CYTOTOXIC AND WILD TYPE P53-PROMOTING ACTIVITIES OF SELECTED ZINGIBERACEAE SPECIES IN COLON CANCER CELL LINES

FATHIAH ABDULLAH

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
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FATHIAH ABDULLAH

DISSERTATION SUBMITTED IN FULLFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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The Zingiberaceae family is distributed worldwide and is acknowledged not only as an essential food but has been used throughout history for their medicinal effects. In the present study, 30 crude petroleum ether, chloroform and methanol extracts of ten selected Zingiberaceae species namely *Alpinia galanga*, *Boesenbergia rotunda*, *Curcuma aeruginosa*, *Curcuma domestica*, *Curcuma mangga*, *Curcuma xanthorhiza*, *Kaempferia galanga*, *Zingiber montanum*, *Zingiber officinale* and *Zingiber zerumbet* were studied. The crude extracts of these plants were screened for their cytotoxic activity (with and without 5-fu) against human colon cancer derived- HCT-116 and HT29 cells and for their wild type p53 (wt-p53) promoting activity in HCT-116 and HT29 cell lines.

The crude extracts of selected Zingiberaceae were screened at varying concentrations ranging from 1 μg/ml to 100 μg/ml for their cytotoxic activity using the neutral red (NR) cytotoxicity assay. Results indicated that the percentage of inhibition in HCT-116 and HT29 cells increased with the increasing concentration of extracts.

The best extracts from selected Zingiberaceae extracts that showed active cytotoxic activity were further tested with 5-Fluorouracil (5-Fu) against HCT-116 and HT29 cells using neutral red (NR) assay. The combination of 5-Fu with all the selected extracts
exhibited better cytotoxic effect and gave the better values of IC$_{50}$ as compared to 5-Fu alone. Synergy with Zingiberaceae extracts was also seen at low 5-Fu concentrations.

In the current study, crude petroleum ether, chloroform and methanol extracts of ten selected Zingiberaceae species were analyzed qualitatively for wt-p53 proliferation in colon cancer cell lines, HCT-116 and HT29. The cells were then treated independently with each of the crude extracts at varying concentrations of 0, 1, 10, 25, 50 and 100 μg/ml for 72 hours at 37°C in the 5 % carbon dioxide (CO$_2$) incubator. The 3-step indirect avidin-biotin immunoperoxidase technique was subsequently used to examine the expression of wt-p53 in both cell lines. Results showed that the expression of wt-p53 increased in HCT-116 and HT29 cells treated with crude extracts of the selected Zingiberaceae species.

The results of the present study indicated that *Zingiber zerumbet*, *Alpinia galanga* and *Zingiber officinale* extracts showed significant cytotoxic effect and wt-p53 promoting activity. Further studies should be carried out to isolate and identify the active compound(s) and to elucidate their mechanism of action.
AKTIVITI SITOTOKSIK DAN PENINGKATAN AKTIVITI WT-P53 OLEH SPESIS ZINGIBERACEAE TERPILIH KE ATAS LELUHUR SEL KANSER KOLON

ABSTRAK


Ekstrak petroleum eter, kloroform dan metanol tumbuhan Zingiberaceae terpilih dikaji aktiviti ketoksikannya menggunakan esei sitotoksik Neutral Red pada kepekatan yang berbeza bermula dari 1 μg/ml hingga 100 μg/ml. Keputusan daripada ujian ini menunjukkan bahawa peratusan kematian sel kanser HCT-116 dan HT29 meningkat dengan peningkatan kepekatian ekstrak yang digunakan.

Ekstrak Zingiberaceae yang nilai IC₅₀ nya menunjukkan bacaan sitotoksik yang aktif seterusnya diuji dengan 5-Fluorouracil (5-Fu) terhadap sel kanser HCT-116 dan HT29
menggunakan esei neutral red (NR). Kombinasi 5-Fu bersama ekstrak terpilih jelas menunjukkan kesan sitotoksik yang lebih baik dengan memberikan nilai IC₅₀ yang lebih rendah jika dibandingkan dengan sel kanser yang hanya diuji dengan 5-Fu sahaja.

Dalam kajian yang dijalankan ini, 10 tumbuhan terpilih daripada famili Zingiberaceae telah diekstrak menggunakan petroleum eter, kloroform dan metanol dan seterusnya dijalankan ujian kualitatif untuk mengesan kehadiran wt-p53 dalam leluhur sel kolon, HCT-116 dan HT29. Sel ini kemudiannya diuji dengan ekstrak tumbuhan pada kepekatan 0, 1, 10, 25, 50 dan 100 μg/ml untuk tempoh 72 jam pada suhu 37°C dalam inkubator yang mengandungi 5% karbon dioksida (CO₂). Teknik imunositokimia kemudiannya dijalankan ke atas keduadua sel tersebut. Keputusan yang diperolehi menunjukkan bahawa aktiviti wt-p53 dalam keduadua sel yang terhasil daripada tindakan ekstrak Zingiberaceae adalah berbeza dan tidak tetap di antara satu sama lain.

Keputusan kajian ini menunjukkan bahawa spesis Zingiber zerumbet, Alpinia galanga dan Zingiber officinale mempunyai kesan toksik yang baik dan juga meningkatkan aktiviti wt-p53. Kajian yang lebih lanjut diperlukan untuk memencil dan mengenalpasti sebatian aktif yang terdapat di dalam tumbuhan tersebut serta mekanisme di sebalik aktiviti yang ditunjukkan akan juga dikaji.
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CHAPTER 1

INTRODUCTION

Colorectal cancer represents one of the most important challenges in the field of cancer chemotherapy and one of the most common malignancies worldwide (Gabriella et al., 1988; Chinery et al., 1997). Colorectal cancer that ranks fourth in cancer incidence in the United States (National Cancer Institute of USA, 2004) is also one of the five most frequently reported cancers in Malaysia. Colorectal cancer has become the number one diagnosed cancer in men and the third most common cancer among women in Malaysia (National Cancer of Registry, 2004).

The existing therapies employed for colorectal cancer are surgery, chemotherapy and radiotherapy. However, these 3 methods can cause serious side effects to the patients and this form the basis in the growing interest in herbal medicine as substitute therapeutic approach for colon cancer and other cancers. Even though major advances have been achieved in the treatment of malignancy through radiotherapy and chemotherapy, the search for naturally occurring cancer chemoprevention compounds is still the focus of research worldwide.

Malaysia is one of the twelfth mega diversity countries in the world. These mega diversity countries harbour more than 70% of the world’s biodiversity (McNeely et al., 1990). These forests as well as Malaysia’s forest are the largest source of the natural products and have served as a major source of drugs for centuries (Clark, 1996). There are a large number of edible plants; especially that eaten for purposes other than nutrition
which may be promising sources for biologically active properties including anticancer, 
antiviral and antioxidant.

Many components that are derived from medicinal or dietary plants possess potential 
chemopreventive properties (Seong et al., 2002). Cancer chemoprevention is a concept 
defined as the prevention of cancer by the administration of natural or synthesized pure 
chemical, or by daily foods enriched with cancer preventive components (Chauhan, 
1997).

Epidemiological data also suggest that dietary manipulations play an important role in the 
prevention of many human cancers (Chauhan, 1997). Herbs and spices usually contain 
essential oils, which show antioxidant activity and also carry flavour. Members of the 
Zingiberaceae are famous for their use as spices or as medicinal herbs. As Zingiberaceae 
is known to be widely used as a food additive, there may be high potentiality for its 
application as a cancer preventive agent.

The Zingiberaceae family has had an extremely long use in some South East Asian 
countries including Malaysia. Rhizomes of Boesenbergia rotunda and Zingiber 
montanum are employed as a traditional medicine for anti-inflammation, while the young 
shoots and inflorescence are used as condiments (Murakami et al., 1999). Rhizomes of 
some members of the Zingiberaceae family such as galangal (Alpinia galanga), ginger 
(Zingiber officinale) and turmeric (Curcuma domestica) have been extensively used as 
condiment for flavouring and local medicines for stomach ache, carminative and treating
various illnesses (Jirawan et al., 2005). Galangal is also used as a medicine for curing stomach ache in China and Thailand (Yang and Eilerman, 1999). Ginger is the most common herb that is used to treat nausea and vomiting of pregnancy, either recommended by providers or used as self-treatment by women (Burkill, 1966; Allaire, 2000). Meanwhile, the rhizomes of Curcuma mangga are traditionally used as a stomachic for the cure of chest pain, fever and to aid wound healing (Burkill, 1966; Faridah et al., 2005).

5-Fluorouracil (5-Fu) is a chemotherapy drug that interferes with the growth of cancer cells. It can be used to treat many types of cancers, including cancer of the colon and rectum. For more than four decades, 5-Fu has been the standard drug used in the treatment of colorectal cancer. It has demonstrated its value in terms of survival in advanced diseases when compared to best supportive care alone (Scheithauer et al., 1993). Moreover, 5-Fu therapy begun at the time of diagnosis of advanced disease improves survival compared to therapy begun at the onset of symptoms (Nordic Gastrointestinal Tumor Adjuvant Therapy Group, 1992).

