PHYTOCHEMICAL SCREENING AND ANTI-CANCER EFFECT OF *Clinacanthus nutans EXTRACT TOWARDS CERVICAL CANCER CELL LINE, HELA*

by

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CERTIFICATE

This is to certify that the dissertation entitled

" **Phytochemical Screening and Anti-cancer Effect of *Clinacanthus nutans* Extract towards Cervical Cancer Cell Line, HeLa**"

is the bona fide record of research work done by

Ms Lim Wei Yee

during the period from September 2012 to May 2013

under my supervision.

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For any errors or inadequacies that may remain in this work, of course, the responsibility is entirely my own.

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LIST OF SYMBOL, ABBREVIATION AND ACRONYMN

ATCC	American Tissue Culture Collection
CDC	Centers for Disease Control and Prevention
CO_2	Carbon dioxide
C.nutans	*Clinacanthus nutans*
DMSO	Dimethyl sulfoxide
dH_2O	Deionized water
EDTA	Ehtylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal bovine serum
FDA	Food Drug Administration
$FeCl_3$	Ferric chloride
HeLa	Human Cervical Cancer Cell Lines
HPV	Human Papillomavirus
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid,
IC	Inhibition concentration
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
mg/mL	milligram/ milliliter
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride

nm	Nanometer
OD	Optical density
PBS	Phosphate buffered saline
PSM	Plant secondary metabolite
RONS	Reactive oxygen/nitrogen species
rpm	Revolutions per minute
SD	Standard deviation
Vero	Normal Kidney Cell Lines
v/v	Volume/volume
w/v	Weight/volume
w/w	Weigth/weight
WHO	World Health Organization
µg/mL	Microgram/ milliliter
%	Percentage
°C	degree Celsius

ABSTRAK

Bidang penyelidikan kini banyak memberikan tumpuan terhadap penemuan bahan terepeutik berasakan sumber semulajadi yang mempunyai kesan sitotoksik kepada manuasia. *Clinacanthus nutans* (*C.nutans*) atau lebih dikenali dengan nama tempatannya Belalai gajah di Malaysia telah digunakan secara tradisional untuk jangkitan virus herpes di Thailand. Di Malaysia, kini *C.nutans* popular dalam perubatan tradisional untuk merawat kanser. Objektif utama kajian ini adalah untuk mengenalpasti aktiviti anti-kanser aktiviti daripada ekstrak *C.nutans* secara *in vitro*. Ekstrak kasar akues dan metanol telah diekstrak dari daun *C.nutans* dan pemeriksaan fitokimia dilakukan untuk mengenalpasti metabolit sekunder. Aktiviti sitotoksik daripada kedua-dua ekstrak kasar telah disiasat secara *in vitro* ke atas sel kanser seviks, HeLa dengan menggunakan asai MTT. Kesan *C.nutans* ke atas cara kematian sel diperiksa dengan menggunakan pewarnaan Hoechst 33258. Hasil kajian menunjukkan metabolit sekundar yang terdapat dalam ekstrak kasar akues ialah terpenoid dan flavonoid, manakala metanol ekstrak mengandungi terpernoid, alkaloid and flavonoid. Hasil daripada ujian asai MTT menunjukkan ekstrak akues *C.nutans* memberi kesan anti-proliferasi yang ketara kepada sel HeLa (IC_{50}=13±0.82 µg/mL), tetapi ekstrak metanol tidak memberikan kesan dengan tiada nilai IC_{50} diperolehi. Hasil kajian juga menunjukkan tiada kesan yang ketara terhadap sel Vero dirawat dengan ekstrak akues and metanol. Walaubagaimanapun sel HeLa dan sel Vero yang dirawat dengan tamoxifen menunjukkan kesan sitotoksik yang ketara dengan nilai IC_{50} masing-masing 3.8±0.19 µg/mL dan 2.2±0.029 µg/mL. Pewarnaan Hoechst 33258 menunjukkan ekstrak akues *C.nutans* megaruh kematian sel HeLa secara apoptosis. Akues ekstrak *C.nutans* menunjukkan aktiviti anti-kanser terhadap sel HeLa dengan bersifat sitoselektif dan

mengaruh kematain sel secara apoptosis di mana sifat ini mampu mencadagkan *C.nutans* sebagai bahan terapeutik alternatif dalam pencegahan dan rawatan kanser.

ABSTRACT

Nowadays many researches are focusing on the discovery of new therapeutic substance of natural origin for the treatment of cancer based on its cytoxicity to human cells. *Clinacanthus nutans (C.nutans)* or locally known as Belalai Gajah in Malaysia have been used in Thailand as a folk medicine for the Herpes virus infection. Recently *C.nutans* had become popular folk medicine in the treatment of cancer around Malaysia. In the present study, we aim to examine anti-cancer activities of *C.nutans* extracts for the treatment of cancer *in vitro.* Aqueous and methanol crude extracts were extracted from the leaves of *C.nutans* and phytochemical screening was performed to study the plant secondary metabolites. The cytotoxic activity of both crude extracts were investigated *in vitro* against human cervical cancer cell lines, HeLa by using MTT assay. The mode of cell death induced by the crude extracts of *C.nutans* was examined by Hoechst 33258 stain. The secondary metabolism constituents in aqueous crude extract are terpenoids and flavonoids, whereas methanol crude extract contains terpernoids, alkaloids and flavonoids. Our results showed that *C.nutans* in aqueous extract exerted a significant antiproliferative effect on HeLa cells (IC_{50}=13±0.82 μg/mL) and but no IC_{50} was detected by methanol extract on HeLa cells. No significant activities (IC_{50} = not detected) were present in vero cells treated with both aqueous and methanol crude extract. Whereas HeLa and Vero cells treated with control drug, tamoxifen shows a significant cytotoxicity effect with IC_{50} values of 3.8±0.19 μg/mL and 2.2±0.029 μg/mL respectively. Hoechst 33258 stain showed that aqueous extract of *C.nutans* induce cell death on HeLa cells via apoptosis. The aqueous extract of *C.nutans* exert the anti-cancer activity against cultured human cervical cancer cell lines with cytoselectivity property and induces cell death by apoptosis pathway , suggesting a promising alternate therapeutic substance for cancer prevention and treatment.

