EFFECTS OF TWO DIFFERENT MATURITY OF FREEZE DRIED COCONUT WATER ON CERVICAL CANCER CHEMOPREVENTION USING IN VITRO ANALYSIS

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UNIVERSITI SAINS MALAYSIA

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LIST OF ABBREVIATIONS

oC	Degree celcius
%	Percentage
g	Gram
g/mL	Gram per milliliter
mg/mL	Milligram per milliliter
mL	Milliliter
mM	Millimolar
µg	Microgram
µg/mL	Microgram per millimeter
µL	Microliter
CO2	Interleukin
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
DMEM	Dulbecco's Minimum Essential Medium
FBS	Fetal Bovine Seum
PBS	Phosphate Buffered Saline
RPM	Revolution Per Minute
SD	Standard Deviation
TBEA	Trypan Blue Exclusion Assay

KESAN ANTARA DUA KEMATANGAN AIR KELAPA BEKU KEATAS KEMOPENCEGAHAN MENGGUNAKAN ANALISIS IN VITRO

ABSTRAK

Dengan kebimbangan yang semakin meningkat tentang kemopencegahan di Malaysia, penggunaan produk semula jadi dan bahan buangan daripada produk botani mungkin mempunyai kesan yang baik ke arah kajian kanser kemopencegahan. Kemopencegahan merujuk kepada usaha-usaha untuk mencegah dan melambatkan pembentukan kanser dengan menggunakan agen semula jadi atau sintetik. Di Malaysia, kelapa adalah tanaman perindustrian penting keempat selepas kelapa sawit, getah dan padi. Kepelbagaian utama yang ditanam ialah Malayan Tall, MATAG, MAWA, PANDAN dan Malayan kerdil. Kajian ini melibatkan perbandingan kesan kemopencegahan antara MATAG muda iaitu 5 hingga 6 bulan umur dan MATAG Lama yang 6-9 bulan umur menggunakan analisis *In Vitro* pada sel kanser serviks (HeLa). IC_{50} kedua-dua tempoh matang pada sel-sel HeLa ditentukan dengan kaedah trypan biru exclusion assay (TBEA) dengan IC_{50} daripada 100µg / mL. Percambahan sel assay dipantau dan menunjukkan kesan yang merencat berkesan ke atas sel-sel HeLa. Kepekatan yang merencatkan pendedahan dos antara MATAG muda dan lama menunjukkan bahawa terdapat perbezaan yang signifikan di dalam MATAG muda mengurangkan percambahan sel-sel HeLa dalam kepekatan dan cara bergantung masa berbanding MATAG lama. Ini menunjukkan bahawa terdapat min yang berbeza dalam merencatkan sel setiap dos di MATAG muda $p<0.001$ manakala tidak ada yang berbeza dalam MATAG lama $p> 0.05$. Pemerhatian mikroskopik assay percambahan sel dan Hoescht 33342 noda menunjukkan bahawa rawatan MATAG muda mendorong ciri apoptotik sel. Ini adalah satu kajian awal air kelapa pada titisan sel kanser dan analisis selanjutnya bahagian sel pada peringkat molekul perlu diteruskan pada masa hadapan.

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ABSTRACT

With the increasing concerns about chemoprevention in Malaysia, the utilizing of natural and waste from botanical product might have beneficial effects towards cancer chemoprevention study. Chemoprevention refers to the efforts to prevent and delay the development of cancer by using natural or synthetic agents. In Malaysia, coconut is the fourth important industrial crop after oil palm, rubber and paddy. The major variety grown is the Malayan Tall, MATAG, MAWA, PANDAN and Malayan Dwarfs. This study involves comparative chemoprevention effect between MATAG young which is 5 to 6 months age and MATAG Old which is 6 to 9 month age using In Vitro analysis on cervical cancer cell line (HeLa). The IC_{50} of both maturities on HeLa cells were determined with Trypan Blue Exclusion Assay (TBEA) method with an IC_{50} of 100µg/mL. Cell proliferation assay was monitored and showed an effective inhibitory effect on HeLa cells. The inhibitory concentration on dose exposure of *Cocus nucifera* shows that there are significant different in MATAG young in reduces the viability of HeLa cells in a concentration and time dependent manner compared to MATAG old. It shows that there are mean different in cell viability of each dose in MATAG young $p<0.001$ while there are no different in MATAG old $p>0.05$. Microscopic observation of cell proliferation assay and Hoescht 33342 stain shows that *Cocus nucifera* treatment induces apoptotic features of cells. This is a preliminary study of coconut water on cancer cell line and further analysis of the cell proportion on molecular levels need to be continue in future. It was concluded that the MATAG young extracts exhibited a better chemoprevention effect on HeLa cells.