P53 acts as a tumour suppressor in many tumour types; induces growth arrest or apoptosis depending on the physiological circumstances and cell type. P53 is involved in cell cycle regulation as a trans-activator that acts to negatively regulate cell division by controlling a set of genes required for the process. It responds to diverse cellular stresses to regulate target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair,
or changes in metabolism. P53 protein is expressed at low level in normal cells and at a high level in a variety of transformed cell lines (Poremba et al., 1995).

The present study was undertaken to scientifically investigate the bioactive properties of crude petroleum ether, chloroform and methanol extracts of 10 selected local Zingiberaceae sp. The specific objectives of the present study are as follows:

1) to screen for cytotoxic activity of the crude petroleum ether, crude chloroform and crude methanol extracts of ten selected Zingiberaceae species by an in vitro growth inhibition assay system against human colon cancer cell lines, HCT-116 and HT29;

2) to screen for cytotoxic activity of 5-Fluorouracil (5-Fu) with and without selected Zingiberaceae extracts using Neutral Red (NR) cytotoxicity assay in HCT-116 and HT29 cells; and

3) to screen for wild type-p53 promoting activity (wt-p53) in HCT-116 and HT29 cells after treatment with selected Zingiberaceae crude extracts using the 3-step indirect avidin-biotin immunoperoxidase technique.

Plants of ginger family are among the most frequently and heavily consumed dietary condiments throughout the world. Thus, this study that combined Zingiberaceae extracts together with 5-Fu would be worthwhile to investigate since there are no such study yet and might be of potential use in chemotherapy regime.
CHAPTER 2

LITERATURE REVIEW

2.1 Herbal Medicine

Malaysia is rich with natural forest and has been identified as one of the twelfth mega diversity countries besides Mexico, Colombia, Ecuador, Peru, Brazil, Zaire, Madagascar, China, India, Indonesia and Australia, with high levels of species endemism. These countries harbour more than 70% of the world’s biodiversity (McNeely et al., 1990; Elmqvist, 1999). The tropical rainforest of Malaysia is diverse in trees and contains the largest source of the natural products that can be used for medicinal purpose.

Natural products have served as a major source of drugs for centuries and about half of the pharmaceuticals in use today are derived from natural products (Clark, 1996). In Western countries, there is now an increased use of herbal medicines, largely due to the belief that powerful synthetic agents used in Western medicine can exert more unwanted side effects and are too often used indiscriminately and irrationally. Nowadays, most of our present medicines are derived directly or indirectly from higher plants (Fontanarosa, 2000; Ebadi, 2002).

Patients who are using conventional health care are also seeking for herbs and related products. The earliest evidence of human use of plants for healing dates to the Neanderthal period. In the 16th century, botanical gardens were created to grow medicinal plants for medical schools. Herbal medicine practice flourished until the 17th century when more scientific pharmacological remedies were favoured (Thomas, 1997; Fontanarosa, 2000; Ebadi, 2002).

Since ancient times, people have been exploring nature, in the search for new drugs. This has resulted in the use of a large number of medicinal plants to treat a variety
of diseases. Many of these plants have been shown to be active, and a few drugs of modern medicine are based on the traditional use of plant drugs. The traditional uses of medicinal plants remain a significant lead for drug discovery. Ginger, galanga and turmeric are among the plants that have been used in traditional medicine since ancient time and both of them are the members of Zingiberaceae family.

### 2.2 Zingiberaceae

Zingiberaceae is one of the largest families in the order of Zingiberales (Halijah, 1988). The word ‘ginger’ refers to the edible ginger, also known as *halia* in Malay. While ‘gingers’ is a term for members of the ginger family. The gingers are an important part of the tropical flora, and appreciated and used worldwide, whether as ornamentals, spices, condiments, flavours, salads (locally known as *ulam*) or in medicinal preparations (Larsen *et al*., 1999).

The Zingiberaceae comprises about 1,200 species, and 1,000 of them occur in tropical Asia. There are more than 160 species under 18 genera in Peninsular Malaysia, consisting mostly of large herbs (Larsen *et al*., 1999) and the herbaceous ground flora of the rainforest (Habsah *et al*., 2000). The eight main genera of Zingiberaceae that have been cultivated widely are shown in Figure 2.1.
Figure 2.1: The main genera of Zingiberaceae (Larsen et al., 1999)

Zingiberaceae species are perennial herbs where the rhizome (the general term for an underground stem), may be short or long, may be above ground or subterranean. A real stem is present in many species, but it is usually short, while a pseudo stem (false stem) formed by the leaf sheath may reach as high as 8 m (Larsen et al., 1999).

Zingiberaceae has an extremely long use in Malaysian traditional medicine, and is widely distributed throughout the tropics particularly in South East Asia. Gingers have been a part of the Asian culture as long as those cultures can be recalled. Species of Zingiberaceae are often spicy to the taste, and many of them have been used as medicines and flavourings. The most important commercial spices in the family are ginger, turmeric and cardamom (Polunin, 1998; Larsen et al., 1999).

Zingiberaceae is used for abdominal bloating, coughing, vomiting, diarrhoea, and rheumatism. Many species of the family have been reported to be known as a multi
purpose medicine throughout Asia. *Zingiber officinale* (*halia*), *Curcuma xanthorriza* (*temu lawak*) and *Curcuma domestica* (*kunyit*) are among the important Zingiberaceae, which are used extensively in traditional system of medicine. Burkill (1966) has reported that several species of Zingiberaceae such as *cekur* and *lengkuas* were used mainly as post-partum protective medicine. The presence of essential oils such as limonene and eugenol, colouring and pungent compounds have made some species important since the ancient times (Hasnah, 1991; Vimala et al., 1991).

The young rhizomes of *Curcuma mangga*, *Boesenbergia rotunda*, and *Zingiber zerumbet* and young inflorescences of *Curcuma domestica* and *Alpinia galanga* are also consumed as fresh vegetables or *ulam* (salad) by village folk (Ibrahim, 1992). Primary dietary prevention by edible plants could play a role in slowing down if not inhibiting the carcinogenesis of colon cancer (Mason, 2002).

### 2.2.1 *Alpinia galanga*

*Alpinia galanga* (Figure 2.2), mostly referred to simply as greater galangal or *lengkuas* (local name), is a very popular spice in whole of South East Asia and especially typical for the cuisine of Malaysia. It is also known and used in Thailand, Indonesia, Cambodia, Vietnam and Southern China. Chinese five spice powder is sometimes enhanced with galangal. In Western countries, however, galangal is not well known.

*A. galanga* is a ginger-like rootstock (rhizome). It is built up from cylindrical subunits (circular cross-section), whose pale-reddish surface is characteristically cross-striped by reddish-brown, small rings. The interior has about the same colour as the skin and is hard and woody in texture. The pseudo stem is non-woody, soft, watery, smooth
and green in colour, while the true stem is a fleshy thick rhizome that grows in the soil (Joseph et al., 2005).

Galangal may be used fresh or dried, which makes a great difference in flavour. Fresh galangal has a pure and refreshing odour and a mildly spicy flavour. The young shoots of the rhizome are pale pink and light yellow, and are more flavourful and tender than the older rhizomes. Galangal is too spicy to be eaten raw, and is used in slices, chunks or pounded to a paste for various curries and side dishes.

The chemical constituents of *A. galanga* reported by Malaysian Herbal Monograph (1999) are β-bisabolene, borneol, camphene, curcumene, limonene, linalool, quercetin, kaempferol and galangin.

![Figure 2.2: Fresh specimen of *Alpinia galanga* rhizome](image)

### 2.2.2 *Boesenbergia rotunda*

*Boesenbergia rotunda* formerly known as *B. pandurata* (Figure 2.3) is locally known as *temu kunci*. It is a herb with a rhizome of a few globose joints, with lateral subclavate roots. *B. rotunda* is not native to Peninsular Malaysia and is probably indigenous to India and south China. It is cultivated throughout Malaysia as food and
spices (limited to certain areas of Peninsula), and for traditional medicine such as treatment of colic disorder and as antiseptic (Larsen et al., 1999). It is also used to cure diarrhoea and dysentery. The leaves are reputed to be an antidote to certain poisons (Hutton, 1998).

Fingerroot is the best English name that can be devised for this South East Asian spice, which has become generally known in the West only in recent years. Fingerroot is used as a medicine, not for cooking in China, and it is a rare spice in the cuisines of Vietnam and Indonesia.

Figure 2.3: Fresh specimen of Boesenbergia rotunda rhizome

2.2.3 *Curcuma aeruginosa*

*Curcuma aeruginosa* (Figure 2.4) or locally known as *temu hitam* is herb with beautiful white-purplish flowers. *C. aeruginosa* is widely distributed throughout South East Asia (Larsen et al., 1999). The optimum ground altitude for growing is 1,750 meters above sea level and also can be found at lower altitudes. The rhizome can be used for treatment of obesity. *C. aeruginosa* is one of the main ingredients in jamu preparation and research done by Kikuzaki (2000) stated that *C. aeruginosa* can be used as one of the
ingredients in food for women in confinement after birth and as a treatment for cough and asthma.

