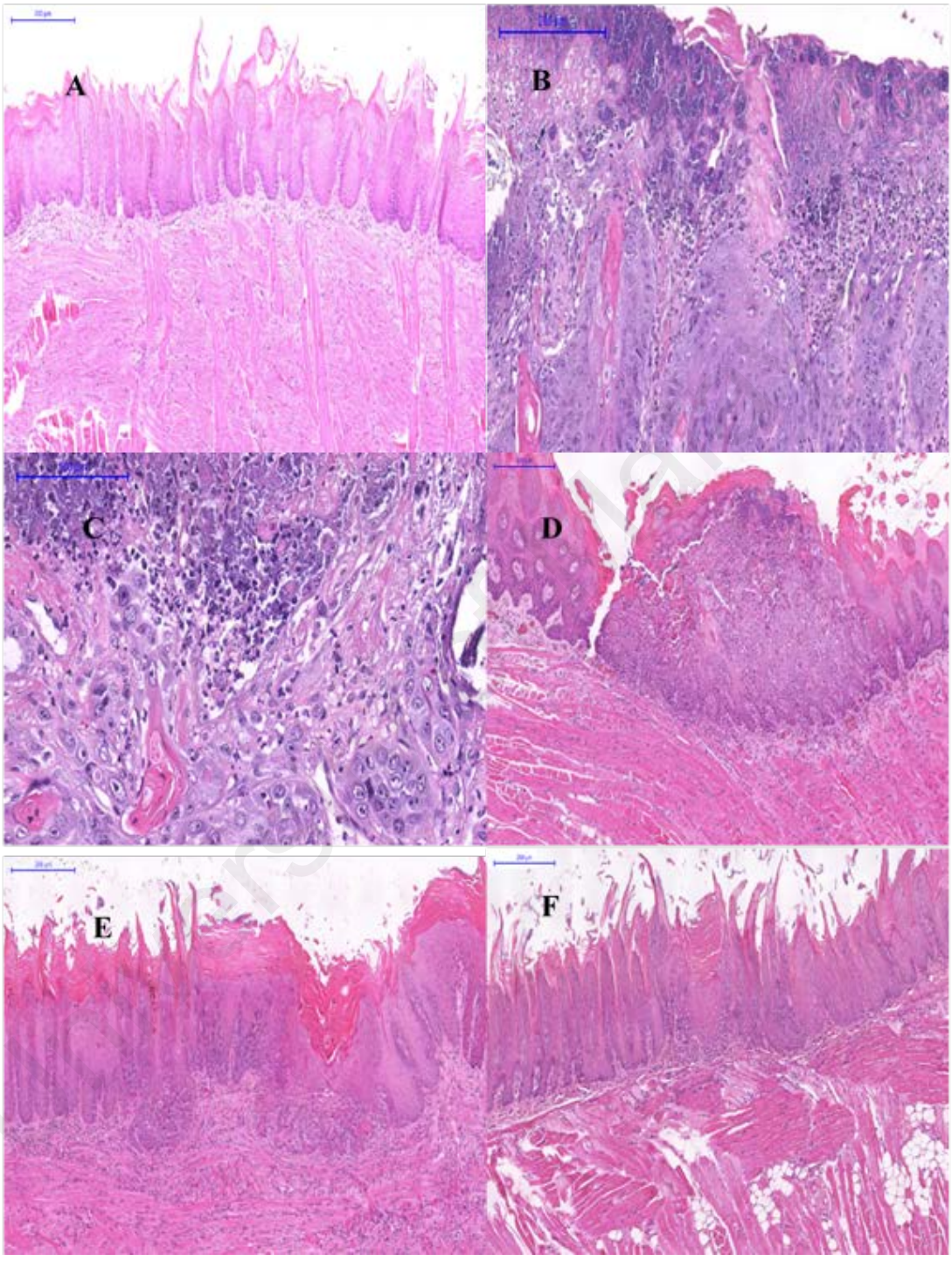
Table 5.7: Effect of GTN on Tongue Tumour Size in 4NQO-induced oral cancer in rats

Group	Tongue Tumour Size (Mean± SD)	*P* value
(G 2) Induced cancer	105.22±17.76	0.000*
(G 3) GTN 25 mg/kg	75.41±31.05	
(G 4) GTN 50 mg/kg	30.86±36.45	
(G 5) GTN 100 mg/kg	34.36±30.21	
(G 6) GTN 150 mg/kg	38.67±35.50	
(G 7) Cisplatin	29.23±36.02	
(G 8) GTN-Cisplatin	24.25±33.38	

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

5.3.4 Effect of GTN 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). The 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 were observed in tongue tissues of the normal animals (group 1) (Figure 5.1).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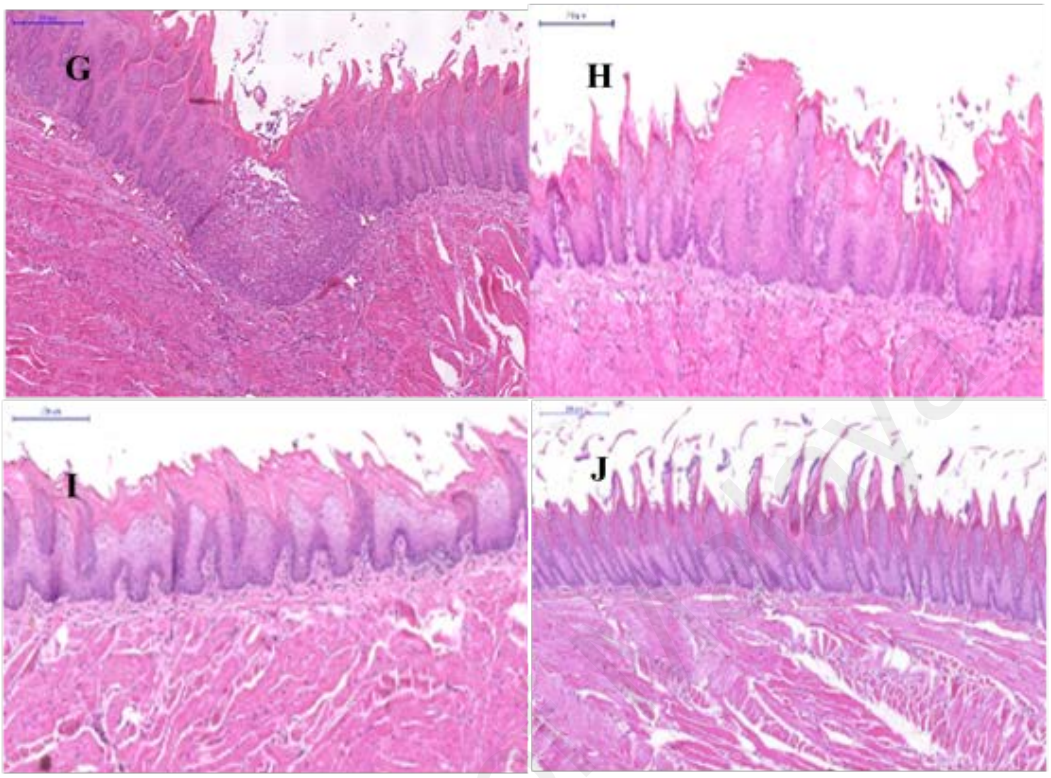