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

Cancer has been known since human societies first recorded their activities. It was well known to the ancient Egyptians and to succeeding civilizations but as most cancers develop in the latter decades of life until the expectation of life began to increase from the middle of the nineteenth century onwards the number of people surviving to this age was relatively small. Nowadays, the infectious diseases are the major cause of death in the past have been controlled by improvements in public health and medical care, the proportion of the population at risk of cancer has increased dramatically. Although disease of the heart and blood vessels are still the main cause of death in our aging populations now cancer are the major problems (Underwood, J.C.E, 2004).

For this reason, cancer chemoprevention and control are major health issues. However, cancer research has wider significance. Cancer is not confined to man and the higher mammals but affects almost all multicellular organisms, plant as well as animals. Since it involves disturbances in cell proliferation, differentiation and development, knowledge of the processes underlying this disease help us to understand the very basic mechanisms of life (Underwood, J.C.E, 2004).

Despite the availability of diagnostic tools and current treatments of the disease, cervical cancer still remains the second most common cancer amongst women in the world. National Cancer Registry (NCR) 2007 report states that cervical cancer are the third most common amongst women's in Malaysia. Since the intervention of Pap Smear have been introduced in clinical practice guidelines, the conditions have been improved due to inexpensive and simple diagnostic tools can be used in early detection stage (Malaysian National Cancer Registry Report, 2003). Conventional therapeutic and surgical approaches have not been able to control the occurrence of cervical cancer. Therefore, there is an urgent need to develop mechanisms based approaches for the management of cancer.

Chemoprevention via non – toxic agents have shown cancer chemoprevention potential in a variety of assays and animal model having relevance to human disease. It is appreciated that an effective and acceptable chemoprevention agent have certain properties of little or non – toxic effects in normal cells known mechanisms or action, low cost and acceptance by Malaysian populations. There is an overwhelming amount of information in the press and on the internet that suggests certain food, supplements or medications that can prevent cancers. The term chemoprevention refers to the efforts to prevent and delay the development of cancer by taking medicines, vitamins or other agents (Jemal A, Siegel R, Ward; et al, 2008).

With the increasing concerns about cancer chemoprevention in Malaysia, the utilizing of natural , waste from botanical product might have beneficial effect towards cancer chemoprevention study. Chemoprevention is divided into 3 groups that is primary, secondary and tertiary prevention. It starts with the strategies seek to prevent malignancies in an otherwise healthy population then the prevention involves pre-malignant lesion utilizing medications in an attempt to prevent the progression of these lesion into cancers and finally it focuses on the prevention of new cancers in patients cured of an initial cancer or individuals who have been treated for pre-malignant lesion (Thompson IM, Goodman PJ, Tangen CM, et al,2003).

Therefore, this study will take an initiative to observe and monitor the potential of these multi-purpose products towards cancer chemoprevention activity in HeLa model of cervical cancer cell line.

The coconut palm *(Cocus nucifera*) is aptly described as the 'Tree of Life' with multifarious uses. It is socially and culturally linked besides providing jobs and income to millions of people. In many island economies, it is the major source of revenue and is an integral part of the livelihood of the population. Malaysia is located in the South East Asian region and covers an area of 329,961 km^2. It shares borders with Singapore, Thailand, Indonesia and Brunei. The South China Sea separates West and East Malaysia, West Malaysia being a peninsular region of the Asian continent whereas East Malaysia is located on the island of Borneo (National Cancer Registry, 2003).

In Malaysia, coconut is the fourth important industrial crop after oil palm, rubber and paddy in terms of total planted area. It is also on the oldest agro-based industries in Malaysia. The major variety grown is the Malayan Tall followed by the hybrid MATAG, MAWA, aromatic dwarf (PANDAN) and the Malayan Dwarfs (MARDI, 2012). This study involve comparative chemoprevention effect between MATAG Young and MATAG Old freeze dried water using *In Vitro* analysis on cervical cancer cell line (HeLa) will be used in this study.