![Curcuma aeruginosa rhizome](image)

**Figure 2.4: Fresh specimen of Curcuma aeruginosa rhizome**

### 2.2.4 Curcuma domestica

In Malaysia, *Curcuma domestica* (Figure 2.5) or turmeric is locally known as *kunyit*. The Malays use it medicinally in association with many other herbs. It is widely grown throughout the warmer parts of the world including Malaysia and is a native of southern Asia, probably India (Larsen *et al.*, 1999). In India, the rhizomes are dried and crushed to form powdered turmeric, but in South East Asia, the fresh rhizome is generally preferred.

*C. domestica* is a perennial herb with a tufted leaves and short stem (Joseph *et al.*, 2005). Turmeric from the rhizome of *C. domestica* has been known for its colouring, flavouring and digestive properties since ancient times. Turmeric has a long list of uses compared to other species of the Zingiberaceae family, ranging from flavour to cultural beliefs and rites. It is a constituent of curry powder and contributes to its characteristic colour and odour (Tang *et al.*, 1992). The juice extracted from crushed turmeric is
favoured for giving a bright yellow colour to ceremonial rice dishes (pulut kuning) in South East Asia. It was also widely used as a dye for cloth, but has been replaced these days by commercial dyes.

The broad aromatic leaves of *C. domestica* are used for wrapping fish before steaming or baking in some Malay and Indonesian dishes. In Thailand, young shoots and inflorescences can also be cooked with eggs to make an unusual omelette. The rhizomes contain curcumeneol, curcumenone, eugenol, limonene, linalool, terpineol, curcumin and demethoxy curcumin (Ismail *et al*., 1999; Asai *et al*., 1999; Ebadi, 2002).

The phenolic yellowish pigments of turmeric have been suggested to have antioxidative and hypocholesterolemic activities. Turmeric has been proposed as antioxidative food supplements because of its curcuminoids.

![Fresh specimen of Curcuma domestica rhizome](image)

**Figure 2.5: Fresh specimen of Curcuma domestica rhizome**

### 2.2.5 Curcuma mangga

In Malaysia, *Curcuma mangga* (Figure 2.6) is locally known as *temu pauh*, a name for one of the Mangiferas because of its smell like the mango. *Temu pauh* is grown
in the Peninsular Malaysia by the Malays and is used medicinally as a stomachic and also seasoning for food (Burkill, 1966). *C. mangga*, which is grown for medicine and as a spice, is an almost stem less plant which is the stems usually short and replaced by pseudo stems formed by leaf sheaths. The leaves distichously, simple, those toward base of plant usually bladeless and reduced to sheaths (Burkill, 1966).

*C. mangga* is also used in food preparations for women in confinement after birth and to treat fever (Larsen *et al*., 1999; Kikuzaki, 2000).

![Figure 2.6: Fresh specimen of Curcuma mangga rhizome](image)

### 2.2.6 Curcuma xanthorriza

*Curcuma xanthorriza* (Figure 2.7) is locally known as *temu lawak*. It is also known as ‘temu raya’ in Java and ‘koneng gede’ in Sundanese. *C. xanthorriza* is a big species, often exceeding a man’s height and having a large rhizome (Burkill, 1966). It has been traditionally used in many Indonesian jamus and for food as well. The smell of the rhizomes is pungent and the taste is bitter.
It is applied to the body after childbirth in the form of a paste. The fresh rhizomes of *temu lawak* have been used in Indonesian folk medicine as a general tonic, stomachic, anti-constipation, carminative, anti-diarrhea, analgesic and antibacterial (Larsen *et al.*, 1999; Kikuzaki, 2000). The compounds present in *temu lawak* are curcuminoids such as curcumin and desmethoxycurcumin (Zhari *et al.*, 1999).

**Figure 2.7: Fresh specimen of *Curcuma xanthorriza* rhizome**

### 2.2.7 *Kaempferia galanga*

*Kaempferia galanga* (Figure 2.8), locally known as *cekur* is also called as ‘chekur java’ in Java, ‘kenchur’ in Sundanese and ‘pro hom’ in Thailand. It is a herb from India and is cultivated throughout Malaysia. The plant is native to South India, but today mainly cultivated in South East Asia and China. As a spice, *K. galanga* is nearly unknown outside the Malesian region (Malaysia, Singapore and Indonesia). In Europe, it is available only in The Netherlands, where a large Indonesian community is living. It consists of a spicy, white, central body with several little tubers below it. It resembles ginger in shape in that the subunits are flat (elliptical cross-section), but it is much
smaller (5 cm). It has a dark reddish-brown skin, and the soft interior is nearly white. The rhizomes are short and stout; consist of dense small tubers adhering to one another to form a larger tuber.

*K. galanga*, which is grown for medicine and as a spice, is an almost stemless plant, spreading out close to the ground and develops its few short-lived leaves and the flower at ground level. The leaves and rhizomes are chewed for coughs, making lotions and poultices for almost all ailments (Burkill, 1966). The leaves are finely sliced and used as herb in salads. In Thailand, both the rhizomes and leaves are added to fish curries, and the young leaves are also eaten as a raw vegetable with a spicy shrimp paste sauce, ‘kapi kua’. In Malaysia, the rhizome and the young leaves of *K. galanga* are eaten raw or finely sliced, used in *kerabu* and *nasi ulam* (Joseph *et al*., 2005).

Chemical constituents in *K. galanga* are now being actively screened. The chemical components so far isolated are cinnamic acid, ethyl cinnamate, carene, camphene, borneol, cineol, kaempferol and few others (Zhari *et al*., 1999). The rhizome has been used as treatment for diarrhea, rheumatism, indigestion, gastritis, cough and sore throat (Larsen *et al*., 1999; Kikuzaki, 2000).

![Fresh specimen of Kaempferia galanga rhizome](image)

*Figure 2.8: Fresh specimen of Kaempferia galanga rhizome*
2.2.8 Zingiber montanum

*Zingiber montanum* or *bonglai* (Figure 2.9) is a herb with stem 1 to 6 feet high, arising from a thick rhizome. It possesses a strong camphoraceous odour and has spicy and bitterish taste (Burkill and Haniff, 1930). It is found throughout Malaysia.

*Z. montanum* often used as an anticonvulsant and as a treatment for swelling, rheumatism, stomachache and jaundice (Larsen *et al*., 1999; Kikuzaki, 2000). Such medicines or tonics are available in the form of jamu; a traditional Javanese herbal medicine from Indonesia. It has been used in food preparation for women in confinement after birth.

![Figure 2.9: Fresh specimen of Zingiber montanum rhizome](image)

2.2.9 Zingiber officinale

*Zingiber officinale* (Figure 2.10) is known locally as *halia*, ‘atuja’ in Java and ‘sunti’ by Sundanese (Burkill, 1966). The Thais called it as ‘khing’, ‘jahe’ by the Indonesian and ‘luya’ by the Filipino. Ginger seems to originate from Southern China and is cultivated in India, Brazil, Jamaica and Nigeria. Today, it is cultivated all over tropic and subtropics Asia and 50% of the world's harvest is produced in India.
The ginger grown in Malaysia is used fresh as flavouring in food and the young part of the rhizome are eaten raw (Burkill, 1966). In the fresh state, it has a characteristic stag horn-like appearance. The rhizome is light yellow in whorl, has an aromatic smell and slightly hot in taste. The stem grows from the rhizome and may reach to 50 cm tall. Dried ginger is usually sold in the form of white to very light brown powder. Ginger leaves are occasionally used for flavouring in ginger producing countries.

Ginger is among the most important and valued spices, as the many synonyms indicate. Today, the plant grows in tropic regions all over the world and plays a part in the local cuisines. In Europe, however, it is not common, although it had been an important spice during Roman times. Fresh ginger (also called green ginger) is now easily available in Western countries and still in demand as one of the ingredients in food, confectionaries, bakeries, beverages and traditional medicine.

Surh et al., (1998) and Surh (2002) have reported that ginger extracts are rich in gingerols and shogaols which exhibit antioxidant, antiinflammatory and anticarcinogenesis properties under both in vitro and in vivo systems. It is officially listed in Chinese Pharmacopoeia and used in Chinese medicine as stomachic and antidiarrheal (Tang et al., 1992). Ginger has been used traditionally as a carminative, diaphoretic and anti-spasmodic (Langner et al., 1998). It is also used particularly to improve digestion and the most commonly used to counteract nausea and vomiting (Hutton, 1998; Allaire et al., 2000; Bryer, 2005).

Ginger is well known for their numerous chemical constituents, such as zingiberol, citronellal, camphene, [6]-gingerol, [6]-paradol, zingerone, zingiberene and zingiberone (Zhari et al., 1999).
2.2.10 Zingiber zerumbet

*Zingiber zerumbet* (Figure 2.11) or locally known as *lempoyang* or wild ginger is a widely cultivated medicinal plant that is probably native to south India and Sri Lanka although in some areas it can be found growing in secondary forests or at the village edges. This plant which has been described as the flower of the first is a pale yellow in colour, paler towards the apex and light yellow at the base (Burkill, 1966). It grows in damp spots in woods and has been widely cultivated in tropical and subtropical areas around the world, and has naturalized in some areas.