Figure 5.1: Photomicrographs shows (A) GTN-treated normal tissue showing normal oral mucosa with papillae having surface keratinized epithelium, underlying connective tissue and skeletal muscle bundles following oral carcinogenesis induced by 4NQO and treated with GTN 50 mg/kg (stain: H&E; 50x magnification) (B) untreated group (4NQO-Induced cancer) showing invasive squamous cell carcinoma (stain: H&E; 100x magnification) (C) untreated group (4NQO-induced cancer) showing squamous cell carcinoma invading the underlying connective tissue (stain: H&E; 200x magnification) (D) GTN 25 mg/kg group showing severe dysplasia (stain: H&E; 100x magnification) (E) GTN 50 mg/kg group showing moderate dysplasia (stain: H&E; 100x magnification). (F) GTN 100 mg/kg showing moderate dysplasia (stain: H&E; 200x magnification) (G) GTN 150 group showing moderate dysplasia (stain: H&E; 200x magnification) (H) GTN 50 mg/kg group showing hyperplasia of the surface epithelium (stain: H&E; 200x magnification) (I) GTN-Cisplatin combination group showing hyperplasia of the surface epithelium (stain: H&E; 200x magnification). (J) Normal tissue.

For the animals that subjected to 20 ppm of 4NQO in their drinking water, different histopathological changes such as hyperplasia, dysplasia, and SCC appeared in all groups. The incidence of SCC was detected by the presence of discontinuation of the basement membrane and submucosal invasion of epithelial tumor cells as islands, nests, and sheets. The epithelial tumor cells show nuclear and cellular pleomorphism, hyperchromatic nuclei, and altered nucleus: cytoplasmic ratio and keratin pearls in connective tissue.

In the present study, the incidence of dysplasia 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 GTN at 25 (group 3), 50 (group 4), 100 (group 5) and 150 mg/kg (group 6) as a result of 4NQO administration (Table 5.8). Mild histological changes including basal cell hyperplasia, few mitoses, hyperchromatic nuclei and the level of atypia in the lower 1/3 with normal epithelial stratification were evident in the tongue epithelia of GTN treated groups (group 3 to 6). These incidences were less than those seen in the other. 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 GTN-treated groups (group 3 to 6). 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 4NQO group (group 2). The hyperplasia with clearly defined basement membrane was seen in some samples in groups treated with GTN (group 3 to 6).

As shown in Table 5.8, GTN significantly inhibited the tumor growth and minimized the OSCC incidents induced by 4NQO. In the 4NQO (induced cancer) group (group 2), the incidence of OSCC was 71.4%, while in rats treated with GTN at 25 (group 3), 50 (group 4), 100 (group 5) and 150 mg/kg (group 6) the incidence were 57.1%, 28.6%, 14.3% and 14.3%, respectively, while the incidence of OSCC in Cisplatin group (group 7) was also14.3%. Finally, in the combination group of GTN + Cisplatin (group 8), no incidence of OSCC was detected as the incidence was 0%, offering the best protection to the rats exposed to 4NQO. The anticancer activity of GTN was confirmed as a statistically significant decrease in OSCC incidence (Table 5.8).

Preneoplastic lesions incidence were also produced in the present study. Sever dysplasia were 28.6% in the animal group of 4NQO (cancer group) (group 2), while in GTN treated with 25 (group 3), 50 (group 4), 100 (group 5), 150 mg/kg (group 6) were 42.9%, 28.6%, 28.6% and 42.9%, respectively. In the Cisplatin group (group 7) the incidence of severe dysplasia was 28.6%. Finally, in the combination group of GTN and Cisplatin (group 8) the incidence was 57.1%. Moderate dysplasia incidence in the group of GTN treated with 25 (group 3), 50 (group 4), 100 (group 5), 150 mg/kg (group 6) were 28.6%, 28.6% and 14.3%, respectively. In Cisplatin group (group 7) the incidence was 28.6%. Mild dysplasia incidence was observed only in 100 mg/kg GTN treated group (group 5) (14.3%). Hyperplasia incidence in the group of GTN treated with 50 (group 4), 100 (group 5), 150 mg/kg (group 6) were 28.6%, 14.3% and 28.6%, respectively. In Cisplatin group (group 7) the incidence of hyperplasia was 28.6% while in GTN-Cisplatin combination group (group 8), the incidence of hyperplasia was 42.9%.

Dysplastic lesions demonstrated both cytological and architectural changes extending to various thicknesses of the epithelium; severe dysplasia, moderate dysplasia and mild dysplasia (Figure 5.1).

Table 5.8: Effect of GTN on the 4NQO-induced oral cancer in rats

Group (n=7)	A total tumor	Pre-neoplasms and neoplasms lesions incidence (%)				
		Hyperplasia	Dysplasia			SCC
			mild	moderate	sever	
(G2) 4NQO	**7**	0 (0)	0 (0)	0 (0)	2 (28.6.)	5 (71.4)
(G3) GTN 25	7	0 (0)	0 (0)	0 (0)	3 (42.9)	4 (57.1)
(G4) GTN 50	7	2 (28.6)	0 (0)	2 (28.6)	2 (28.6)	1 (28.6)
(G5) GTN 100	7	1(14.3)	1 (14.3)	2 (28.6)	2 (28.6)	1 (14.3)
(G6) GTN 150	7	2 (28.6)	0 (0)	1 (14.3)	3 (42.9)	1 (14.3)
(G7) Cisplatin	7	2 (28.6)	0 (0)	2 (28.6)	2 (28.6)	1 (14.3)
(G8) GTN + Cisplatin	7	3 (42.9)	0 (0)	0 (0)	4 (57.1)	0 (0)

5.3.5 Gene Expression Evaluation

A custom RT² PCR array was carried out to evaluate the alteration of gene expression between the untreated group of animals (group 2) (the induced cancers group that given 4NQO alone) and the GTN-treated group that given GTN after the end of 4NQO administration. The Cisplatin and combination of Cisplatin GTN groups (group 8) were included in the gene analysis. Gene expression analysis of the selected genes is shown in Table 5.9

The GTN-treated group (group 3) showed a significant increase in the expression of both *bax* and *casp3* genes compared to the untreated group. Whereas *tp53*, *bcl-2*, *cox-2*, *cyclinD1* and *egfr* genes were decreased when compared with untreated group (group 2). Statistical significant difference ($p < 0.05$) was observed between the groups in all selected genes except in *cyclinD1* and *egfr* (Table 5.9). Interestingly, the combination group of

Cisplatin and GTN (group 8) showed superior result than either Cisplatin (group 7) or GTN alone (group 3).