The nut is the most marketable part is the inner part of the nut has two edible part that are a white kernel and a clear liquid coconut water. (Alexiaprades et al, 2012). The cavity within the kernel contains coconut water this part begins to form as a gel when the coconut is 5 to 6 months old. It becomes harder, whiter and the inside is filled with coconut water called Young coconut (Oliveria et al, 2003). Mature coconuts between 6 to 9 months contain about 750 mL of water that eventually becomes the flesh called Old coconut. The coconut fruit takes 11 to 12 months to reach full maturity (Oliveria et al, 2003).

Freeze drying also known as 'Lyophilization' is a method of processing a liquid product in a dry solid product. With freeze-drying, biological product can be dried at low temperature. The avoidance of high temperature helps to reduce the extent of decomposition or loss of activity in biological product and can also circumvent the alteration of taste and sensory qualities in samples. Freeze dried product have a high surface area which enables them to be reconstituted quickly and easily with the re-introduction of the solvent usually water (Labconco Corporation, 2010).

Freeze drying involves removing the water from a material which is involves three stages. First, the product is frozen to a defined temperature then the free ice is removed during "primary drying" by sublimation under vacuum. Finally, in secondary drying typically by higher temperature and lower pressure much of the remaining which is unfrozen water may be desorbed under vacuum. A typical freeze dried product will occupy the same volume as the original sample even if it was initially a liquid (Labconco Corporation, 2010).

Food benefited from being freeze dried as their sensory qualities such as color, size, smell and taste are only minimally affected in comparison to other drying processes. Besides that, Freeze drying is also more compatible with the production in comparison to dry powder filing. In addition, solutions can be sterile filtered immediately before being transferred to vials and freeze dried. Freeze drying can increase product viable storage time at more economical and practical temperatures (Labconco Corporation, 2010).

1.2 OBJECTIVES

1.2.1 GENERAL OBJECTIVE:

To understand the chemoprevention effect of *Cocus nucifera* freeze dried water between MATAG Young and MATAG Old on cancer cell line

1.2.2 SPECIFIC OBJECTIVES:

1. To determine the Inhibitory Concentration (IC_{50}) of *Cocus nucifera* freeze dried water with different maturity on cancer cell line.

2. To identify the anti – proliferative effect of *Cocus nucifera* freeze dried water with different maturity on cancer cell line.

3. To observe any morphological changes upon treatment with different maturity of *Cocus nucifera* freeze dried water on cancer cell line.

1.3 HYPOTHESIS

- Different maturity of freeze dried coconut water will give different cancer chemoprevention activities on cancer cell line.

- Young green coconuts have a high composition of nutrition that plays an essential role in cancer chemoprevention activities.

CHAPTER 2

LITERATURE REVIEW

2.1 CERVICAL CANCER

2.1.1 ANATOMY AND PHYSIOLOGY

The cervix is the lower fibromuscular portion of the uterus. It is cylindrical or conical in shape and measures 3 to 4 cm in length and 2.5 cm in diameter. It is supported by the cardinal and uterosacral ligaments which stretch between the lateral and posterior portions of the cervix and the walls of the bony pelvis. The lower half of the cervix called portio vaginalis protrudes into the vagina through its anterior wall and the upper half ramains above the vagina. The portion vaginalis opens into the vagina through an orifice called the external os (Tortora,2009).

The cervix varies in size and shape depending on the woman's age, parity and hormonal status. In parous women, it is bulky and the external os appears as a wide, gaping, transverse slit. In nulliparous women, the external os resembles a small circular opening in the centre of the cervix. The supravaginal portion meets with the muscular body of the uterus at the internal cervical os. The portion of the cervix lying exterior to the external os is called the ectocervix. This is the portion of the cervix that is readily on speculum examination (Tortora,2009).