CHAPTER 1

1.0 INTRODUCTION

1.1 Introduction

Cancer is one of the predominant killers in the world. According to WHO, cancer accounted for 7.6 million deaths (around 13% of all deaths) in 2008. The number of global cancer deaths is projected to increase 45% from 2007 to 2030 (from 7.9 million to 11.5 million deaths), influenced in part by an increasing and aging global population.

In most developed countries, after cardiovascular disease the second largest cause of death is cancer whereas epidemiological evidence points to this trend emerging in the less developed world. This is particularly true in countries in "transition" or middle-income countries, such as in South America and Asia.

Cervical cancer is second most frequent cancer in women worldwide, accounting for 15% of all cancer related deaths in women (Boyle and Ferlay, 2005). More than 85% of the global burden occurs in developing countries, where it accounts for 13% of all female cancers (GLOBOCAN 2008). Even the treatment for cervical cancer is in advance, the development and search of novel and effective anticancer agents have become very important issues as the side effect of non-specific cytotoxicity of drugs and resistance to treatment still represents a great problem in cervical cancer management (Cameron and Bell, 2004).

Natural products serve as vital source of drugs since ancient times. Large fractions of the current pharmacopeia were come from plant origin (Kingston, 2011). Currently, natural compounds have provided many effective anticancer agents. Approximately

over 50% of drugs isolated from natural sources or are related to them were used in clinical trials for anticancer activity (Newman and Gragg, 2007). With the rise of synthetic molecules and pharmaceuticals in past century, natural products were touted as "alternative" medicines. Proponents of nutritional supplements, find greater purpose for natural products besides as adjunctive agents and supportive care. In recent years, the use of nutritional supplements has showed an upward trend (Dennis *et al.*, 2009). The surveys have shown approximately 80% of cancer patients reported utilizing nutritional supplements worldwide in 2002 (Cassileth and Deng, 2004; Hyodo *et al.*, 2003).

Clinacanthus nutans (*C.nutans*) is a well-known folk medicinal plant especially in Thailand (Thai name: phaya yaw). Fresh leaves of *C.nutans* have long been traditionally used in Thailand to treat skin rashes, insect and snake bite, herpes simplex virus (HSV), and varicella-zoster virus (VZV) lesions (SantiSakdarat, *et al*, 2008). Extracts from the leaves were reported to possess analgesic and anti-inflammatory activities (Satayavivad, *et al.*, 1996), antiviral activities against varicella-zoster virus (Thawaranantha, *et al.*, 1992) , herpes simplex virus type-2 (Jayavasu, *et al.*, 1992a) and antioxidant activity (Pannangpetch *et al.*, 2007) . In Malaysia, *C.nutans* leave traditional to cure, sore throat, kidney problems, uric acid, gout, prostate inflammation, skin problems likes shingles. Currently, the rumor about *C.nutans* in treatment of various cancers has spread throughout the country. However, its anticancer properties are poorly defined. In this study, we examined aqueous and methanol crude extracts from leaves of *C.nutans* for presence of anticancer activity against human cervical cancer cells, HeLa. Based on the previous study done by other research, among breast cancer cell line (MCF-7), cervical cancer cell lines (HeLa), and bladder cancer cell lines (T24), HeLa cells treated with

C.nutans extract shows the most significant cytotoxicity effect. The mode of cell death was then measured by Hoechst 33258 staining.

This study would confirm the cytoselective activity of *C.nutans* towards human cervical cancer cell line, HeLa and its mode of action. This will provide a scientific data and prove the anti-cancer property of *C.nutans.* Then the further study of *C.nutans* against anti-cancer can be done. For example, isolation and identification of active compound of *C.nutans*, improvement of extraction method to produce stronger effect of *C.nutans*, and proteins involved in apoptosis pathway. In our study, half maximal inhibitory concentration (IC_{50}) was measured. IC_{50} values derived from cell-based assays help drive the medicinal chemistry efforts toward improved drug design. Therefore, *C.nutans* can be commercialized used as an anticancer agent in future.

Today several distinct chemicals derived from plants are important drugs, which are currently used in one or more countries in the world. Secondary metabolites are economically important as drugs, flavor and fragrances, dye and pigments, pesticides, and food additives (Saba, *et al* 2012). Secondary metabolites are also of interest because of their use as dyes, fibres, glues, oils, waxes, flavouring agents, drugs and perfumes, and they are viewed as potential sources of new natural drugs, antibiotics, insecticides and herbicides (Croteau *et al.* 2000; Dewick 2002). Because of the variety of secondary metabolites in plants and their effects on living organisms, secondary metabolites compounds in *C.nutans* extract is analyzed qualitatively.

1.2 Research objectives

- To study the effect of *C.nutans* extract in suppression of human cervical cancer cell line, HeLa .
- To investigate the selective cytotoxic effect of *C.nutans* on human cervical cancer cell line, HeLa by comparing it to non-malignant cell lines, Vero
- To determine the secondary metabolite substance present in aqueous and methanol extract of *C.nutans* extract.
- To determine the mode of cell death induce by *C.nutans* towards human cervical cancer cell lines, HeLa by Hoechst 33258 stain

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Cancer

A Greek physician Hippocrates (460 to 370 BC) had coined the word "Cancer" who is also considered as the "Father of Medicine" (Nema and Garg, 2011). Cancer is a general term applied to malignant diseases characterized by rapid and uncontrolled abnormal cells formation which may mass together to form a growth or proliferate throughout the body and it may progress until it causes death. According to National Cancer Institute, cancer is an unrestrained proliferation of cells which become structurally abnormal and possess the ability to detach them from a tumor and establish a new tumor at a remote site within the host (Nema and Garg, 2011).

Deaths from cancer worldwide are projected to continue rising, with an estimated 13.1 million deaths in 2030. Overall, the mortality: incidence ratio is 52%, and cervical cancer is responsible for 275 000 deaths in 2008, about 88% of which occur in developing countries: 53 000 in Africa, 31 700 in Latin America and the Caribbean, and 159 800 in Asia (GLOBOCAN 2008). The main reason contributed to the higher rates of cervical cancer in developing countries compared with developed countries are lack of effective screening and treatment strategies (Ashford and Collymore, 2005).