Table 5.9: Gene expression evaluation

	Bax	Bcl-2	Casp3	Tp53	Ptgs2	Ccnd1	EGFR
(G2) Untreated cancer ($2^{-\Delta C_t}$)	0.008	0.045	0.011	0.037	0.410	0.122	0.290
(G4) GTN ($2^{-\Delta C_t}$)	0.033	0.012	0.033	0.011	0.174	0.046	0.122
Fold Regulation	4.103	3.102	-3.638	-3.364	-2.362	-2.633	-2.367
***P* value**	0.014*	0.038*	0.034*	0.005*	0.009*	0.075	0.123
(G7) Cisplatin ($2^{-\Delta Ct}$)	0.036	0.009	0.033	0.008	0.124	0.057	0.129
Fold Regulation	4.428	3.146	-5.028	-4.490	-3.294	-2.129	-2.250
***P* value**	0.001*	0.012*	0.024*	0.002*	0.003*	0.095	0.117
(G8) Cisplatin + GTN ($2^{-\Delta Ct}$)	0.048	0.008	0.047	0.008	0.111	0.039	0.111
Fold Regulation	5.911	4.408	-5.376	-4.902	-3.681	-3.081	-2.609
***P* value**	0.000*	0.033*	0.023*	0.001*	0.003*	0.058	0.105

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

5.3.6 Immunohistochemical evaluation

Immunohistochemical evaluation of the selected tumor markers was conducted to assess and compare positive (brown) stained percentage area between the untreated group (group 2) that given 4NQO alone and GTN-treated groups (group 3) after the end of 4NQO administration. The Cisplatin (group 7) and the combination of GTN and Cisplatin groups (group 8) were also included in the immunohistochemical expression analysis.

As shown in Table 5.10, GTN-treated groups (group 3) showed low positive stained percentage area for the tumor markers; cyclind1, cox2, ki67, bcl-2 and p53 compared to

untreated group (group 2). However, β-catenin and E-cadherin tumor markers showed higher expression in GTN-treated groups (group 3) when compared with the untreated group (group 2). A significant difference ($p< 0.05$) was observed between the groups in all selected markers except in β-catenin and E-cadherin. A post hoc Tukey test was used to detect the differences between the groups as presented in Table 5.11 Cyclind1 positive stained percentage area was slightly higher in the Cisplatin group (group 7) than GTN-treated (group 3) and GTN-Cisplatin combination groups (group 8). Confirming the RT2 PCR array result, the GTN-Cisplatin combination group (group 8) showed a better result than either GTN-treated (group 3) or Cisplatin group (group 7) alone.

Table 5.10: Immunohistochemical evaluation of the effect of GTN on 4NQO-induced oral cancer in rats (one-way ANOVA).

Tumour Markers	**(G2) Untreated cancer (% area)**	**(G4) GTN-treated (% area)**	**(G7) Cisplatin (% area)**	**(G8) GTN-treated + Cisplatin (% area)**	***P* value**
cyclind1	9.55±3.44	1.24±0.88	1.64±1.13	0.82±0.54	0.001*
ki-67	4.22±1.32	1.55±0.25	1.23±0.54	0.59±0.18	0.001*
bcl-2	10.95±1.26	4.04±1.12	2.53±1.71	1.11±0.82	0.001*
p53	0.55±0.22	0.10±0.07	0.07±0.01	0.04±0.03	0.001*
β-catenin	24.18±3.08	26.42±9.47	27.02±7.36	28.61±6.69	0.268
e-cadherin	24.55±5.43	26.32±3.52	25.13±4.24	24.23±6.56	0.253

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

Table 5.11: Immunohistochemical evaluation of the GTN effect on 4NQO-induced oral cancer in rats. A post hoc Tukey test

Dependent Variable	(I) IHC expression	(J) IHC expression	Mean Difference (I-J)	*P* value
Cyclin D1	(G4) GTN-treated	Untreated cancer	-8.31	0.001*
	(G7) Cisplatin		-7.91	0.001*
	(G8) GTN-treated + Cisplatin		-8.73	0.001*
Ki-67	(G4) GTN-treated	Untreated cancer	-2.67	0.001*
	(G7) Cisplatin		-2.99	0.001*
	(G8) GTN-treated + Cisplatin		-3.63	0.001*
Bcl-2	(G4) GTN-treated	Untreated cancer	-6.91	0.001*
	(G7) Cisplatin		-8.56	0.001*
	(G8) GTN-treated + Cisplatin		-9.84	0.001*
p53	(G4) GTN-treated	Untreated cancer	-0.45	0.001*
	(G7) Cisplatin		-0.48	0.001*
	(G8) GTN-treated + Cisplatin		-0.51	0.001*
β-catenin	(G4) GTN-treated	Untreated cancer	2.24	0.932
	(G7) Cisplatin		2.84	0.873
	(G8) GTN-treated + Cisplatin		4.43	0.646
E-cadherin	(G4) GTN-treated	Untreated cancer	1.77	0.913
	(G7) Cisplatin		0.58	0.996
	(G8) GTN-treated + Cisplatin		-0.32	0.999