The portion proximal to the external os is called the endocervix and the external os needs to be stretched or dilated to view this portion of the cervix. The endocervical canal which transverse the endocervix connects the uterine cavity with the vaginal and extends from the internal to the external os where it opens into the vagina. It varies in length and width depending on the woman's age and hormonal status. It is widest in women in the reproductive age group when it measures 6 – 8 mm in width. The space surrounding the cervix in the vaginal cavity is called the vaginal fornix. The part of the fornix between the cervix and the lateral vaginal walls is called the lateral fornix while the portions between the anterior and posterior walls of the vagina and the cervix are termed the anterior and posterior fornix (Tortora,2009).

The stroma of the cervix is composed of dense fibromuscular tissue through which vascular, lymphatic and nerve supplies to the cervix pass and form a complex plexus. The arterial supply of the cervix is derived from internal iliac arteries through the cervical and vaginal branches of the uterine arteries. The cervical branches of the uterine arteries descend in the lateral aspects of the cervix at 3 and 9 o'clock positions. The veins of the cervix run parallel to the arteries and drain into the hypogastric venous plexus. The lymphatic's vessels from the cervix drain into the common, external and internal iliac nodes, obturator and the parametrial nodes. The nerve supply to the cervix is derived from the hypogastric plexus. The endocervix has extensive sensory nerve endings while there are very few in the ectocervix.

The cervix is covered by both stratified non – keratinizing squamous and columnar epithelium. These two types of epithelium meet at the squamous columnar junction (Tortora,2009).

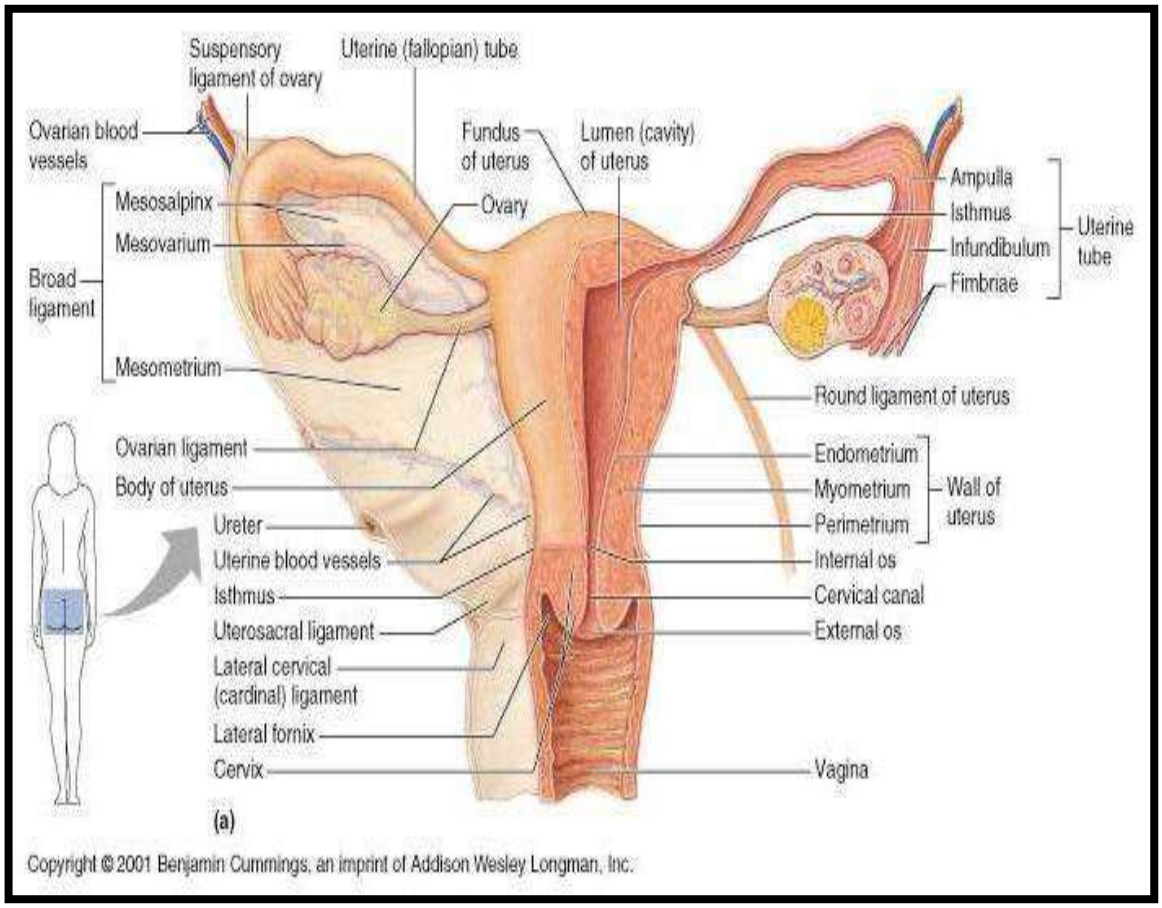

Figure 1.1 Cross-section anatomy of female reproductive system (Tortora, 2009)