The rhizome of *Z. zerumbet* has been used traditionally as an appetizer and treatment for stomach ache, diarrhoea, cure for swelling sores, anorexia and asthma (Burkill, 1966; Kikuzaki, 2000). The juice of the boiled rhizomes has also been used as a medicine for worm or ascaris in children (Saadiah *et al.*, 1995).
2.3 Colorectal Cancer

2.3.1 Colorectum

The colon and rectum are parts of the digestive system (Figure 2.2). The food is processed in the intestine to create energy and rid the body of waste matter. After food is chewed and swallowed, it travels down to the stomach. There it is partly broken down and sent to the small intestine. The colon is the first 4 to 5 feet of the large intestine. The waste matter moves from the colon into the rectum, the final 6 inches of the large bowel. From there the waste passes out of the body through the opening called the anus during a bowel movement. The part of the colon that joins to the rectum is the sigmoid colon. The part that joins to the small intestine is the cecum (Osteen, 1986). The cecum, transverse colon, and sigmoid loop are mobile structures that lie free in the peritoneal cavity and are completely covered with serosa (visceral peritoneum).
2.3.2 Cancer

Cancer is the general term for a series of neoplastic diseases that are characterized by changes in a cell leading to abnormal (unordered and uncontrolled) cellular proliferation (Pettit, 1977). Cancer is well known in all human populations and has probably been with us from the beginning of time.

In general, there are many factors that cause cancers. These factors include tobacco, alcohol, ultraviolet radiation (UV), ionizing radiation, occupation, pollution, medications, viruses and genetic susceptibility (Fraumeni, 1990). Their control will rely on public education and health policies. Hormonal factors and infections are among risk markers and typical examples of viruses correlated with cancers are hepatitis B virus (HBV), human papilloma virus (HPV) and *Helicobacter pylori*, which infect liver, cervix and stomach respectively.
Carcinogenesis has been recognized to consist of three stages: initiation, promotion, and progression. Initiation is a process stimulated by some carcinogens causing point mutation(s) of the H-\emph{ras} gene of the cellular DNA, which alters a normal cell into a dormant tumour cell (Baillul \textit{et al.}, 1989). Promotion is considered to be a consecutive process caused by some tumour promoters accelerating the proliferation of the initiated cells, and the repetitive attack of tumour promoters to the initiated cells results in the formation of visible, benign tumour cells. Progression is a process-involving invasion of tumour cells into the surrounding tissues or metastasis to distant organs.

In the broadest sense, neoplastic disease can be divided into benign and malignant categories. A benign tumour is generally contained within a membrane of connective tissue. Histologically all cells appear alike and derived from one tissue source. Unlike malignant tumours, benign tumours do not metastasize. In the beginning, malignant tumour cells may maintain some degree of their original specialized function, structure, and relationship to the tissue cells of origin. By maintaining this degree of differentiation, some cancer cells can still perform some limited useful activity at an early stage. However, as the disease progresses, the histological changes become more obvious until the cells are no longer identifiable. At this point dramatic structural and functional changes are noticeable.

The aims in cancer chemoprevention are to prevent, arrest, or reverse either the initiation phase of carcinogenesis or the progression of neoplastic cells to cancer (Greenwald, 2002). Many chemopreventive agents are derived from vegetables and fruits, such as lycopene in tomatoes, lutein in spinach, quercetin in apples, resveratrol in grapes, anthocyanins in berries, and polyphenols in tea (Kucuk, 2002).
Many compounds, belonging to diverse structural and functional chemical classes, have been identified as potential chemopreventive agents. These include vitamins and minerals such as folate, vitamin E, vitamin D, calcium, and selenium; naturally occurring phytochemicals such as curcumin, genistein, indole-3-carbinol, and L-perillyl alcohol; and synthetic compounds such as retinoids, selective oestrogen receptor modulators, and cyclooxygenase-2 inhibitors (Greenwald, 2002).

Soybean phytochemicals such as genistein may inhibit the growth of prostate tumours through reduced cell proliferation and angiogenesis and increased apoptosis (Zhou et al., 1999; Davis et al., 1998). Potential chemopreventive agents selected for testing in people with high risk in developing cancer must have low toxicities compared with the drugs used to treat existing cancer (Greenwald, 2002).

### 2.3.3 Colorectal Cancer

Cancer is typically a disease of old people, and this is undoubtedly true for colorectal cancer (Golfinopoulos et al., 2005). Cancer that begins in the colon is called colon cancer, and cancer that begins in the rectum is called rectal cancer. Cancers affecting either of these organs may also be called colorectal cancer. Colon and rectal cancers begin in the digestive system that is also called the gastrointestinal system.

The colon has 4 sections that are colon, rectum, cecum and small intestine, as shown in the Figure 2.13. Cancer can start in any of the four sections of the colon, or in the rectum. The wall of each of these sections and rectum has several layers of tissues. Cancer starts in the inner layer and can grow through some or all of the other layers.

Cancer that starts in the dissimilar areas may cause different symptoms. In most cases, colon and rectum cancers develop slowly over a period of several years. These
cancers begin as a polyp, a growth of tissue into the centre of the colon or rectum. A type of polyp known as adenoma can become cancerous. Removing the polyp early may prevent it from becoming cancer. Over 95% of colon and rectal cancers are adenocarcinomas. These are cancers of the cells that line the inside of the colon and rectum (Kern and Vogelstein, 1990).

When colorectal cancer spreads outside the colon or rectum, cancer cells are often found in nearby lymph nodes. If cancer cells have reached these nodes, they may also have spread to other lymph nodes such as liver or any other organs. When cancer metastasizes from its original place to another part of the body, the new tumour has the same kind of abnormal cells and the same name as the primary tumour. For example, if colorectal cancer spreads to the liver, the cancer cells in the liver are actually colorectal cancer cells. The disease is metastatic colorectal cancer, not liver cancer and it is treated as colorectal cancer (Kern and Vogelstein, 1990). The staging of colorectal cancer is defined in Table 2.1.
Table 2.1: Staging in colorectal cancer

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Cancer confined to the inner lining of the colon or rectum</td>
</tr>
<tr>
<td>II</td>
<td>Cancer spreads through the wall of the colon or rectum</td>
</tr>
<tr>
<td>III</td>
<td>Cancer spreads to nearby lymph nodes</td>
</tr>
<tr>
<td>IV</td>
<td>Cancer spreads to distant parts of the body, such as the liver or lungs</td>
</tr>
</tbody>
</table>

Colorectal cancer provides an excellent example of a human tumour type that can be effectively studied. It is widespread and progress through easily recognizable stages ranging from very small benign polyps called adenomas, to large malignant cancers called carcinomas (Vogelstein et al., 1994).

People with colorectal cancer may develop a number of non-specific symptoms which include diarrhoea, bright red or very dark blood in or on the stool, a change in bowel habits, stools that are thinner than usual, unexplained stomach discomfort, unexplained weight and appetite loss, abdominal pain, and chronic fatigue (Eisenberg et al., 1982). However these symptoms can also be connected with many other health conditions.

The occurrence of colorectal cancer is higher in developed countries, with approximately half of the Western population developing an adenoma by the age of 70 (Fodde et al., 2001). The low incidence of large bowel cancers in India can be attributed to their diet that is high in natural antioxidants including turmeric (Reddy et al., 2003).

Most colorectal malignancies are sporadic, but a fraction of colon cancers occur in an inherited fashion. Familial adenomatous polyposis (FAP) is one of the best-characterized inherited colon cancers, with patients developing hundreds to thousands of preneoplastic colonic polyps in early adulthood (Kinzler et al., 1996).
Colon cancer is inevitable in FAP patients who decide against prophylactic surgical options. In addition to colonic lesions, FAP patients can develop other malignancies, including ampullary carcinomas and hepatoblastomas (Kinzler et al., 1996; Lal et al., 2000). Molecular cloning of the FAP locus identified the tumour suppressor adenomatous polyposis coli (APC) as the causative gene for this disease (Groden et al., 1991; Kinzler et al., 1991).

FAP patients harbour germ line mutations in one allele of the APC gene, and upon loss of function of the wild type APC allele, they develop intestinal polyps. Soon after the discovery of APC gene and its role in FAP, somatic mutations in APC gene were identified in most sporadic colorectal cancers (Powell et al., 1992). This finding further emphasized both the participation of APC gene in colorectal homeostasis as well as the consequences associated with altered function of this tumour suppressor gene. (See Figure 2.14).
APC - adenomatous polyposis coli

Figure 2.14: The progression of colon carcinoma (Groden et al., 1991)
Molecular basis of colorectal cancer

All colon cancer cases, sporadic or hereditary, are thought to arise through two different pathways. In 1990, Fearon and Vogelstein published a model pathway for the transition of normal epithelia cells to carcinoma cells. In this model, colorectal cancer was hypothesized to occur by a multi-step process in which four genes are mutated in a specific order. It is now widely accepted that the multistep carcinogenesis process for colon cancer involves mutational events that ultimately give the cancer cells a growth advantage (Patrizia et al., 2002).