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

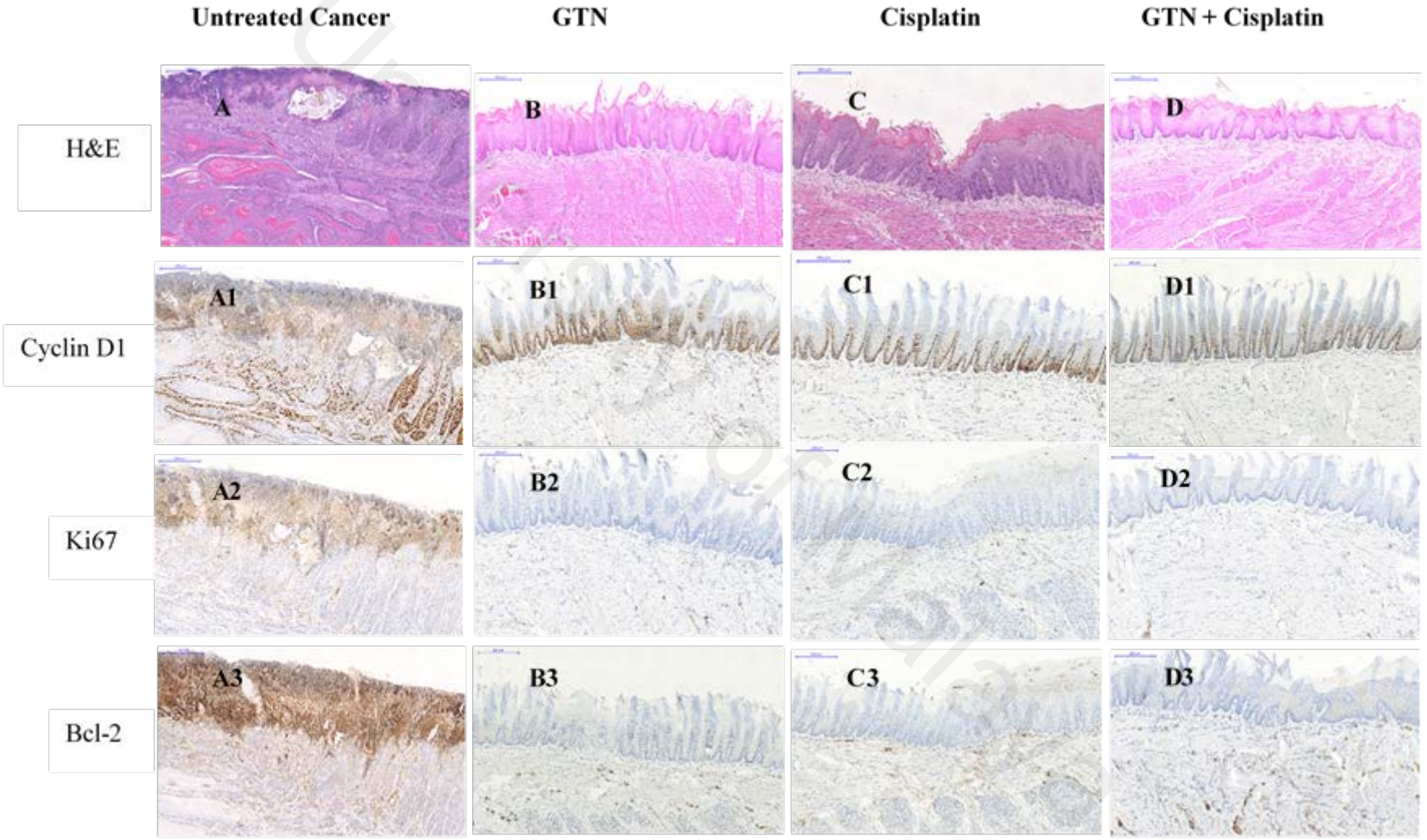
Photomicrographs of haematoxylin and eosin stained tissue sections of GTN-treated (group 3), Cisplatin (group 7) and the combination of GTN-Cisplatin (group 8) (Figure 5.2.B, C and D, respectively) showed decreased proliferation indices in GTN-treated (group 3) when compared to induced cancer (4NQO alone) (group 2) (Figure 5.2).

In the induced cancer (4NQO alone) group (group 2), there was an increased in the proliferation as represented with high cyclind1 and ki-67-labeled epithelial cells/per optical field. The cyclind1 and ki67 expression were found in the basal and parabasal compartment of the keratinized stratified squamous epithelium of induced cancer rats (group 2) and extended into the epithelial tumor cells that have invaded the underlying connective tissue as shown in (Figure 5.2 A1 and A2) experiments. Inversely, administration of GTN-treated (group 3) as well as Cisplatin (group 7) and the combination of GTN-treated + Cisplatin (group 8) decreased the cyclind1 expression (Figure 5.2 B1, C1 and D1) which was restricted to the basal compartment of the keratinized stratified squamous epithelium, whereas the staining was almost negative for ki-67 (Figure 5.2 B2, C2, and D2), indicating strong anti-proliferative effects.

bcl-2 and p53 proteins expression showed a characteristic cellular localization. In induced cancer group (group 2), high positive bcl-2 and p53 stained cells found in basal and parabasal layers and extended into the epithelial tumor cells that have invaded into the connective tissue as shown in Figure 5.2 A3 and A4. However, few positive bcl-2 and p53 stained cells detected in GTN-treated (group 3), Cisplatin (group 7) and the combination of GTN-treated + Cisplatin (group 8) groups (Figure 5.2 B3, C3 and D3 and Figure 5.2 B4, C4 and D4 respectively).

A membranous pattern of β-catenin and E-cadherin protein expression with no staining in the cytoplasm and/or nucleus was showed in the groups GTN-treated (group 3), Cisplatin (group 7) and GTN-treated + Cisplatin (group 8) (Figure 5.2 C5 and D5 for β-catenin and Figure 5.2 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 (group 2) 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 a tumour as illustrated in Figure 5.2 A5 and A6.


Untreated Cancer
GTN
Cisplatin
GTN + Cisplatin
H&E
Cyclin D1
Ki67
Bcl-2
A
B
C
D
A1
B1
C1
D1
A2
B2
C2
D2
A3
B3
C3
D3