In Malaysia, cancer of the cervix was the second most cancer among Malaysian women from the statistic of GLOBOCAN 2008 (Figure 2.1.0). Whereas from Malaysia cancer statistics in Peninsular 2006, cervical cancer was the third most common cancer

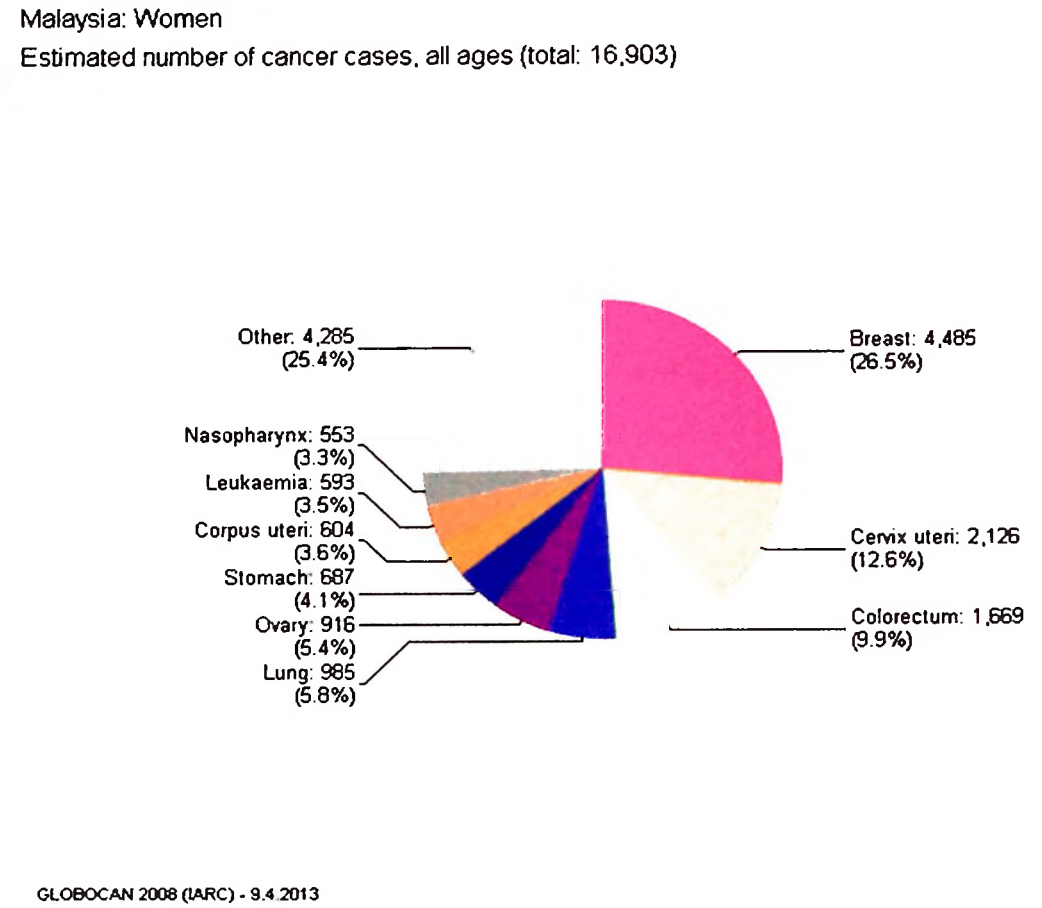

Figure 2.1.0 : Pie charts shows the incidence of various cancer among women in Malaysia (GLOBOCAN 2008)

among women in Penincular Malayisa. Cervical cancer incidence rate increased with age after 30 years and has its peak at ages 60-69 years. According to Malaysia cancer statistics in 2006, compared among the major races, Chinese (Figure 2.1.1) women had the highest incidence for cervical cancer followed by Indian (Figure 2.1.2) and Malay (Figure 2.1.3).

The cervix is the lower part of the uterus (womb). It connects the body of the uterus to the vagina. Squamous cells (on the **exocervix**) and glandular cells (on the **endocervix**) are two main types of cells covering the cervix (Figure 2.1.4). The place where these two cell types meet are called transformation zone, most cervical cancers start at this zone (America Cancer Society).

Human Papillomavirus (HPV) is considered as the etiologic agent of cervical cancer (Biswas, 2000). The close relationships between HPV infection and cervical cancer development have shown in epidemiological and biological studies. In 94 - 100% of cervical precancerous lesions and cancer has been detected with the present of high risk HPV, such as HPV16 and HPV18 (Castellsagué *et al.*, 2006). HPV is a common virus that passed from one person to another during sex. At least half of sexually active people will have HPV at some point in their lives, but few women will get cervical cancer. Cervical cancer may not present any signs and symptoms at early stage, but an advanced cervical cancer may cause bleeding or discharge from the vagina during or after sex (CDC, 2012). Pap test to detect cervical cell changes is a critical way to prevent cervical cancer. Women who have not had regular Pap smears are found to present invasive cervical cancer (Leyva *et al.*, 2006).

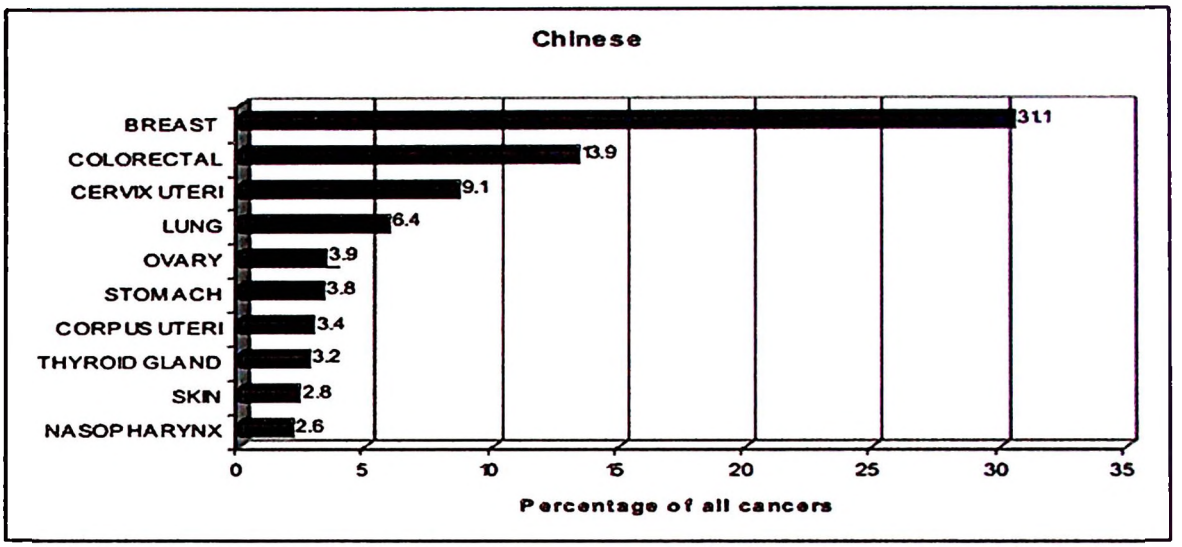

Figure 2.1.1 : Ten most frequent cancers in females by ethnic groups, Peninsular Malaysia 2006: **Chinese** (Malaysia cancer statistics (Peninsular), 2006)