The first step to occur is the loss of the APC tumour suppressor gene. APC is thought to be a gatekeeper gene for colorectal cancer because when it becomes non-functional, there is subsequent loss of other growth control genes. After the loss of the APC gene, there is thought to be activation of the K-ras oncogene (Jen et al., 1994; Sikora et al., 1997). This is followed by loss of gene function on chromosome 18q and inactivation of p53, leading to eventual carcinoma formation. Mutations in the p53 gene are the most common genetic alterations in human cancers. Although p53 was first heralded as an oncogene, later research discovered that only mutant p53 was oncogenic, and that wild type p53 (wt-p53) functioned as a tumour suppressor (Gangopadhyay et al., 1997).

This pathway is characterized by chromosomal instability and leads to specific genes or entire portions of chromosomes being deleted or lost, resulting in aneuploidy (abnormal amounts of DNA per cell). Indeed, 75% to 80% of colon tumours show abnormalities at both p53 alleles: one allele is often deleted, and the other has point mutations that are usually missense mutations that yield an altered protein product. This
pathway accounts for approximately 85% of all sporadic colorectal cancer cases and all cases of colorectal cancer associated with FAP (Fearon and Vogelstein, 1990). (See Figure 2.15).

It was realized now that some colorectal cancers occur via different pathway, one that is characterized by microsatellite instability due to mismatch repair gene mutations. Chromosomal instability and aneuploidy are not seen in this pathway. This pathway has been called the Mutator Pathway and it accounts for approximately 15% of all sporadic cancer cases and most cases of Hereditary Non-Polyposis Colorectal Cancer (HNPCC). A composite diagramme of the two pathways is showed in Figure 2.16. However, the HNPCC patients are at increased risks also of other tumour types, among which is ovarian cancer (8-15% life-time risk). Increased risks apply also to rare tumour types such as cancer of the upper urinary track, the small intestine, the skin and the brain (Mlander et al., 2005; Aarnio et al., 1999; Hampel et al., 2005; Rijcken et al., 2003).

Figure 2.15: Pathway from normal epithelium to cancer cell
Figure 2.16: Two different pathways for the formation of colorectal cancer

(Adapted from Lynch et al., 2003 and Robbins et al., 2002)
2.3.4 Epidemiology of Colorectal Cancer

Cancers of the colon and rectum are among the most common causes of illness and death not only in Europe but also in Malaysia. It is listed as one of the top five cancers occurring in both males and females in Malaysia. National Cancer of Registry (2004) indicated that colorectal cancers account for 14.2% of male cancers, making it the number one cancer among men and the third most common cancer among women (10.1% of female cancers). (See Table 2.2).

Table 2.2: Five most frequent cancers in Peninsular Malaysia 2003

(National Cancer of Registry, 2004)

<table>
<thead>
<tr>
<th>No.</th>
<th>Percentages of five most frequent cancers in Peninsular Malaysia</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Colorectal - 14.2%</td>
<td></td>
<td>Female breast – 31%</td>
</tr>
<tr>
<td>2</td>
<td>Lung – 13.8%</td>
<td></td>
<td>Cervix uteri – 12.9%</td>
</tr>
<tr>
<td>3</td>
<td>Nasopharynx – 8.8%</td>
<td></td>
<td>Colorectal – 10.1%</td>
</tr>
<tr>
<td>4</td>
<td>Leukaemia – 7.1%</td>
<td></td>
<td>Corpus uteri – 4.3%</td>
</tr>
<tr>
<td>5</td>
<td>Prostate glands – 6.4%</td>
<td></td>
<td>Ovary – 4.1%</td>
</tr>
</tbody>
</table>

The age specific incidence for both colon and rectal cancers increased exponentially with age. The Chinese had a higher incidence of colon cancers than other races in Malaysia. Comparing the crude rates between the Chinese and the Malays, the Chinese had more than 5.1 times the incidence colon cancer amongst males, and 4.6 times amongst females. With regard to rectal cancers, the Chinese had the highest incidence rate of rectal cancers which was 2.8 times of the Malay male incidence and 3.4 times of the Malay females.

Studies done around the world found that the lowest rates of colon cancer are in the African and Asian countries, where fibre-rich diets are highly consumed. The highest
colon cancer rates are in western societies, where refined carbohydrates have commonly replaced naturally occurring fibre-rich foods and where intake of fibre is consequently low (Cohen et al., 1993).

It has been frequently demonstrated that religious groups practice lifestyles different from the general population (vegetarian, abstaining from tobacco, alcohol, tea and coffee) experience lower morbidity and mortality from colorectal cancer. Furthermore, colorectal cancer is found somewhat more frequently in higher socio-economic classes. This difference is larger in areas with an overall lower risk. Sedentary occupations have also been associated with a high colorectal cancer risk.

Data on Japanese migrants to the United States and European migrants to the United States or Australia demonstrate that the migrant population approaches the (high) incidence and mortality figures of the new country of residence. This is taken as strong evidence for the fact that environmental risk factors play an important role in the etiology of colorectal cancer. Within one country there can be large differences in the incidence of colorectal cancer between different racial subgroups, for example there is a three fold higher incidence in non-Maori New Zealanders compared to the inborn Maori population (Cohen et al., 1993).

Colorectal cancer is the third most common cancer among males and females in both United States of America (USA) and United Kingdom (UK). Here in Malaysia, in the group of young adults (15–49 years old), the common cancers were nasopharynx, leukaemia, lymphoma, lung, colon and rectum in men, and cancers of the breast, cervix, ovary, uterus, thyroid gland and leukaemia in women. In older subjects (50 years old and above), cancers of the lung, colon, rectum, nasopharynx, prostate and stomach were
predominant among men, while cancers of the breast, cervix, colon, uterus, lung and rectum occurred commonly in women (Lim et al., 2004).

The most common cancer in males in the year 2003 was cancer of the colorectal (14.2% of all male cancers in Peninsular Malaysia). Whereas among females, the colorectal cancer sits at the second place with 10.1% of all female cancers in Peninsular Malaysia. There is variation of cancer incidence rate between the different ethnic groups. Chinese had a higher incidence of colon cancers than the other races. Comparing the crude rates between Chinese and Malays, Chinese had more than 5.1 times the incidence of male colon cancer, and 4.6 times the incidence of female colon cancer. With regard to rectal cancers, Chinese had the highest incidence rate of rectal cancers which was 2.8 times the Malay male incidence and 3.4 times the Malay females.

Chinese men had the highest colorectal cancer incidence of 58.9% per 100,000 population, followed by Malays with 35.6 per 100,000 population and Indians with 5.5 per 100,000 population. For women, Chinese had the highest colorectal cancer incidence per 100,000 population (59.5%), followed by Malays (34.75 per 100,000 population) and Indians (5.8 per 100,000 population).

For Malay men, colorectal cancer is the second most common cancer after lung cancer but for Chinese men, the colorectal cancer is the number one followed by lung and nasopharynx cancers. Among Indian men, colorectal cancer is the second most common cancer after stomach cancer and the prostate gland cancer sits at the third place.

On the other hand, colorectal cancer is the third frequent cancer among both Malay and Chinese women after breast cancer and cervical cancer. However for Indian women, the colon cancer is the tenth most common cancers (Lim et al., 2004).
2.3.5 Treatment of Colorectal Cancer

The treatment of colorectal cancer depends on the specific location and extent of the disease. Surgery is the most common treatment for colorectal cancer, and aims to remove the tumour and part of the healthy intestine and the nearby lymph nodes. Since colorectal carcinoma usually arises in adenomas, removing adenomas should prevent most cases of cancer. Some patients may require a colostomy, which is a surgical opening through the abdomen to provide a pathway for the waste to exit the body into a bag worn by the patient. This opening is usually temporary but may be permanent in some cases.

In colorectal cancer, chemotherapy is often used to kill cancer that may have spread to the other parts of the body from the original tumour. It has been shown to reduce the risk of the cancer returning in the future. Chemotherapy also can help people with advanced stage cancer to live longer and more comfortably. The early use of chemotherapy in asymptomatic patients prolongs both symptom-free survival and overall survival. This treatment may cause some side effects, but they are usually short lived and manageable. Clinical counseling of patients and their families has been based on the pathologic and/or clinical stages of colorectal cancer. For example, adjuvant therapy with 5-Fu alone or in several combinations and routes of administration with leucovorin, irinotecan or oxaliplatin is increasingly used for advanced colorectal cancer. The common drugs used for colon cancer treatment are 5-Fluorouracil, leucovorin, irinotecan, oxaliplatin, bevacizumab, bortezomib and topotecan (de Gramont et al., 2000; Bogliolo et al., 2000; Grizzle et al., 2002; Golfinopoulos, 2006).

Radiotherapy, which uses high-energy radiation to kill cancer cells, is commonly used in rectal cancer. It is painless and does not involve electrical currents on patients.