2.1.2 EPIDEMIOLOGY AND STATISTICS

The Malaysian National Cancer Registry Report (NCR) 2003 found that most frequently occurring cancers in Malaysian women are cancers of the breast, cervical, colon, ovary, leukemia and lungs. Cervical cancer caused about 12.9% of all female cancers in Malaysia. This was higher than other Asian and Western countries and even globally (National Cancer Registry, 2003). Death from cervical cancer are rare amongst young women but its incidence increased from the age 30 years and peaked at 60 – 69 years. Half of the cases involved women ages 40 – 59 years. Incidence rates were in general highest among Chinese women (28.8/100,000) followed by Indians (22.4/100,000) and the lowest amongst by Malays (10.5/100,000) (Malaysian National Cancer Registry Report, 2003).

The Ministry of Health reported an average of 2000 to 3000 hospital admissions of cervical cancers cases per year in the country and most of them presenting late into the disease (Devi Beena CR, Tang TS, Gerard LC, 2008). The annual cervical cancer death rate is 5.6 per 100,000 (Cervical Cancer Incidence and Mortality Rates 2011). The mortality rate due to cervical cancer in Malaysia is more than two times higher than the Netherlands, United Kingdom, and Finland. Even with the introduction of screening program and immunization against cervical cancer, the mortality rate has not decreased to a desirable level. The economic burden due to cervical cancer is enormous. It costs about RM312 million (USD76 million) to manage cervical cancer from prevention to managing invasive disease annually in Malaysia. A big proportion which is 67% of this is spent to manage invasive cancer cases (Sharifa Ezat WP, Syed Aljunid, 2010).

Syed M AlJunid have discussed the burden of cervical cancer in Malaysia and the potential costs and consequences of Human Papilloma Virus (HPV) vaccination (Syed M Aljunid, Zafar A, Saperi S, *et al*,.2010). Since cervical cancer is treated primarily within regional hospitals while pre-cancerous lesions are treated within an ambulatory care set up, the burden was estimated as the direct, indirect and total annual costs associated with cervical cancer and pre-cancerous lesions in Malaysia (Syed M Aljunid, Zafar A, Saperi S, *et al* .2010). Data from World Health Organization (WHO) and Global cancer incidence, mortality and prevalence (GLOBOCAN) estimated 4696 prevalent cases of cervical cancer annually in Malaysia. Based on this, Syed M AlJunid found the estimated treatment cost to be RM 37,652,528 for inpatient and outpatients. There were 1372 cases estimated for pre-cancerous lesion with the cost of outpatient treatment calculated as RM 1,501,171 making the direct total management of HPV related disease to be RM 39, 153, 699 with and additional RM12.4 million in indirect cost due to lost productivity (Syed M Aljunid, Zafar A, Saperi S, *et al* .2010).

2.1.3 RISK FACTOR OF CERVICAL CANCER

The World Health Organization (WHO) had linked persistent high risk HPV infection particularly HPV 16 and 18 to cervical cancer. Human Papilloma Virus (HPV) infections are cleared within two years in most women. It is the persistent high risk HPV infection that puts the infected individual at high risk to develop CIN 3 and cervical cancer. Many studies in Malaysia also showed this association (Cheah PL, 2011). An independent, prospective, multi – centered, hospital – based cross – sectional studies involving Malaysia, Vietnam, Singapore, South Korea and the Philippines evaluate the prevalence of Human Papilloma Virus (HPV) in women older than 21 years old with Invasive Cervical Cancer (ICC) and high – grade pre-cancerous lesions (Quek SC, Lim BK, Domingo E, *et al.*2013).