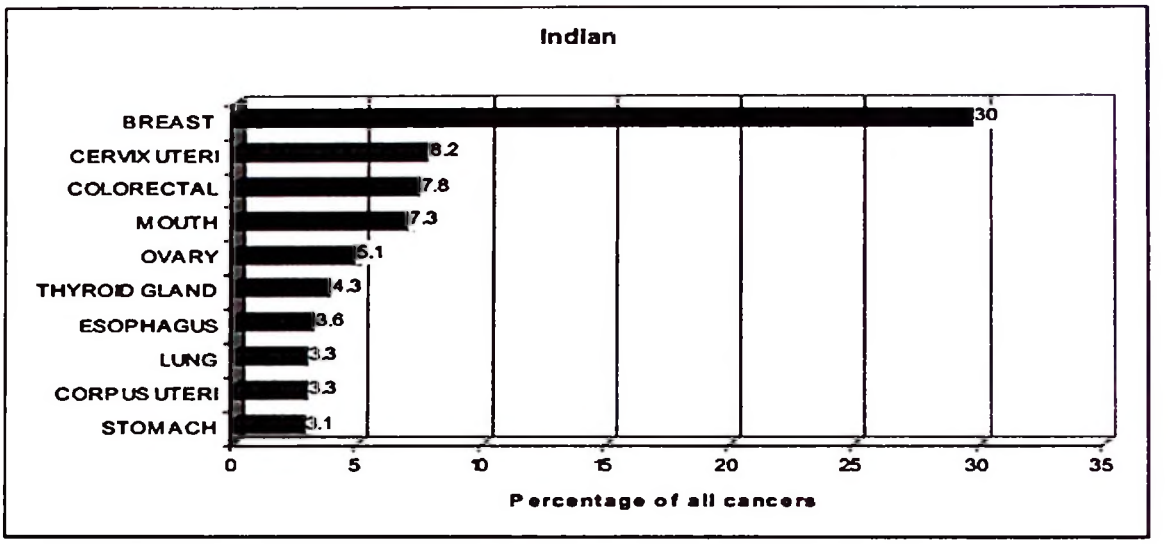

Figure 2.1.2 : Ten most frequent cancers in females by ethnic groups, Peninsular Malaysia 2006: **Indian** (Malaysia cancer statistics (Peninsular), 2006).

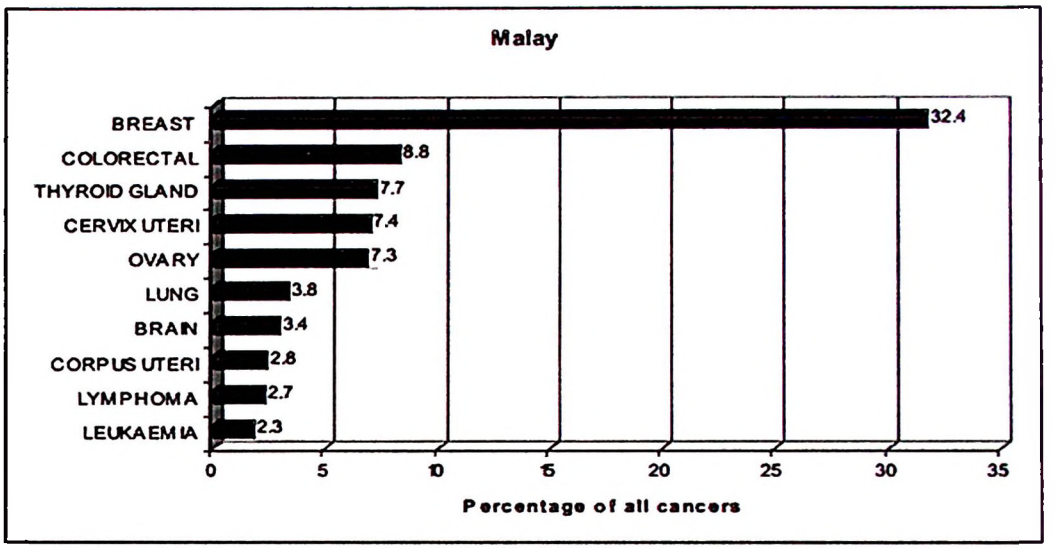

Figure 2.1.3 : Ten most frequent cancers in females by ethnic groups, Peninsular Malaysia 2006: **Malay** (Malaysia cancer statistics (Peninsular), 2006).