A huge number of observational and randomized trials have shown the preventive properties of various drugs and micronutrients such as aspirin, sulindac, anti COX-2 and other NSAIDs, calcium and vitamin D, selenium, folic acid, HRT (hormone replacement therapy), soy, curcumin and green tea polyphenols (Veronesi et al., 2005). The potential preventive effect of dietary agents may be very appealing to many. Irinotecan is one of the plant derived drug sample which has been developed from a plant alkaloid discovered in the tree *Camptotheca acuminata*. It has been approved by the FDA for the treatment of metastatic colorectal cancer and is currently in clinical trials for a variety of other cancers.
2.3.6 5-Fluorouracil (5-Fu)

Fluorouracil is one of the most commonly drugs used to treat cancer and 5-fluorouracil (5-Fu) is one of the oldest chemotherapy drugs. 5-Fu (Figure 2.17) is a fluorinated analog of uracil and the only agent with significant activity in colorectal cancer (Tebbuttt et al., 2002). The U.S. Food and Drug Administration (FDA) approved it for cancer chemotherapy as an antineoplastic and antimetabolic agent. 5-Fu has now become the standard chemotherapeutic regimen for patients with Stage III colon carcinoma. It has been around and is used for decades in the treatment of many types of cancer including breast, head and neck, anal, stomach, pancreas, colon and some skin cancers. 5-Fu is part of a group of chemotherapy drugs known as the anti-metabolites (Chen et al., 1994; Tebbutt et al., 2002).

Anti-metabolites are similar to normal body molecules but they are slightly different in structure. These differences mean that anti-metabolites stop cells working properly instead of helping them. Anti-metabolites often stop cells synthesizing and repairing DNA. Cancer cells need to synthesize and repair DNA in order to grow and multiply. The side effects associated with 5-Fu are fatigue, nausea, mouth sores, diarrhoea and temporary effect on the bone marrow (Chau and Cunningham, 2002).

Fluorouracil is a cell cycle-specific cytotoxic agent used in colorectal cancer that induces DNA damage, both by inhibition of thymidilate synthase and by direct fragmentation of DNA. The tumour cell may respond to DNA damage by undergoing G1 arrest or by inducing cell death by apoptosis. This response may rely on a normal p53 gene and functional p53 protein. Therefore, normal p53 protein is essential for optimal cytotoxic drug action (Kressner et al., 1996).
The cytotoxic effects of 5-Fu occur following its conversion (through the *de novo* pyrimidine pathway) to 5-fluoro-deoxyuridine monophosphate (5-FdUMP). 5-FdUMP is an irreversible inhibitor of thymidylate synthase and hence of DNA synthesis through deoxythymidine triphosphate (dTTP) deprivation.

**Formula**: C₄H₃FN₂O₂  
**Molecular Weight**: 130.1

![Figure 2.17: The structure of 5-Fu](image)

5-Fu is normally administered by intravenous route. The life span of 5-Fu in blood and body tissues is very short and limited to minutes. 5-Fu binds to an enzyme inside of the cancer cells called thymidylate synthase and thereby exert its anti cancer effect on the cells. Leucovorin enhances the binding of 5-Fu to this enzyme and as a result prolongs the life span of 5-Fu within the cancer cells, resulting in a greater anti cancer effect.

5-Fu exerts its cytotoxicity mainly following its conversion, by a two-step route, to 5-fluoro-uridine monophosphate (5-FuMP). 5-FuMP is further transformed to 5-FdUMP, an irreversible inhibitor of thymidylate synthase and results in dTTP starvation and subsequent apoptosis. 5-Fu can also follow another enzymatic pathway where it is
primarily degraded to nontoxic β-alanine (www.invivogen.com-assessed on 26th December 2005).

2.4  **In vitro Cytotoxicity Screening**

*In vitro* cytotoxicity screening is a useful tool in screening plants for anti-cancer agents. Cytotoxicity is the ability of plants derived compound or natural products to arrest the proliferation of cells. Cytotoxicity assays measure drug-induced alterations in metabolic pathways or structural integrity, which may or may not be related directly to cell death. Some cytotoxicity assays offer instantaneous interpretation, such as the uptake of a dye by dead cells, or release of $^{51}$Chromium or fluorescein from pre-labelled cells (Wilson, 2000). There are a number of fluorescent probes now available for measuring membrane integrity as shown in Table 2.3 (Wilson, 2000).

Pre-screens, screens, monitors and secondary testing are four major types of bioassays as suggested by Suffness and Pezzuto (1990). Pre-screens are applied to large numbers of initial samples such as certain plants. It must have high capacity, low cost and give rapid answers. On the other hand, screens are used to select material for secondary testing whereas monitors are used to guide fractionation of a crude material towards isolation of the pure active substances. In the secondary testing, the lead compounds are evaluated in multiple models and test conditions in order to select compounds for development towards clinical trials. It is usually described as a low capacity, expensive and slow bioassay (Vlietinck, 1999).

**Table 2.3: Commercially available kits for cytotoxicity assays**
**Kit** | **Method**  
---|---  
Quantos Cell proliferation assay kit | Measures fluorescence of a dye when bound to DNA  
MTT-based assay kit | Metabolism of tetrazolium salts to insoluble formazan products by mitochondrial enzymes  
XTT-based assay kit | Metabolism of tetrazolium salts to soluble (XTT) formazan products by mitochondrial enzymes  
Neutral red-based assay kit | Neutral red taken up by lysosomes and Golgi bodies  
Lactate dehydrogenase-based assay kit | Measures membrane integrity  

**In vitro Neutral Red Cytotoxicity Assay**

The neutral red (3-amino-<em>m</em>-dimethylamino-2-methylphnazine hydrochloride) (NR) cytotoxicity assay is performed in a modified form as described by Borenfreund and Puerner (1985). In vitro neutral red cytotoxicity assay is often favoured for screening of anti-tumour activity as it is rapid, inexpensive and only a small amount of extract is needed for screening. This assay quantifies the number of still viable, uninjured cells after their exposure to plant extracts or natural products. It is based on the uptake and subsequent lysosomal accumulation of the supervital dye, neutral red by viable cells. The amount of dye extracted from the cells has been shown to be linear with numbers of viable cell. Quantification can be done both by direct cell counts and by protein determinations of cell populations.

Cell sensitivity may also be defined by the IC<sub>50</sub> and IC<sub>90</sub> values (i.e. drug concentration required to inhibit viability of the cells by 50% or 90%). These values may also be denoted as ID (inhibitory dosage), ED (effective dosage), LC (lethal concentration), or GI (growth inhibition) (Wilson, 2000).
The NR cytotoxicity assay is one of the most common assay used worldwide besides MTT, LDH leakage and protein assay. Luminescence and colourimetric-based assays measure samples directly in the 96-well plates by using a microtiterplate reader or ELISA plate reader. This miniaturization allows numerous samples to be analyzed rapidly and simultaneously. NR assay and MTT assay were the most sensitive assay in detecting cytotoxic events compared to the LDH leakage and protein assay (Fotakis and Timbrell, 2005). Weyermann et al. (2005) has reported that different results are obtained from the different cytotoxicity assay, depending on the test agent used and the assay employed.

The advantages of the NR cytotoxicity assay are clearly documented by Ciapetti et al., (1996). They include easy handling and high number of replicates; inexpensive reagents and common laboratory instruments required; and good sensitivity in a short assay time which is less than three hours.

Borenfreund and Babich (1987) recommended that the cells should achieve 60-70% confluence by the time of the addition of test agent. In this study, the degree of confluence achieved was 70% before starting the NR test. This is the log phase for the growth cycle of the cells. Moreover at this phase, the cells are most active metabolically and the results obtained will most certainly indicate the degree of activity of the test agent (Suffness and Pezzuto, 1990). For an agent that requires metabolic activation, the cells tested should be incubated for about 3-6 days to allow the metabolic conversion to take place and for this study, the cells have been incubated for 3 days.
2.5 P53

P53 was first heralded as an oncogene because of its vigorous expression in human tumours. However it was later discovered that only mutant p53 is oncogenic and the wild type p53 function as a tumour suppressor. Oncogene is a term for a gene that when mutated and expressed at abnormally high levels, will cause a normal cell to convert into cancer cell. Tumour suppressor gene is a gene that protects a cell from becoming cancer one. P53 has appeared as a key tumour suppressor and important target for novel cancer therapy. It plays a major role in preventing tumour development (Mills, 2006).

Cellular tumour antigen p53 is a transcription factor that regulates the cell cycle and functions as a tumour suppressor. P53 is a nuclear protein and also known as tumour protein 53 (TP53), tumour suppressor p53, phosphoprotein p53 and antigen NY-CO-13. It has been described as ‘the guardian of the genome’- referring to its role in conserving stability by preventing genome mutation and selectively inhibit the growth of or eradicate damaged cells (Lane, 1992).