Saini R studied compared the performance nested MY/CP Polymerase Chain Reaction (PCR) and FDA approved – Hybrid Capture II (HCII) using clinical cervical scrapings from 40 patients. It was found that PCR was more sensitive compared to CHII in detecting HPV but the specificity of HCII was much higher than PCR. The Negative Predictive Value (NPV) of both the techniques was quite similar but Positive Predictive Value (PPV) of HCII was much higher compared to PCR. While the HCII method showed good specificity for HPV detection it is less sensitive than PCR
(Saini R, Shen TH, Othman NH, *et al.*2007).

Tay et al studied the prevalence of high – risk HPV DNA among 2364 women. The overall prevalence of high risk HPV DNA showed the peak age to be in women between 20 – 24 years old and a second peak prevalence in women 50 – 54 years. This pattern of peak prevalence is similar to the general trend published from other parts of the world. Therefore it is recommended that the implementation of a comprehensive cervical cancer screening and anti – HPV-16 and 18 vaccination program is an important and urgent measure for reducing the burden of cervical in Malaysia and Singapore (Tay SK, Tay YK, 2009).

2.1.4 DIAGNOSIS OF CERVICAL CANCER

In Malaysia, Pap Smear screening has been available since the 1960s, yet cervical cancer remains the second most genital cancer afflicting Malaysia women it was the eighth leading cause amongst medically certified death in 1998 (Kasri, 1993).A variety of devices are available to obtain cervical samples such as the spatula with cytobrush, Accelon Combi cervical biosampler, and Papette (Figo Committee on Gynaecological Oncology, 2000). The specimen once taken is generally smeared on a glass slide, wet fixed in 95% ethanol or cytospray and sent to the laboratory for staining and screening. Recently, the advent of liquid based cytology has improved the sensitivity of cytologic screening (Austin et al, 1998). Computerized screening devices like Papnet and AutoPap for primary screening or for re-screening of negative cases have offered the possibility of greater sensitivity and specificity albeit with an added cost (CPG Cervical Cancer, 2003). The use of Human Papilloma Virus (HPV) typing as a potential primary screening tool is currently under evaluation (Figo Committee on Gynaegological Oncology, 2000).

Another study of 200 cervical swab samples by Chong also concluded that PCR was a reliable method to detect HPV. Both studies concluded that PCR was an ideal and reliable method for detecting HPV from clinical samples (Chong PP, Asyikin N, Rusinahayati M, *et al.*2010). A study that compared conventional Pap smears to split sampling using ThinPrep smears found split sampling from discarded sampling devices after conventional Pap smears retain adequate sample cells for diagnostic purposes (Norodiyah O, Othman NH. 2012).

2.2 CHEMOPREVENTION

Carcinogenesis is generally recognized as a multistep process in which distinct molecular and cellular alterations occur. From the experimentally induced carcinogenesis in rodents, tumor development is considered to consist of several separate but closely linked stages for instances tumor initiation, promotion and progression. Although these divisions are an oversimplification of carcinogenesis but it is useful to think in these stages when considering possible opportunities for chemoprevention (Young,2003).

Initiation is a rapid and irreversible process that involves a chain of extracellular and intracellular events. These includes the initial uptake or exposure to a carcinogenesis agents, its distribution and transport to organs and tissues where metabolic activation and detoxification can occur and the covalent interaction of reactive species with target cell DNA leading to genotoxic damage. In contrast to initiation, tumor promotion is considered to be a relatively lengthy and reversible process in which actively proliferating pre – neoplastic cells accumulate. Progression is the final stage of neoplastic transformation which involves the growth of a tumor with invasive and metastatic potential (Young,2003).

According to the conventional classification originally proposed by Lee Wattenberg, chemoprevention agents can be divided into main categories which are blocking agents and suppressing agents. Blocking agents prevent carcinogens from reaching the target sites from undergoing metabolic activation or from subsequently interacting with crucial cellular macromolecules such as DNA, RNA and proteins. On the other hand, suppressing agents inhibit malignant transformation of initiated cells in either the promotion or the progression stage. Chemoprevention phytochemicals can block or reverse the pre – malignant stage which are initiation and promotion of multistep carcinogenesis (Young,2003).