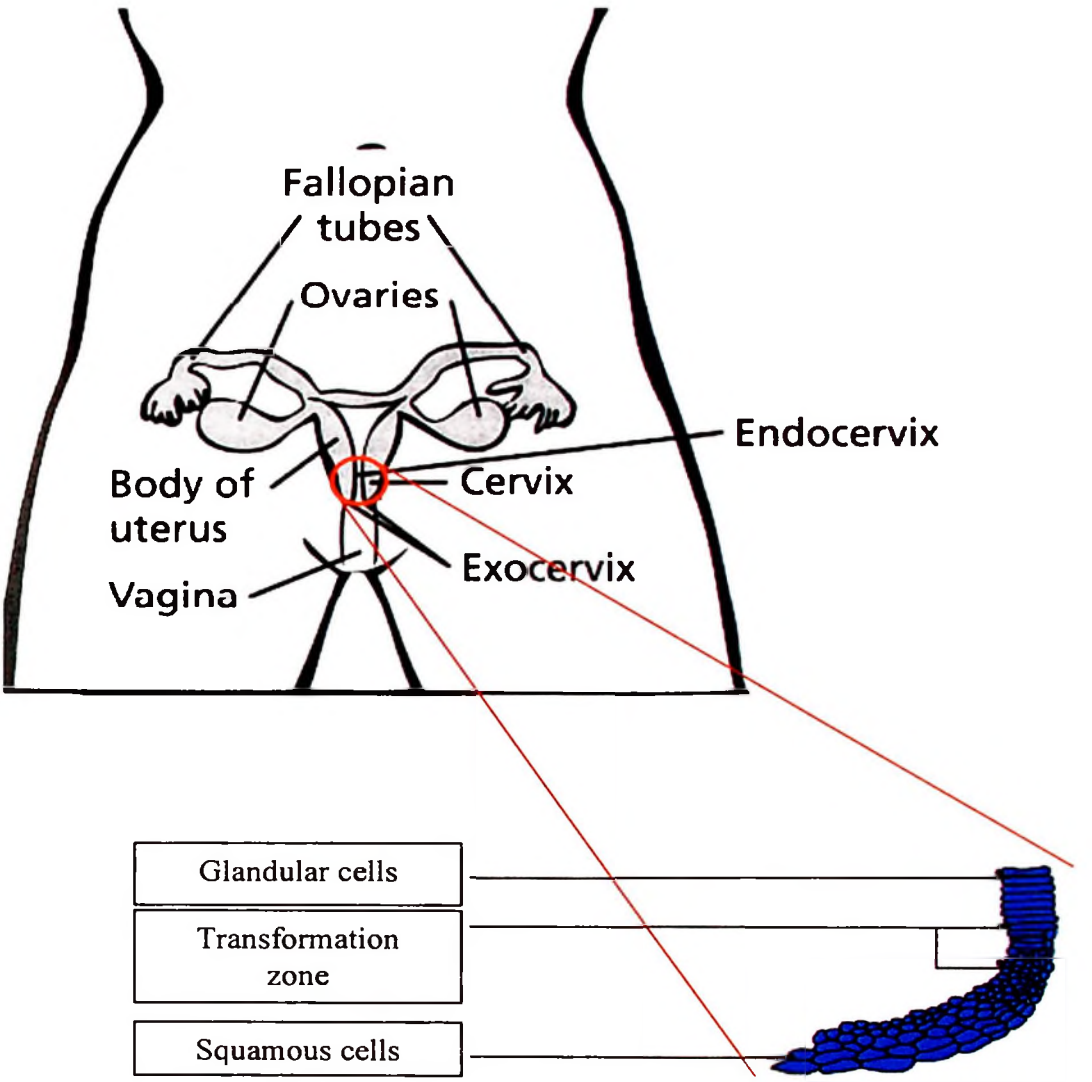

Figure 2.1.4 : The female reproductive system (American Cancer Society).

2.2 Medicinal plants

Plant-derived compounds and their semi-synthetic, as well as synthetic analogs, serve as major source of pharmaceuticals for human diseases. Since 1978 WHO has established the use of medicinal plants as an alternative method to cure cancer. Several plant-derived compounds are currently successfully employed in cancer treatment with plant products as the main sources of drugs (Hernandez-Ceruelos *et al.*, 2002). Despite great development of organic synthesis, currently 75% of prescribed drugs worldwide are derived from plant sources (Tan *et al.*, 2006), showing that plant species are still an important source of new drugs for diseases that continue to lack a cure, such as cancer. Hence, for the treatment of disease states where in drug therapy is a rational approach, plant materials represent legitimate starting materials for the discovery of new agents. In the case of human cancers, nine plant derived compounds have been approved for clinical use as anticancer drugs in the United States. They are vinblastine,(Velban), vincristine (Oncovin), vinorelbine (Navelbine®),etoposide (VP-16®), teniposide (VM-26®), paclitaxel (Taxol®), docetaxel (Taxotere®), topotecan (Hycamptin®),and irinotecan (Camptosar®) (Wang *et al.*,1997; Lee 1999).

2.3 *Clinacanthus nutans* (*C.nutans*)

Clinacanthus nutans (Burm.f.) Lindau (Thai name: Phaya Yo or Phaya Plong Thong) belongs to the family of Acanthaceae (Figure 2.3). This plant is small shrub, native to tropical Asia, and is often cultivated (SantiSakdarat *et al*, 2008). *C.nutans* is erect, sometimes rambling shrubs. It stems are cylinder shape, when dry, it will turn to yellow color. Petiole 5-7cm or more; leaf blade lanceolate to ovate-lanceolate , 5-11×1-4 cm, papery, secondary veins 5 or 6 on each side of midvein and convex on both surface

when dry, base oblique, margin subentire, apex caudate-acuminate. Stamens and pistal glabrous and capsule not seen. (Pieroni and Vandebroek , 2007).

Clinacanthus nutans is a medicinal plant used in Thailand and Malaysia in recent years as a folk medicine for cancer treatment (Yuann *et al*, 2012). *C. nutans* cream was later clinically shown to be as efficacious as acyclovir in relieving pain and healing herpes simplex and herpes zoster without causing a burning sensation, the side effect experienced by some patients using acyclovir (Sangkitpporn *et al.*, 1993; Jayavasu, 1998).In traditionally in Thailand this plant were used as treatment of skin rashes, dysentery, insect and snake-bite, herpes simplex virus (HSV), and varicella-zoster virus (VZV) (SantiSakdarat *et al*, 2008). This plant was used to treat diabetes in Indonesia (Gloobinmed).

In 1995, the toxicity studies carried out by Chavalittumrong *et al*, ethanol extract of *C.nutans* leaves shows no acute toxic effect in mice and rats with no abnormalities of the internal organs were observed. The methanol extract toxicity studies on rats were also proven safe without causing liver and kidney damage (Xiu *et al.*, 2012).