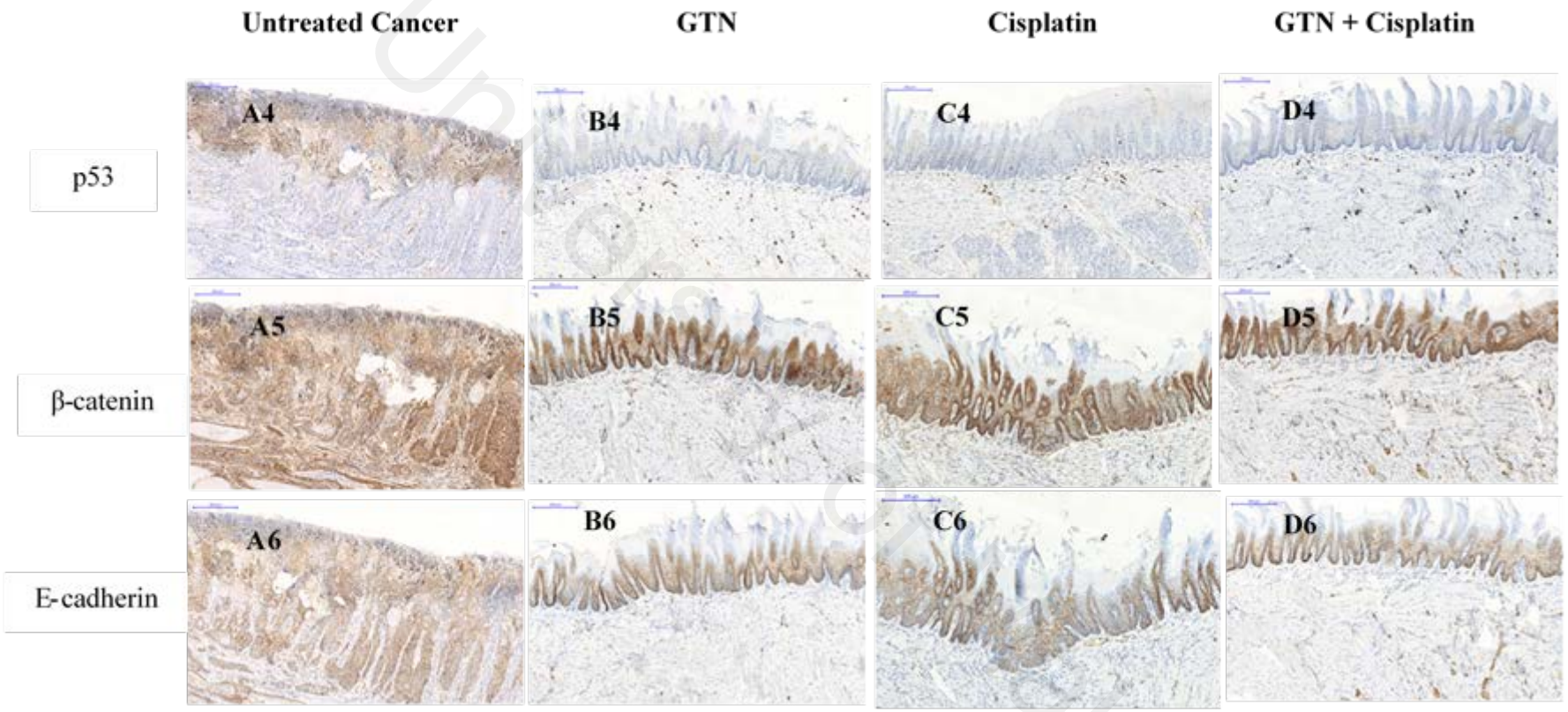


Figure 5.2: Photomicrographs of H&E stained slides. (A) Untreated cancer group. (B) GTN-treated. (C) Cisplatin. (D) GTN-treated + Cisplatin. Photomicrographs of immunohistochemical expression of cyclin d1: (A1) Untreated cancer group. (B1) GTN-treated. (C1) Cisplatin. (D1) GTN-treated + Cisplatin. Ki-67: (A2) Untreated cancer group. (B2) GTN-treated. (C2) Cisplatin. (D2) GTN-treated + Cisplatin. Bcl-2: (A3) Untreated cancer group. (B3) GTN-treated. (C3) Cisplatin. (D3) GTN-treated + Cisplatin. p53: (A4) Untreated cancer group. (B4) GTN-treated. (C4) Cisplatin. (D4) GTN-treated + Cisplatin. β-catenin: (A5) Untreated cancer group. (B5) GTN-treated. (C5) Cisplatin. (D5) GTN-treated + Cisplatin. E-cadherin: (A6) Untreated cancer group. (B6) GTN-treated. (C6) Cisplatin. (D6) GTN-treated + Cisplatin, (100× magnification).

5.4 Discussion

In cancer development, there are three phases involves initiation, promotion, and progression (Aggarwal et al., 2006; Hahn & Weinberg, 2002). The oral carcinogenesis develops from mild dysplasia to moderate and severe dysplasia and then to OSSC (Bascones-Martínez et al., 2012; Sudbø et al., 2003). Animal models are regarded as essential parts in investigating how the disease progresses and how diagnostic or therapeutic protocols develop.

The present study was performed to determine the anticancer activity of GTN on oral cancer. In this study, 4Nitroquinoline 1-oxide (4NQO), a potent chemical carcinogen, was used to induce oral cancer in rats.

The present study findings indicated that oral cancer was successfully induced into Sprague Dawley rat model using 20 ppm of 4NQO agent dissolved in their drinking water for 12 weeks. The importance of rat tongue and Syrian hamster buccal pouch is being currently recognized by surrogate animal models in recapitulating the conditions of the human oral cavity (Kanojia & Vaidya, 2006; Mognetti et al., 2006). In the most representative mode used for studying squamous cell carcinoma of the oral cavity is the animal model of chemical carcinogenesis, which is induced locally or systemically by 4-nitroquinoline-1-oxide (4NQO) (Dayan et al., 1997). The carcinogenic agent 4NQO has been extensively used to induce oral cancer in rodents (Lee & Choi, 2011; McCormick et al., 2015; Patel & Damle, 2013; Ribeiro et al., 2007; Tanaka et al., 1995). The 4NQO carcinogenic induces oral epithelium ulcers in the rat in which these ulcers develop from hyperplasia to mild dysplasia and severe dysplasia and carcinoma in situ, and to SCC (Tanaka et al., 1995). Moreover, chronic administration of 4NQO in water plays an important role in stimulating tongue carcinogenesis in rats in a similar way to its human counterpart (Minicucci et al., 2009). Administration of this agent into rodents leads to

damaging DNA. It also involves a number of dysplastic lesions in the epithelium oro-pharyngeal, depending on the dose and duration of treatment, with morphological and molecular alterations of oral pre-neoplastic and neoplastic lesions which are similar to those in human (Wilkey et al., 2009).

It was also reported that exposure to 4NQO results in molecular and cellular changes, which causes histopathological changes that are similar to those seen in human oral cancer (Kanojia & Vaidya, 2006). Because of the similarity between the oral mucosa of these animals and that of human oral mucosa particularly of the palate and tongue as well as injuries, the carcinogenesis model in rat and mouse is more appropriate for studying the oral cancer (McDonald, 1981; Nauta et al., 1995). It was regarded as the best carcinogen that is currently available for producing oral carcinogenesis in mice by induction of the formation of sequential stages of carcinogenesis (initiation, promotion, and progression). Results of previous research demonstrated that administration of 4NQO resulted in histological and molecular changes that are similar to those occurring in humans, thus, promoting the formation of well-differentiated tumors (Kanojia & Vaidya, 2006). It was also argued by some researchers (Nauta et al., 1995; Tang et al., 2004; Yuan et al., 1997) that the 4NQO models are best systems used for experiments and for examining histological and molecular changes occurring during oral carcinogenesis in humans and cancer progression.

Moreover, liquid solutions represent the most important routes of oral administration of carcinogens (Okazaki et al., 2002; Ribeiro et al., 2005; Vered et al., 2005). They are also being based in the multistep process of carcinogenesis characterized by the initiation, promotion, and progression of a tumor. Currently, chronically administering 4NQO that is associated with drinking water is a good simulator that resembles the carcinogenic process occurring in humans. This model is also advantageous because it is possible to

systematically administer chemical agent for a long-term and at lower doses, which leads to a reduction of any potential additional adverse effects (Vered et al., 2005). It is stated despite the wide variety of experimental protocols, carcinogen concentrations used tend to be similar (Nauta et al., 1995; Steidler & Reade, 1986; Vered et al., 2005). During carcinogenesis, histopathological changes occurring to the lingual epithelium is not static, but they pass from mild to moderate and severe dysplasia. Initiation in combination with irreversible cell changes emerges during the 4 weeks after systemically administering 4NQO through the drinking water (Steidler & Reade, 1986).