Chemoprevention phytochemicals can also halt or at least retard the development and progression of pre – cancerous cells into malignant ones. Recent advances in understanding of the carcinogenesis process at the cellular level shown this blocking and suppressing categorization to be an oversimplified and a lot of cellular molecules and events that can be potential targets of chemoprevention agents have been more specifically identified. Therefore, the ability of any single chemoprevention phytochemical to prevent tumor development should be recognized as the result of the combination of several distinct sets of intracellular effects rather than a single biological response (Manson, 2003, Milner, 2001).

The cellular and molecular events affected or regulated by these chemoprevention phytochemicals including carcinogen activation or detoxification by Xenobioitic metabolizing enzymes, DNA repair, Cell cycle progression, Cell proliferation, Differentiation and apoptosis expression, Functional activation of oncogenes or tumor suppressor genes, Angiogenesis, Metastasis , Hormonal and growth factor activity

2.3 *COCUS NUCIFERA*

2.3.1 BACKGORUND

Cocus nucifera is a large palm, growing up to 30 meters (98ft) tall, with primate leaves 4-6 meters (13-20ft) long and pinnae 60-90 cm long, old leaves break away, leaving the trunk smooth. Coconuts are generally classified into two general types which is tall and dwarf (Pradeepkumar et al.,2008). It is found across much of the tropic and sub-tropic area, the coconut is known for its great versatility as seen in the young domestics, commercial and industrial uses of its different parts. Coconuts are parts of the daily diet of many (Alzebroek, 2008). When young, the entire fruits are used as melons. When mature, only seeds are used as nuts. Its endosperm is initially in its nuclear phase suspended within the coconut water.

As development continue, the cellular layers of endosperm deposits along the walls of the coconut becoming the edible coconut “flesh”. When dried, the coconut flesh is called copra (Alzebroek, 2008). The oil and milk derived from it are commonly used in cooking and frying, coconut oil is also widely used in soaps and cosmetics. The clear liquid coconut water within is a refreshing drink and can be processed to create alcohol. The husks and leaves can be used as materials to make a variety of products for furnishing and decorating (Adkins et al., 2006). The coconut (Cocus nucifera) is an important fruit in the tropical regions and the fruit can be made into a variety of foods and beverages.

The edible part of the coconut fruit either coconut meat and coconut water is the endosperm tissue. Endosperm tissue undergo one of the three main modes of development which are the nuclear, cellular and helobial modes and the development of coconut endosperm belongs to the nuclear mode (Patrick J.W,2001). Initially, the endosperm is a liquid containing free nuclei generated by a process in which the primary endosperm nucleus undergoes several cycles of division without cytokinesis. After cytokinesis occur, progressing from the periphery towards the centre thus forming the cellular endosperm layer.

At first, the cellular endosperm is translucent and jelly-like, but it later hardens at maturity to become white flesh (coconut meat). Unlike the endosperms of other plants, the cellularization process in a coconut fruit does not fill up the entire embryo sac cavity, but instead leaves the cavity solution-filled. This solution is commonly known as coconut water and it is of cytoplasmic origin (Janick. J, 2001). Nutrients from coconut water are obtained from the seed apoplasm (surrounding cell wall) and are transported symplasmically (through plasmodemata, which is the connection between cytoplasms of adjacent cells) into the endosperm (Patrick J.W,2001). Coconut water should not be confused with coconut milk although some studies have used the two terms interchangeably (Sandhya V.G, 2008).

The aqueous part of the coconut endosperm is termed coconut water whereas coconut milk also known as “santan” in Malaysia and Indonesia and “gata” in the Philipines refers to the liquid products obtained by grating the solid endosperm with or without addition of water (APCC, 1994). Coconut water is served directly as a beverage to quench thirst while coconut milk is usually used as a food ingredient in various traditional cooking recipes. The main components of coconut milk are water, fat and protein whereas coconut water contains mainly 94% water (Seow, 2007). Unlike coconut water, coconut milk which is the source of coconut oil is generally not used in plant tissue culture medium formulation (George, 2004).