2.3.1 Antiviral activity

Leaves of *C.nutans* had been traditionally used to treat herpes infections in Thailand. Laboratory investigations in Thailand have indicated that the extract of this herb exhibits anti-viral properties against the herpes simplex virus (HSV) (Thawaranantha *et al.*, 1992) and varicella zoster virus (VZV) (Jayavasu *et al.*, 1992). They found that the extract from

Table 2.3 : Vernacular names of *C.nutans* (Globinmed)

Malayisa	**Belalai gajah**
English	Sabah snake grass
Indonesia	Ki tajam (Sunda); Dandang gendis (Java); Gendis (Central Java)
Thailand	Saled pangpon tua mea (Saliva of the femle mongoose) (Watson *et al*, 2008)

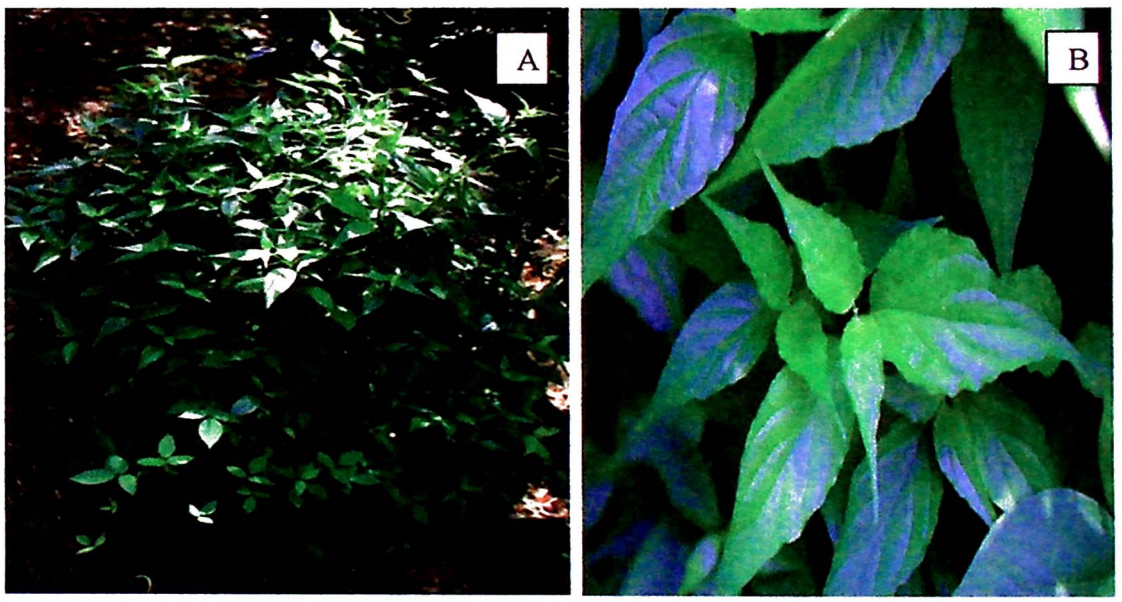

Figure 2.3 : Morphology illustration of *Clinacanthus nutans* (A) Shrub and (B) Leaves

(ASEAN Tropical Plant Data Base)

the leaves of *C.nutans* was able to inhibit plaque formation by HSV type 2 in baby hamster kidney cell line. But in 1996, Yoosook *et al* found that the anti HSV type 2 strains against organic solvent extract did not show any anti-HSV type 2 activity.

2.3.2 Immune response activity

Some study had been done to focus on the activity of *C.nutans* extract on the immune system. The study shows that lymphocyte proliferation was able to increase significantly and the activity of natural killer cells (NK cells) also can be reduced after treated with *C.nutans* extract (Sriwanthana *et al,* 1996). From their study, they found that the Cell-mediated Immune Response (CMIR) activity of this plant extract was partially caused by the release of Interleukin 4 (IL-4) from the peripheral blood mononuclear cells.

2.3.3 Anti-inflammatory activity

Methanol extract of *C.nutans* found to possess significant anti-inflammatory properties as seen in both the rat paw oedema model induced by injection of carrageenan and the Ethyl phenylpropiolate (EPP)-induced rat ear oedema mode (Panthong *et al.*, 2008). One of the active ingredients in *C.nutans* was identified as a flavonoid compound (Satayavivad *et al.*, 1996).

2.3.4 Antioxidant

Many studies had been done and suggest that oxidative damage to cell components has an important role in pathophysiology of human disease. Antioxidant activity and protective effect against free radical-induced haemolysis had found in ethanol extracting from leaves of *C.nutans.* (Pannangpetch *et al.*, 2007). Increasing evidence has

demonstrated the close association between tumorigenesis and elevated level of intracellular free radicals, the reactive oxygen/nitrogen species (RONS), which trigger cancer initiation and progression (Wiseman and Halliwel, 1996; Kamiya, 2003; Shi *et al.*, 2012). An uncontrolled level of RONS in endogenous system will cause damage to cellular protein, DNA, and leading to genomic instability, and ultimately promotes cancer formation (Halliwell, 1999; Xu *et al.*, 2002; Klaunig and Kamendulis, 2004). Therefore, antioxidant supplement could salvage cells from oxidative stress and prevent cancer growth and expansion (Yoke *et al.*, 2013)

2.4 Anti-cancer agents

Traditionally natural products have played an important role in drug discovery and were the basis of most early medicine (Buss, 2003).Since 1950s, the search for anti-cancer agents from plants sources started with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins. Vinca alkaloids, vinblasine (VLB) and vincristine (VCR), isolated from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apocynaceae) were the first agents to advance into clinical used. The mechanisms of these agents were inhibition of tubulin polymerization and primarily combined with other cancer chemotherapeutic drugs for the treatment of various types of cancers including lymphomasn leukemias, breast and lung cancer (Newman and Gragg, 2007).

Triterpenoids and diterpenoids under the class of terpenoids also found to possess anti-cancer properties. Several tirterpene extract from the hooks of *Uncaria rhynchophylla* (Rubiaceae) showed anti-proliferative effect on cancer cell (Lee *et al.*, 2000). The representative diterpene anticancer compounds are taxol and its derivatives, Paclitaxel

clinical therapeutic agent against certain human solid tumor, such as breast cancer and ovarian cancer (Kim and Park, 2002). The discovery of paclitaxel (Taxol®) from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. (Taxaceae), is another evidence of the success in natural product drug discovery (Mohammad, 2006). Paclitaxel act through the promotion of tubulin polymerization and stabilization of the resultant microtubules (Newman and Gragg, 2007).

In Chinese traditional treatment, genus Selaginnela which is rich in biflavonoids is used extensively to treat cancer, gastritis, hepatitis, and cardiovascular disease. Mild cytotoxicities against lymphoma and leukemia cell lines were shown in four new robustaflavone derivatives which isolated from *S.delicatula* (Shelaginellaceae)(Lin *et al.,* 2000). Other examples of plant-derived compounds currently under investigation are flavopiridol, homoharringtonine, ß-lapachone and combretastatin A4 (Nobilia *et al.,* 2009).