Regarding the method of application of 4NQO, the present study 4NQO was applied intraperitoneally according to studies previously published. It was reported that best results gained especially when dissolving 4NQO carcinogens in the drinking water of animals (Lu et al., 2006; Tang et al., 2004). Moreover, in a study by Tang et al. (2004), the researchers examined tumor incidence in mice given 4NQO diluted in water or brushed on the tongue. Their results showed that at the end of the 28 weeks of the experiment, while tumors were developed by100% of the animals that received the diluted drugs in water when applying the brushing technique, tumors were developed by only 5% of them (Tang et al., 2004). Thus, this methodology is established as the main form of experimental oral carcinogenesis (Fracalossi et al., 2011; Noguti et al., 2012).

The current study findings revealed that GTN, inhibits the oral malignancy development in the rat oral cancer model, significantly reduced tumor size, tumor multiplicity and the incidence of hyperplasia, dysplasia and minimally invasive SCC.

In agreement with the present study findings, Vendramini-costa, et al. (2010) investigated the antitumor activity of GTN on mice solid Ehrlich a tumour at doses 30, 100 and 300 mg/kg and they reported that GTN inhibited 48.5 ± 9.4%, 40.9 ± 9.5%, and

66.7 ± 15.9%, respectively, of tumour growth compared to the control group with no significant difference, was found between the different doses (Vendramini-Costa et al., 2010). They concluded that GTN has anti-inflammatory and antiproliferative activity and they confirmed its potential as a therapeutic agent. On the other hand, many previous in vitro studies reported the anticancer activity against various cancer cells such as cervical (HeLa), gastric (HGC-27), kidney (768- 0), breast carcinomas (MCF-7, T47D and MDA-MB-231) and leukemia (HL-60, Jurkat and CEM-SS) (Alabsi et al., 2012; Inayat-Hussain et al., 2010; Rajab et al., 2005).

Administration of 4NQO produced a significant body weight loss in animals due to the intraoral cancer development accompanying with malnutrition that caused by lack of appetite, unable to eat, increased metabolic rate (Thandavamoorthy et al., 2014). Patient with cancer cachexia usually suffers from body weight loss which contributes to patient morbidity and mortality (Fearon & Preston, 1990). In the current study, 4NQO produced a significant reduction of body weight gain among the induced cancer animals and also among animals treated with Cisplatin. These weight reductions were controlled by GTN treatment with or without a combination of Cisplatin. Herein, the result based on the body weight gain revealed that the combination of GTN with Cisplatin may reduce the toxic effect of Cisplatin by gaining of body weight as nearly as that in the normal animal group. This result is in a like manner with a study performed by Patel and Damle (2013), who demonstrated that loss of body weight caused by Cisplatin could be controlled by treatment with a combination of telmisartan with Cisplatin (Patel & Damle, 2013).

In the present study, a significant reduction in hemoglobin levels, WBC, neutrophil, and monocyte counts observed in the induced cancer group. Similar findings were reported previously attributed this reduction to the effect of 4NQO on the immune system (Gannot et al., 2004).

Chemotherapy is one of the most important causes of anemia in cancer patients and the association between dose and duration of chemotherapy with anemia is well known (Coiffier et al., 2001). The negative impact of anemia on the cancer patient in terms of 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).

The carcinogenic agent 4NQO induces dangerous injuries into liver and kidney and leads to significant changes in the hematological and biochemical parameters. These changes attributed to the increase of renal and hepatic enzymes in the serum of 4NQO-induced animals (Viswanadha et al., 2011). Barcessat et al. (2014), investigated the systemic toxicity effect of 4NQO-induced oral cancer in rats, in which 4NQO was applied topically to the tongue of the rat, and they reported that white blood cell and monocyte counts were significantly reduced while the serum enzymes ALT and AST were significantly increased (Barcessat et al., 2014).

Comparing the present findings to the already established antineoplastic agents Cisplatin, previously when Cisplatin given to the rats twice a week at 2 mg/kg body weight, the lowest hemoglobin value recorded at week 4 after therapy was 5.9 g/dl. This progressive anemia 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 anemia is correlated with the amount of Cisplatin-induced renal damage (Wood & Hrushesky, 1995). In the same manner, the Cisplatin-treated rats (Cisplatin group) showed a significantly lower level of hemoglobin accompanied with lower WBC (with no significant difference) than that of the normal control group.

In regard with the kidney function evaluation, the present study findings showed that GTN significantly reduced the urea, creatinine level at the doses of 50, 100 and 150 mg/kg comparing to those in the 4NQO-induced oral cancer, Cisplatin alone and low GTN-treated group. This result is consistent with a previous study reported that 4NQO significantly increases the creatinine level and reduces the total protein and albumin levels.

In a study performed by Wood and Hrushesky (1995), they 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 (Wood & Hrushesky, 1995).

The alterations in the serum enzymes; 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).

In terms of liver function evaluation in the current study, the GTN-treated animal (50, 100 and 150 mg/kg) showed a significant reduced in the level of biochemical parameters; (ALP, ALT and AST) in compare with untreated animals (4NQO group), Cisplatin alone, and low dose of GTN-treated group. Interestingly, similar results were observed in the combination of GTN-Cisplatin group. These findings are in agreement with the previous study reported that administration of 4NQO in drinking water for 8 weeks significantly increase the level of liver and renal markers (ALP, AST, ALT, urea, creatinine, and uric acid) which reflect a liver and renal toxicity (Viswanadha et al., 2011). Other researchers found that the rats given 4NQO in their drinking water presented with significant increase in the level of liver markers; AST, ALT, ALP and total bilirubin (Mohan et al., 2016).

Comparing the present study result to Cisplatin previously published the result, Palipoch and Punsawad (2013) and Yadav (2015), reported that Cisplatin administration induced a significant increase in serum ALT, AST and ALP and a significant decrease in serum total bilirubin, total protein, and albumin levels (Palipoch & Punsawad, 2013). 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 et al., 2015).

The findings of this study showed a significant alteration in the genes; p53, bcl2, bax, casp3, cox2 and cycline d1.

Apoptosis or programmed or physiological cell death is characterized as a distinct set of morphological and biochemical alterations that include chromatin condensation, fragmentation of internucleosomal DNA and, perhaps most importantly, changes in the cell surface that enable the rapid recognition and embedding of apoptotic cells by the neighbouring phagocytic cells, avoiding the induction of pathological reactions (Kuan et al., 2003)

Apoptosis is an active process that can be triggered by a broad spectrum of stimuli, both intra- and extracellular (Wu & Xue, 2003). The mitochondrial pathway of apoptosis is controlled by the family of Bcl2 proteins (Bcl lymphoma protein 2) that also controls the extrinsic pathway of the death receptor.