The coconut fruit takes between 11 and 12 months to reach full maturity. At 5 month, the kernel begins to form a thin layer of jelly around the inside of the endocarp or shell. The shell encloses the tender water, a clear sweet liquid. At this time the water is under pressure. During the ripening process, the pressure is released and the water is partially replaced by the kernel. Little by little, the kernel grows and replaces the water by cells storing lipid (Henry et al, 2011). Its composition changes as the nut grows (Narayanan, 2007). At full maturity which is 12 months, coconut water represent between 15% and 30% of the weight of the nut. The amount of coconut water that can be harvested from each nut is about 300mL, but depends to a great extent on the stage of maturity and on the variety of coconut. There are only three types of coconut varieties which is tall (allogamous), dwarf (autogomous) and hybrids the last often being a cross between dwarf (mostly mother) and tall (father).

2.3.2 TRADITIONAL USES OF *COCUS NUCIFERA*

Coconut water is a sterile and pure liquid. Coconut water has been a religious symbol for a long time. In Asia and especially in India tender for example immature coconuts are offered as ceremonial gifts and serve as purification media at traditional events (Rethinam, 2001). Centuries ago, Polynesian, Malesian and Micronesian mariners used coconut fruits as reserves of food and drink. Due to this naturally canned beverage they survived on their journeys from one island to another and colonized the entire Pacific Ocean. At present, coconut water from immature is still consumed as a refreshing drink by thousands of inhabitants of tropical regions. Apart from its consumption as a natural drink, one of the most important uses coconut water is medicinal (Nanda, 1990). In the Indian ayurvedic medicine, it is described as "unctuous, sweet, increasing semen, promoting digestion and clearing the urinary path" (Rethinam, 2001).

Coconut water is traditionally prescribed for burning pain during urination, dysuria, gastritis, burning pain of the eyes, indigestion and hiccups or even expelling of retained placenta. In case of emergency in remote region of the world and during World War II, coconut was used as a short term intravenous hydration and resuscitation fluid (Cambell, 2001). In the early 1960, coconut water already known to favor microbial growth and especially *"Nata de coco"* bacterium (Priya, 2014). *Nata de coco* is bacterial cellulose naturally produced at the coconut water. Native to the Philippines, *Nata de coco* has become popular in many other Asian countries. The "Nata" bacterium was later identified as Acetobacter xylinum (Andres, 2004).

Traditionally coconut water is also processed into wine or vinegar due to sugar content and ability to ferment (Augustine, 2007). Coconut water has been shown to induce division of mature cells. For example, the growth of spinach tissue on a medium supplemented with 10% to 15% mature coconut water increased the weight of spinach callus after 5 weeks and accelerated shoot regeneration (4-5 weeks instead of 8-12 weeks without) . Many authors reported that coconut water contains a growth factor that stimulates different bacterial strains and *in vitro* culture of plants (Huang,1992). For this purpose, coconut water from immature fruits was reported to produce better results than water from mature fruits.

2.3.3 CHEMICAL COMPOSITION OF *COCUS NUCIFERA*

Coconut water in its envelope is sterile and composed of both organic and inorganic compounds almost all minerals found in food. Compared with other fruit juices, the dry weight of mature coconut water is very low that is 5 - 6% versus 12 - 15% for apple juice. The main components of coconut water are soluble sugars but it also contains proteins, salts and a very small quantity of soil which contributes to its very low food energy level that is 44 cal L^{-1}. The vitamin C content varies from 20 to 40 mg L^{-1}. This is not high compared with other fruits such as orange or acerola, but it is sufficient to prevent oxidation for a limited period. The vitamin B group is present in coconut water with 0.64 μg mL^{-1} of nicotinic acid and 0.52 μg mL^{-1} of panthotenic acid (Thampan, 2004).

Sugars are the main fraction of soluble solids in coconut water (Pue A.G, 1992). The main sugars in mature coconut water are sucrose, sorbitol, glucose and fructose followed by minor sugars including galactose, xylose and mannose (Pue A.G,1992). The second constituents in terms of quantity are minerals. They account for only 0.4% to 1% of the liquid volume, but nevertheless contribute to its isotonic properties. The osmolarity of coconut water is about 300 mOsmL-1 (Ritcher E.M,2005). Most of authors agreed that potassium is the main mineral element in coconut water (Uphade B.K, 2008). According to Thampan and Rethinam, the major differences in mineral composition between immature and mature coconut water were due to potassium, chloride, iron and sulphur content (Thampan P.K, 2004).