The most common treatment used in traditionally therapies for cancer patient is chemotherapy. Tamoxifen is a synthetic non-steroidal anti-estrogen used for the treatment (Ugwumadu *et al*, 1998) and recommended by "The National Surgical Adjuvant Breast and Bowel Project" (NSAMP) as a chemopreventive (Fisher *et al.*, 1998) drug in high risk population for breast cancer. Tamoxifen also known as (Z)-2-[4-(1, 2-diphenylbut-1-enyl)phenoxy]-N,Ndimethyl-ethanamine was developed in 1967 and approved by the Food and Drug Administration (FDA) in 1978 (Caputo and Copeland, 1996). Even tamoxifen is widely utilized in the management of breast cancer; it also tested as a possible treatment for other types of cancer including melanoma and

cancer of the liver, stomach, kidney, pancreas, cervix of uterus, and ovary (IARC, 1996).

The greatest effect (50% reduction in recurrent disease) is achieves in tamoxifen therapy in estrogen receptor-positive tumors, but it is also effective in estrogen receptor-negative tumors (Fritz and Speroff, 2011). The mechanism of action is tamoxifen binds to the estrogen receptor and form as complex which fails to induce estrogen-responsive gene, and RNA synthesis does not occur. These results in growth-promoting effects of the aromatase inhibitor as it lead down regulation of estrogen receptor (Finkel *et al.*, 2009). Long-term tamoxifen therapy will increased incidence of endometrial changes, including hyperplasia, polyps, and endometrial cancer has been reported. Hot flashes, headache, fatigue, mild nausea, which is transient and unaccompanied by vomiting are adverse effects associated with short-term tamoxifen therapy (Aschenbrenner and Venable, 2009).

2.5 Phytochemical compound in plant

The first person to introduce the term secondary metabolite over one hundred years ago was Kossel, stating that 'whereas primary metabolites are present in every plant cell that is capable of division, secondary metabolites are present only "accidentally" and are not essential for plant life' (Rhodes ,1994). Plant secondary metabolites (PSM), also known as phytochemical; represent a diverse group of natural products (Harborne, 2001; Wink, 2004). Secondary metabolites are substance not essential for the growth and development of a plant, but rather are required for the interaction of plants with their environment (Kutchan and Dixon, 2005). Secondary products are frequently limited to particular organs and to particular cells and tissues within that organ but are

not found uniformly throughout the plant. From the view of human, many of these secondary products have interesting applications in pharmacology, chemical industry, novel materials, agriculture, and forestry. A simple classification of secondary metabolites includes tree main groups: terpenes (such as plant volatiles, cardiac glycosides, carotenoids and sterols), phenolics (such as phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins and lignin) and nitrogen containing compounds (such as alkaloids and glucosinolates). The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolics compounds (Hill, 1952).

2.6 MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay

Cell viability and cytotoxicity assays are used for drug screening and cytotoxicity tests of chemicals to monitor the response and health of cells in culture after treatment with various stimuli. Measurements of surviving and/ or proliferating mammalian cells are requiring in many biological assays. This measurement can be achieved by counting cells that include/exclude a dye, measuring released 5aCr-labeled protein after cell lysis, and measuring incorporation of radioactive nucleotides ([3H]thymidine or [lZSI]iodo- deoxyuridine) during cell proliferation (Mosmann, 1983).

MTT assay is a quantitative colorimetric assay that based on metabolic activity of viable cells. Tetrazolium salts (Smith, 1951) are reduced only by metabolically active cells. Thus, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) can be reduced to a blue colored formazan (Mosmann, 1983).

MTT assay have very wide applicability for measuring survival and/or proliferation of various cells and can potentially be applied to any assay in which living cells must be distinguished from dead cells or a lack of cells (Mosmann, 1983). Monsmann also found that conditions in which components of the medium do not interfere with the measurement of the product. Its simplicity, reproducibility, economy, and versatility make the MTT assay a most advantageous one for toxicity testing and for routine cell culture applications (Jeffrey *et al.,* 1988).

2.7 Apoptotic vs necrotic

Cell death is part of normal development and maturation cycle. Cell death occur when living cell response to xenobiotic agents, for examples microorganism and chemicals, and to endogenous modulation like inflammation and disturbed blood supply (Clavien *et al.,* 2000; Vaupel and Hockel ,2001). In the treatment of cancer, the major approach is the removal, by surgery, of the neoplasm and/or the induction of cell death in neoplastic cells by radiation, toxic chemicals, antibodies and/or cells of the immune system (Kacinski and Flick , 2000 ; Zornig *et al.*,2001 ; Kong *et al.*, 2001 ; Dragan YP *et al.,* 2001). It is now known that there are at least two distinct types of cell death: apoptosis (also known as programmed cell death) and necrosis.

Apoptosis is the major cell death pathway used to remove unwanted and harmful cells during embryonic development, tissue homeostasis and immune regulation (Ellis *et al.,* 1991). In addition, most anti-cancer therapies are based on activation of apoptotic pathways (Sellers and Fisher, 1999).

Characteristic differences also exist in both the structure and the metabolic processes of cells that undergo apoptosis or necrosis (Rosser and Gores 1995). When a cell undergoes apoptosis, the entire cell, including the nucleus, separates into numerous fragments (i.e., apoptotic bodies). Simultaneously, the genetic material of apoptotic cells breaks into a characteristic pattern of pieces of varying sizes. During the breakup of the cell, the cell continues to produce proteins and adenosine triphosphate (ATP), a molecule that is essential for cell functioning and which is required for most of the cell's energy-consuming metabolic processes. As a result, each apoptotic body, which is surrounded by a piece of cell membrane, contains intact, functional cell components (Nanji and Sturmhofel, 1997) (Figure 2.7).

Necrotic cell death, in contrast, is characterized by the loss of metabolic functions and of the integrity of the cell membrane. Thus, cells undergoing necrosis cease their production of proteins and ATP. Structurally, the cells' organelles swell and become nonfunctional during the initial stages of necrosis. In addition, the cell membrane forms bubble like projections (blebs). These blebs, which contain no organelles, fuse and increase in size. Eventually, the cell membrane ruptures, resulting in the release of the cell's components into the surrounding tissue. This process of cell dissolution is called cytolysis. The released cellular content subsequently induces an inflammatory response in the effected tissue (e.g., the liver) (Nanji and Sturmhofel, 1997) (Figure 2.7).