Bcl2 is a protooncogene discovered in human non-Hodgkin's lymphomas that produces the Bcl2 protein, whose biological function is to suppress programmed cell death (apoptosis) induced by various stimuli. Its effectiveness is proportional to the level of protein expression (Batistatou et al., 1993; Fisher et al., 1993; Hockenbery et al., 1990; Jacobson et al., 1993; Sentman et al., 1991; Strasser et al., 1991; Walton et al., 1993).

The expression of Bcl2 presents an inverse relation to the degree of malignancy in cases of glioma (low-grade gliomas express more protein than anaplastic astrocytomas and glioblastomas). The Bcl2 protooncogene encoding the apoptosis inhibitory Bcl2 protein could be involved in both tumorigenesis and tumor cell progression and survival. More than two dozen Bcl2 family proteins have been identified. These can be divided based on amino acid sequence function and similarity in three subfamilies: the anti-apoptosis subfamily, represented by Bcl2 / BclxL that inhibits programmed cell death by distinct mechanisms, such as prevention of mitochondrial protein release and the two proapoptotic subfamilies represented By Bax / Bak (Bcl2 "associated protein" X) and Bid / Bim (Kuan et al., 2003). Bcl-2 family (including bcl-2 and bax) play an important role in the process of apoptosis. They regulate the mitochondrial membrane in association with the intracellular caspases. (Batistatou et al., 1993). Both Bcl-2 and Bax are involved in the apoptotic pathway. The balance between these two markers controls the apoptosis process. The relation between cancer development and apoptosis has been reported (Nishimura, 1999). In this context, bcl2 and bax were evaluated in the present study. These two genes are known to be important in the apoptosis process.

Bcl2 and Bax are important genes in the Bcl2 family. Bcl2 gene, a suppressor of apoptosis, encodes Bcl2 protein that placed in the endoplasmic reticulum and in the nuclear membrane on the inner surface of mitochondria (Nishimura, 1999; Ribeiro et al., 2005). The presence of bcl2 in the basal and suprabasal layers of the normal oral epithelium tissues has been reported by previous studies (Harada et al., 1998; Ribeiro et al., 2005). Bcl2 overexpressed in precancerous lesion and carcinoma of the oral cavity and also overexpressed in other carcinomas such as nasopharynx, lung, colorectum, prostate, stomach, and esophagus (Chen et al., 2000; Juneja et al., 2015). The expression of the anti-apoptotic gene Bcl2 reduces the genetic modification and increases the

survival of the damaged cell and which lead to the appearance of malignant cells (Juneja et al., 2015; Vander Heiden & Thompson, 1999).

In the present study, bcl2 was overexpressed in the 4NQO oral cancer groups that untreated with GTN, while the GTN treated groups showed significantly reduced in the values of bcl2. In contrast, bax expression values were significantly elevated in the GTN-treated animals compared to the untreated animals.

It is known that bax, which is an important effector of apoptosis member in the bcl2 family and its distribution in normal and tumor tissues is in reverse with bcl2 (Krajewski et al., 1994).

In agreement with the present study findings, a study by Lin and Pihie (2003) conducted at Sabah Malaysia University to study GTN ability to induce apoptosis in human ovarian cancer cell using Immunohistochemistry and Western blot analysis reported that GTN increases the expression of Bax and suppressed the expression of Bcl2 (Lin & Pihie, 2003). Innajak et al., (2016) investigated the anticancer activity of GTN on human breast cancer cell line SK-BR-3 and the found that GTN increases Bcl2/Bax ratio and decreases Bcl2 protein expression (Innajak et al., 2016). The authors concluded that GTN induced apoptosis through the mitochondrial pathway. Furthermore, Salakou et al. (2007) reported that Bax/Bcl-2 ratio up-regulates the expression of caspase-3 and modulates apoptosis associated with the development of the illness. In the present study findings, the caspase-3 was significantly highly expressed in the GTN-treated animals in comparison with the cancer control animals (Salakou et al., 2007).

COX-2 plays role in cancer development and progression, was reported to be overexpressed in oral dysplasia and squamous cell carcinoma (Du et al., 2008). Moreover, Ribeiro et al., (2009) reported an increased level of cox2 in 4NQO treated rats comparing

to normal rats (Ribeiro et al., 2009). Later in 2010, a research study was carried out at Illinois Institute of Technology, USA, to study the expression of cox2 in rat oral cancer and oral cancer prevention by cox2 inhibitors, reported that cox2 is strongly expressed in oral carcinoma and 4NQO-induced oral carcinogenesis in rats and this cancer can be prevented by cox2 inhibitors (McCormick et al., 2010). The present study findings showed a reduced level of cox2 in the GTN treated animal comparing to untreated anima. These findings are in agreement with a recent study, researchers from Campinas University in Brazil studied the effect of GTN and Celecoxib anti-inflammatory activity on TRAMP mice prostate cancer and they found that GTN reduced cox2 level comparing to untreated mice (Kido et al., 2017).

Previous studies have shown that cyclin D1 (CCND1) is frequently overexpressed in oral cancer squamous cell carcinoma. In 2003, Yoshida and his research team investigated the activity of troglitazone on rat 4NQO induced tongue carcinogenesis and found that ccnd1 was overexpressed in 4NQO induced tongue carcinoma samples. Moreover, Wilkey et al. (2009) reported that ccnd1 overexpression increases susceptibility to 4NQO-induced oral cancer in mice (Wilkey et al., 2009). Similarly, Naoi et al. (2010) found a significant overexpression ccnd1 and cox2 in the tongue tumors of rats using RT-PCR analysis (Naoi et al., 2010).

In the present study, GTN significantly suppressed the level of ccnd1. In in vitro study conducted at National Cheng Kung University, Taiwan, the researcher studied the effect of GTN on hepatocellular carcinoma-derived cells, the researchers found that CCND1 was downregulated after the cells treated with GTN.

P53 is a tumor suppressor gene, which is well-known as the guardian genome gene. P53 plays a role in repairing the nucleic acid DNA in the cell by directing the cell to

undergo programming cell death process (apoptosis) (Elmore, 2007). P53 is well known to be overexpressed in 4NQO induced tongue oral cancer in rats (Fong et al., 2011; Osugi, 1996). Moreover, a recent study conducted in Faculty of Science and Technology, National University of Malaysia, investigated the p53 level in Sprague Dawley rat mammary tumor samples treated with styryl pyrone derivative (SPD) active compound using western blotting and electrophoresis, reported a prominent accumulation of p53 in all tumors samples treated with SPD comparing to untreated tumor samples. It is known that GTN is a styryl pyrone compound. In contrast with previous study findings, the current study result showed a reduced level of p53 in GTN treated rats when compared to untreated cancer groups.