The human p53 gene is located on the human chromosome 17 (17p13.1). There are 11 exons. There is a very large intron between exons 1 and 2. Exon 1 is a non-coding region in the human p53 gene. It has been demonstrated that this region could form a stable stem-loop structure, which binds tightly to wild type p53 (wt-p53) but not to mutant p53. The location has also been mapped on other model animals such as mouse (chromosome 11), rat (chromosome 10), dog (chromosome 5) and pig (chromosome 12) (Berkson et al., 2005).
P53 protein is a DNA-binding protein. It is 393 amino acids long and has three domains:

i. An N-terminal transcription-activation domain (TAD)

ii. A central DNA-binding core domain (DBD)

iii. A C-terminal homo-oligomerisation domain (OD)

P53 is found in very low levels in normal cells and has a very short half-life; however, in a variety of transformed cell lines, it is expressed in high amounts and believed to contribute to transformation and malignancy (Vojtesek et al. 1992). P53 has many anti-cancer mechanisms as describe in Table 2.4.

**Table 2.4: Functions of p53**

<table>
<thead>
<tr>
<th>No.</th>
<th>Functions of p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>conserving stability by preventing genome mutation</td>
</tr>
<tr>
<td>2</td>
<td>selectively inhibit the growth of or eradicate damaged cells</td>
</tr>
<tr>
<td>3</td>
<td>can activate DNA repair proteins when DNA has sustained damage</td>
</tr>
<tr>
<td>4</td>
<td>can hold the cell cycle at the G1/S regulation point on DNA damage recognition</td>
</tr>
<tr>
<td>5</td>
<td>can initiate apoptosis (programmed cell death) if the DNA damage proves to be irreparable</td>
</tr>
<tr>
<td>6</td>
<td>can induce growth arrest, apoptosis and cell senescence</td>
</tr>
<tr>
<td>7</td>
<td>plays a role in genetic stability and inhibition of angiogenesis</td>
</tr>
</tbody>
</table>

P53 is central to many of the cell’s anti-cancer mechanisms. It can induce growth arrest, apoptosis and cell senescence. In normal cells, p53 is usually inactive, bound to the protein MDM2, which prevents its action and promotes its degradation by acting as ubiquitin ligase. Active p53 is induced after the effects of various cancer-causing agents such as UV radiation, oncogenes and some DNA-damaging drugs.
Oncogenes also stimulate p53 activation, mediated by the protein p14ARF. Some oncogenes can also stimulate the transcription of proteins, which bind to MDM2 and inhibit its activity. P53 has many anticancer mechanisms, and plays a role in apoptosis, genetic stability, and inhibition of angiogenesis (Lane, 1992).

In almost 50% of all human tumours, the p53 gene is mutated (Shiao et al., 1994; Bunz et al., 1999; Olivier et al., 2002). In colon cancer, p53 mutations are less widespread at early stages (polyps or adenomas) but become extremely prevalent at the adenoma-carcinoma transition (Bouchet et al., 2006). The mutations that disable p53 functions in cancer usually occur in the DBD. The mutations destroy the ability of the protein to bind to its target DNA sequences, and thus prevent transcriptional activation of these genes. It is the target of 90% of p53 mutations. It can be found in human cancers and a single mutation within this domain can cause a major conformational change.

Molecules of p53 with mutations in the OD dimerise with wt-p53, and prevent them from activating transcription. Therefore OD mutations have a dominant negative effect on the function of p53. Wt-p53 is a labile protein, comprising folded and unstructured regions that function in a synergistic manner. The wt-p53 induces apoptosis while a mutant p53 loses the property of regulating cell death (Yonish-Rouach et al., 1991).

Inactivation of the p53 gene is essentially due to small mutations (missense and nonsense mutations or insertions / deletions of several nucleotides), which lead to either expression of a mutant protein (90% of cases) or absence of a protein (10% of cases). Alterations of the p53 gene occur not only as somatic mutations in human
malignancies, but also as germ line mutations in some cancer-prone families with Li-Fraumeni syndrome. Poremba et al., (1995) showed that the absence of the growth-inhibitory effect of p53 tumour suppressor gene in gastric carcinoma did play a basic role in tumourigenesis.

Tumour suppression is severely reduced if p53 is damaged. People who inherit only one functional copy of p53 will most likely develop tumours in early adulthood, a disease known Li-Fraumeni syndrome. More than 50% of human tumours contain a mutation or deletion of p53. P53 can also be damaged in cells by mutagens (chemicals, radiation or viruses), increasing the likelihood that the cell will begin uncontrolled division (Berkson et al., 2005).

Certain pathogens can also affect p53 such as human papillomavirus (HPV). It encodes for a protein, E6, which binds p53 and inactivates it. In a healthy person, p53 is continually produced and degraded in the cell. The degradation of p53 is, as mentioned, associated with MDM2 binding. In a negative feedback loop MDM2 is itself induced by p53. However mutant p53s often do not induce MDM2, and are thus able to accumulate at very high concentrations (Gangopadhyay, 1997).

Wild type p53 (wt-p53)

P53 was heralded as an oncogene for about ten years but then later discovered that only mutant p53 was oncogenic, and that wt-p53 functioned as a tumour suppressor
Wt-p53 could inhibit transformation of cells in culture and the p53 gene was mutated in a large fraction in human tumours. Expression of high levels of wt-p53 has two outcomes whether cell cycle arrest or apoptosis. Wt-p53 genes, when introduced into cells, were found to be growth suppressive (Ko and Prives, 1996). The observation that DNA-damaging agents induce levels of p53 in cells led to the definition of p53 as a checkpoint factor. It acts as an ‘emergency brake’ inducing either arrest or apoptosis, protecting the genome from accumulating excess mutations. Cells lacking p53 were shown to be genetically unstable and thus more prone to tumours. The mutations often lead to production of an altered p53 protein that binds to and inactivates the normal, wt-p53, thereby promoting tumourigenesis. Mutant p53 protein is more stable and has an extended half-life in comparison with wt-p53 (Ko and Prives, 1996).

2.6 Immunocytochemistry

In 1955, Coons et al., published the first usable immunocytochemical method. In the beginning, there were many problems of specificity and sensitivity, but these were slowly overcome. Immunohistochemistry describes the detection of antigens in tissues while immunocytochemistry describes detection of antigens in cultured cells. In these techniques, an antibody is used to link a cellular antigen specifically to a stain that can be more readily seen with a microscope. For both, there is a wide range of specimen source, antigen availability, antigen antibody affinity, antibody type, and detection enhancement methods. In the present study, the immunocytochemistry
technique - 3 step indirect avidin-biotin immunoperoxidase with the primary antibody, DO-7 mouse anti-human p53 protein was used.

The early immunohistochemistry approach used polyclonal antibody which was often in short supply and sometimes exerted significant variations among lots. This problem was improved with the introduction of monoclonal antibody which now plays important roles as immunohistological reagent. There are two ways to detect viral antigen; the direct method use only one virus-specific antibody which is directly labelled with an indicator (fluorescein or alkaline phosphatase) and the indirect method which uses two antibodies, one virus-specific antibody to bind the viral antigen and the second antispecies antibody to label with the indicator (Lakeman, 1997).

Three-step indirect avidin-biotin immunoperoxidase is a method which uses the ability of avidin to bind four nonimmunologically and low molecular weight of vitamin biotin. In this method, the test samples were incubated with primary specific antibody to against the viral antigen. Then a secondary biotinylated antibody capable of binding to the primary antibody was added, followed by Horse-raddish peroxidase (HRP) enzyme-conjugated avidin or streptavidin to form a biotin-streptavidin HRP complex. Finally the test samples were exposed to a 3’-diaminobenzidine tetrahydrochloride (DAB) substrate. Upon reaction with HRP, the DAB substrate will form a nondiffusing and insoluble dark brown precipitate at the site of viral antigen (Gay and Docherty, 1986; Espinoza et al., 1992; Forghani and Hagens, 1995). The schematic steps are shown in Figure 2.18.
Figure 2.18: The 3-step indirect avidin-biotin immunoperoxidase

The HRP peroxidase can react with DAB substrate chromogen in the presence of H$_2$O$_2$ to yield brown, alcohol-soluble precipitate at the site of the antigen. The HRP peroxidase will first form an enzyme-substrate complex which will then be oxidized by the electron donor which provides a ‘driving’ force in the continuing catalysis of H$_2$O$_2$. The manipulation of the incubation time and DAB concentrations should be made to control the staining intensity over the entire sample. Overstaining will reduce the contrast between stained and unstained area in the sample and thus may lead to difficulty in interpretation of the result (Gay and Docherty, 1986; Boenisch, 2001b).
CHAPTER 3
MATERIALS & METHODS

3.1  Zingiberaceae Samples
3.1.1  Plant materials

The fresh rhizomes of 10 selected Zingiberaceae species were purchased from a local market in Kuala Lumpur, Malaysia. 2 kg of the rhizomes of each Zingiberaceae species namely, *Alpinia galanga*, *Boesenbergia rotunda*, *Curcuma aeruginosa*, *Curcuma domestica*, *Curcuma mangga*, *Curcuma xanthorriza*, *Kaempferia galanga*, *Zingiber montanum*, *Zingiber officinale* and *Zingiber zerumbet* were collected. Table 3.1 shows the list of the selected Zingiberaceae species with their local names used in the present study.