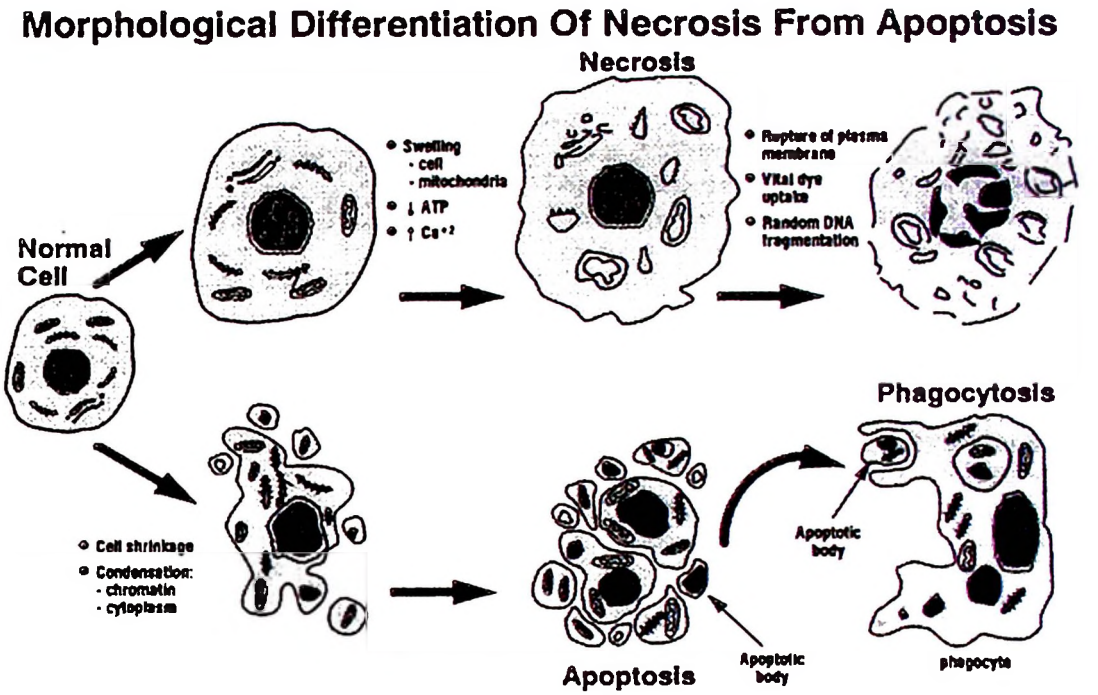

Figure 2.7 : Morphological differentiation of necrosis from apoptosis.

The integrity of the plasma membrane and cell swelling are marked in necrosis, whereas cell shrinkage and fragmentation are most common in apoptosis. Most notable is the discrete process of phagocytosis of the "apoptotic bodies" by resident cells eliminating apoptosis, whereas cell eruption and content leakage (events triggering inflammation) are the final outcome of necrosis (Feuerstein *et al.*, 1997).

2.8 Nuclear staining

Hoechst 33258 (chemical name 4-[5-(4-methyl-1-piperazinyl)-2-benzimidazolyl]-2-benzimidazolyl phenol) (Figure 2.8) is a flouresecent dye and it is cell membrane-permeant, thus is useful as a DNA-binding fluorochrome for staining chromosomes within cell nuclei visualize nuclear changes and apoptotic body formation. It is adenine-thymine-specific dyes that bind to the minor groove of DNA rather than by intercalation (Zhang *et al*, 1999). Their affinity for DNA is sufficiently strong that they will displace bound molecules of variety of intercalating dyes (Shapiro, 2003).

Hoechst stain is widely used for the evaluating the cell cycle, apoptosis, and quantifying viable cells by flow cytometry (Zhang and Kiechle, 1997). DNA stain with nuclear dyes allow the visualization of chromatin condensation, which is a hallmark of apoptosis (ed Kettleworth, 2007). However, only apoptotic cells in the stage of morphological changes can be detect by this type of staining. While apoptotic cells in the earlier stage cannot not be present by Hoechst stain (Fabian *et al.*, 2003). The excitation and emission wavelength of Hoechst-DNA complex are 350nm and 460nm respectively (eds Hughes and Mehmet, 2003).

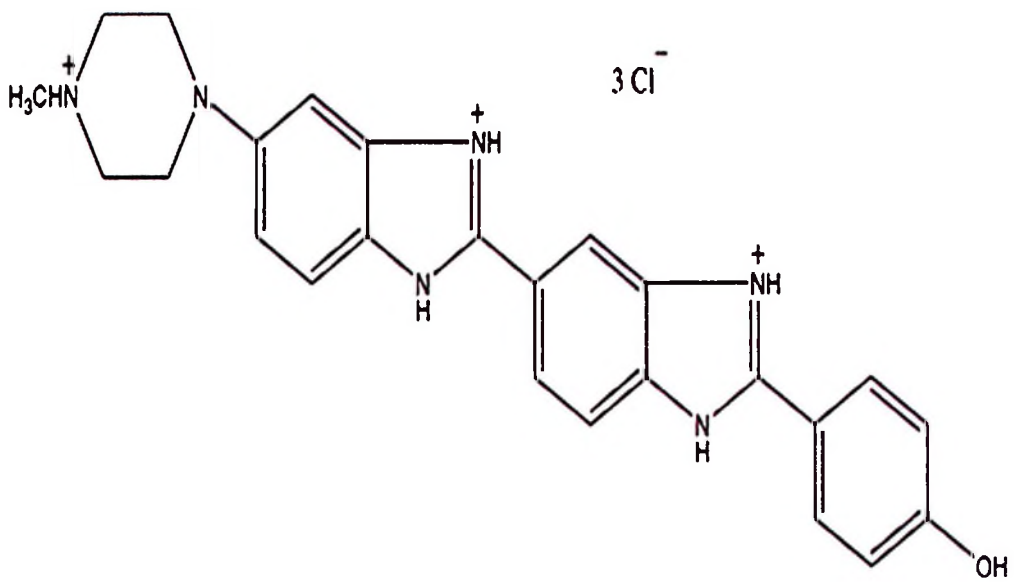

Figure 2.8 : Chemical structure of Hoechst 33258 (Sabnis, 2010)

Molecular Formula: $C_{25}\ H_{27}Cl_3N_6O$

Molecular Weight: 533.88

Physical: Form Dark yellow to tan powder with green cast

(Sabnis, 2010)