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, the administration of GTN-treated reduced the expression of cyclin d1 which is usually overexpressed in head and neck SCC (Perez-Ordonez et al., 2006). similar as the result of this study, and also reduced the expression of Ki-67. These results are 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 4NQO-rats treated compared to the 4NQO-treated group (Wali et al., 2012).

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 detect on G0

phase. There was a direct connection between increasing Ki-67 labeling index and increasing Broders' grade of OSCC (Tumuluri et al., 2002)). In a study that analyzed the anti-tumor effect of Cisplatin with Andrographolide in human OSCC, the result 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, a higher expression of ki-67 showed in the induced cancer group compared to the GTN-treated with or without Cisplatin. The combination of GTN-treated + Cisplatin resulted in more reduction in the expression of ki-67 than other groups. This result indicated that the GTN-treated in combination with Cisplatin produced a synergistic anti-tumor growth activity against OSCC. In addition, this result indicated that GTN-treated reduced the ki-67 and cyclin d1 expression, which in turn decrease the transformation and the aggressiveness toward OSCC.

Previous studies 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 according to the grade of the 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 given 4NQO alone for 8 weeks than the treated group that given GTN-treated. 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 GTN-treated 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, adjunctive treatment that targets Bcl-2 and its family members could be helpful during OSCC treatment with Cisplatin. In the present study, the combination of GTN-treated with Cisplatin reduced the expression of bcl-2 more than GTN-treated or Cisplatin alone. This indicated that GTN-treated 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 shows a mainly membranous β-catenin staining (Pannone et al., 1998), whereas OSCC leaks membrane-bound β-catenin and reveals 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 vehicle untreated group. 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 administrations 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 tumours. 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, leaks membrane-bound and reveals a corresponding increase in cytoplasmic localization as opposed to that in the GTN-treated group. 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 level of β-catenin can result in Cisplatin resistance (Li et al., 2016). In the present study, level of β-catenin in the Cisplatin group was lower than that in induced cancer group; however, it was higher than that in the combination of GTN-treated with Cisplatin group. This result indicated that GTN-treated could restore the β-catenin level to the membranous layer and enhance the role of Cisplatin in regulating the level of β-catenin, which in turn decrease the cell proliferation in the cancer cells and maintaining normal epithelial polarity and inhibiting 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 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 are depended on the degree of tumor differentiation as reported by Mahomed et al. (2007), where a well-differentiated tumor showed with a significantly higher expression of E-cadherin and β-catenin compared with the moderate and poor differentiated tumors (Mahomed et al., 2007). 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 (Kudo et al., 2004). However, a positive E-cadherin expression was observed in the cell membrane of the normal oral mucosa epithelial tissues and also it was 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, leaks membrane-bound and reveals a corresponding increase in cytoplasmic localization as opposed to that in the GTN-treated group. The result of the present study is in corroboration 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 tumor cells (Gao et al., 2005). Therefore, the result of the present study indicated that GTN-treated could restore the E-cadherin to the membranous layer, which in turn decrease 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).

CHAPTER 6: CONCLUSION

6.1 Introduction

The current study provided an insight into the effective inhibition of cell proliferation and induction of apoptotic cell death activity of GTN compound on oral cancer cells *in vitro*. The toxicity profile of GTN compound was also illustrated using body weight, organ weight, hematological, biochemical and histopathological parameters of the vital organs (liver, kidney, heart, spleen, lung, and brain) in SD rats. In addition, the anticancer activity of GTN compound in the 4NQO-induce oral tongue cancer animal model by assessing, body weight, tumor tongue volume and the incidence of OSCC in SD rats.

6.2 Summary of findings

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

1. For the first time, the current study demonstrated that GTN natural active compound showed selective cytotoxic effects against H400 oral cancer cells.
2. GTN compound induces apoptosis in H400 oral cancer cells through mitochondrial-mediated pathway and releases cytochrome c into the cytosol and, as a result, activates caspases 3/7 and 9.
3. GTN natural active compound did not cause any lethality or produce any treatment-related changes in hematological and serum biochemical parameters or important histopathological signs.
4. GTN compound could be well tolerated up to the dose 150 mg/kg body weight.
5. GTN compound is well tolerated at the dose of 42 mg/kg body weight administered daily for up to 14 days.
6. The present investigation demonstrates the safety of GTN suggesting its promising potential for pharmaceutical uses.

7. GTN compound confers statistically significant protection against carcinogenesis in a rat model for OSCC as a marked increase in the body weight gain, controlling the hematological and biochemical parameters and reduction in oral cancer-related mortality and incidence.
8. GTN compound at dose 50 mg/kg showed better result inhibiting the tumor development and reducing the incidence of OSCC when used in combination with the commercially used anticancer drug (Cisplatin).
9. GTN compound 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.
10. The *in vivo* part of the current study confirmed the in vitro study findings in which the GTN compound induced apoptosis by upregulation of Bax and Casp3 genes, and also down-regulation of Tp53, Bcl-2, Cox-2, Cyclin D1 and EGFR genes decreased compared to the untreated cancer group.
11. GTN compound can be used as an add-on therapy with cisplatin for treatment of oral cancer and to control cisplatin toxicity, thus could increase the quality of life of cancer patients.

Taken all together, the results highlighted that the GTN is safe to be used intraperitoneally and possesses anticarcinogenic potency on the oral carcinogenesis and has the potential to be developed as an anticancer agent for oral cancer. This makes GTN a promising candidate for further investigation as a natural anticancer agent.

6.3 Recommendation for future study

GTN compound may potentially represent a significant advance in chemotherapy of OSCC, however, some questions remain to be answered. Future studies will need to assess the followings:

1. GTN doses that used in the current study were selected based on the equivalent doses of GTN compound used on mice in the previous study. Hence, more studies are needed to determine dosing and timing for introduction of GTN compound treatment for achieving maximal effects depending on the stage of oral cancer.
2. An animal model that can produce a high incidence of cancer metastasis is necessary to verify the metastasis obstruction effect of GTN compound.
3. More studies are needed to investigate the exact pathways mechanism of GTN.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

- Published paper: Goniothalamin Induces Cell Cycle Arrest and Apoptosis in H400 Human Oral Squamous Cell Carcinoma: A Caspase-Dependent Mitochondrial-Mediated Pathway with Downregulation of NF-κβ.

Li, L. K., Rola, A.-S., Kaid, F. A., Ali, A. M., & Alabsi, A. M. (2016). Goniothalamin induces cell cycle arrest and apoptosis in H400 human oral squamous cell carcinoma: A caspase-dependent mitochondrial-mediated pathway with downregulation of NF-κβ. *Archives of oral biology, 64*, 28-38.