**Table 3.1: List of selected Zingiberaceae species studied**

<table>
<thead>
<tr>
<th>No.</th>
<th>Scientific name</th>
<th>Local name</th>
<th>Common name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Alpinia galanga</em> (syn. <em>Languas galanga</em>)</td>
<td>Lengkuas</td>
<td>Siamese ginger, galangal</td>
</tr>
<tr>
<td>2</td>
<td><em>Boesenbergia rotunda</em> (syn. <em>B. panduratum</em>)</td>
<td>Temu kunci</td>
<td>Chinese ginger, Chinese key,</td>
</tr>
<tr>
<td>3</td>
<td><em>Curcuma aeruginosa</em></td>
<td>Temu hitam</td>
<td>Curcuma</td>
</tr>
<tr>
<td>4</td>
<td><em>Curcuma domestica</em> (syn. <em>C. longa</em>)</td>
<td>Kunyit</td>
<td>Turmeric</td>
</tr>
<tr>
<td>5</td>
<td><em>Curcuma mangga</em></td>
<td>Temu pauh</td>
<td>Mango ginger</td>
</tr>
<tr>
<td>6</td>
<td><em>Curcuma xanthorriza</em></td>
<td>Temu lawak</td>
<td>Javanese turmeric</td>
</tr>
<tr>
<td>7</td>
<td><em>Kaempferia galanga</em></td>
<td>Cekur</td>
<td>Resurrection lily, sand ginger</td>
</tr>
<tr>
<td>8</td>
<td><em>Zingiber montanum</em></td>
<td>Bonglai</td>
<td>Cassumunar ginger</td>
</tr>
<tr>
<td>9</td>
<td><em>Zingiber officinale</em></td>
<td>Halia</td>
<td>Ginger</td>
</tr>
<tr>
<td>10</td>
<td><em>Zingiber zerumbet</em></td>
<td>Lempoyang</td>
<td>Bitter ginger</td>
</tr>
</tbody>
</table>
3.1.2 Preparation of crude extracts

Rhizomes and root parts of the plants were washed, cut into small pieces and dried in a hot air oven at 50-60°C for three days. The dried samples were then ground into fine powder and extracted with petroleum ether, chloroform and methanol solvents successively. Twenty grammes of powdered rhizome was soaked for a day in 200 ml of each solvent after which the extracts were evaporated using the rotary evaporator (40°C). The crude extracts were weighed and dissolved in dimethyl sulfoxide (DMSO) (Sigma) to form stock solutions of 20 mg/ml for use in the Neutral Red Cytotoxicity Assay. The stock solutions were kept at -20°C until use. The concentration of extract samples was prepared according to the requirements of each assay.

3.1.3 Preparation of culture medium

Basic McCoy’s 5a and RPMI 1640 Medium

Medium was prepared by dissolving one sachet of McCoy’s 5a powder (Sigma) or RPMI 1640 powder (Sigma), 2.0 g of sodium bicarbonate and 0.5206 g 4(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (Sigma) in 1 L of distilled water. The pH of the medium was calibrated to pH 7.4 (Hanna Instruments). The medium was then filter-sterilized through a 0.2 μm filter membrane (Schleicher and Schuell) into sterile bottles and kept at 4°C.

10% Supplemented McCoy’s 5a Medium and RPMI 1640 Medium

One hundred millilitres of 10% supplemented McCoy’s 5a or RPMI 1640 medium were prepared using 90 ml of basic medium, supplemented with 10 ml heat-inactivated foetal
bovine serum (FBS) (PAA Lab.), 1 ml (100 μg/ml) and 1 ml (100 IU/ml) of streptomycin and penicillin (PAA Lab.) respectively and 1 ml of fungizone (PAA Lab.). The medium was filter-sterilized using a 0.22 μm filter membrane and stored at 4°C for up to 2 weeks.

**20% Supplemented McCoy’s 5a Medium and RPMI 1640 Medium**

Fifty-five milliliters of 20% supplemented McCoy’s 5a or RPMI 1640 medium were prepared using 50 ml of 10% supplemented medium and added with 5 ml FBS. The medium was filter sterilized using a 0.22 μm filter membrane and stored at 4°C for up to 2 weeks. This 20% supplemented medium was used to revive cells.

**Freezing Medium**

The freezing medium consisted of 50% inactivated foetal bovine serum, 40% RPMI 1640 or McCoy’s 5a, and 10% DMSO (Sigma). The solution was mixed well by using a 20 ml disposable syringe (Terumo) and filter-sterilized using 0.2 μm cellulose filter membrane and kept at 4°C. Freshly prepared freezing medium was used for cryopreservation of cells.

### 3.1.4 Preparation of solutions

**Phosphate Buffered Saline (PBS) pH7.2**

The phosphate buffered saline (PBS) was prepared using 1.52 g of sodium phosphate anhydrous (NaHPO₄) (Merck), 0.58 g of potassium dihydrogen orthophosphate (KH₂PO₄) (Merck) and 8.5 g of sodium chloride (NaCl) (BDH) that were dissolved in distilled water and the volume was made up to 1 L. The pH of the buffer was adjusted to
7.2 using a pH meter (Hanna Instruments). The buffer was then filtered using a 0.22 μm filter paper (Whatman 541) and autoclaved (P-Selecta) before stored at room temperature.

Phosphate Buffered Saline (PBS) pH7.6

The phosphate buffered saline was prepared using 1.5 g of potassium phosphate anhydrous (K₂HPO₄) (BDH), 0.2 g of potassium dihydrogen orthophosphate (KH₂PO₄) (Merck) and 7.75 g of sodium chloride (NaCl) (BDH), which were dissolved in 1 L of sterile distilled water. The solution was stirred and the pH was adjusted to pH 7.6. The solution was then filtered using filter paper and kept at room temperature.

Trypsin-EDTA

Trypsin-ethylenediaminetetra-acetic acid (EDTA) solution was prepared by dissolving 0.25 g trypsin (Amresco) and 0.03 g EDTA (Sigma) in 100 ml of distilled water. The solution was sterilized by filtration using a 0.22 μm filter membrane and stored at 4°C. Trypsin-EDTA was used for HCT-116 and HT29 cells.

0.4% Tryphan Blue

0.4% tryphan blue was prepared by dissolving 0.2 g of powdered tryphan blue (Sigma) in 50 ml of sterile distilled water. The solution was kept at room temperature.
10% Dimethyl Sulfoxide (DMSO)

The 10% DMSO solution was prepared by mixing DMSO (Sigma) with sterile distilled water at a ratio of 1: 9. Freshly prepared 10% DMSO was used for the neutral red (NR) cytotoxicity assay.

Neutral Red Stock Solution

0.4 g of NR (ICN Biomedical Inc.) was dissolved in 100 ml distilled water. The solution was kept at 4°C.

Neutral Red Medium

The NR stock solution was diluted (80 :1) in treatment culture medium to give a final concentration of 50 μg/ml. Prepared NR medium were incubated overnight at room temperature in the dark. This solution was centrifuged twice at 1000 rpm for 10 minutes (min) before use to remove any fine, needle-like precipitate of dye crystals.

Neutral Red Washing Solution

10% of calcium chloride (CaCl₂) (Sigma) was dissolved in 1ml formaldehyde (Sigma) and 89 ml of distilled water. The solution was kept at 4°C.

Neutral Red Resorb Solution

1 ml of glacial acetic acid (BDH) was dissolved in 50 ml of absolute ethanol (Hamburg) and 49 ml of distilled water. The solution was kept at 4°C.
3% Hydrogen Peroxide

The 3% hydrogen peroxide was prepared by mixing 1 ml of 30% hydrogen peroxide (H₂O₂) (BDH) with 9 ml of distilled water. The solution was wrapped with aluminium foil and kept at room temperature.

3.2 Cell Culture

3.2.1 Cell lines

A cell line is a specific cells culture that will proliferate if given the appropriate fresh medium, conditions and space. Cell lines from human colorectal cancers are useful tools in the study of cell biology and in the growth and testing of novel therapeutic modalities. In general, colorectal cancer cell lines are quite easy to establish. Colorectal carcinoma cell lines can be developed from ascitic effusions, metastatic tissues and primary tumour. There are numerous cell lines that have been identified such as Colo 201, Colo 320, DLD 1, HT29, SW480, HCT15, HCT-116 and SW403 (Brattain et al., 1999). Human colon cancer cell lines, HCT-116 and HT29, were purchased from American Tissue Culture Collection (ATCC, USA). The viability of the cells was checked by the tryphan blue exclusion dye method. Frozen cell stocks were stored in liquid nitrogen (-196°C) prior to revival.

3.2.2 Cryopreservation of cells

Cell stocks were preserved and stored in liquid nitrogen. Cell suspensions were spun down at 1000 rpm for 5 minutes using a bench centrifuge (Clements) after the
medium was discarded. 50% of foetal bovine serum (Sigma) was added to give approximately $1 \times 10^7$ cells/ml, followed by 10% of DMSO as a cryoprotectant. The mixture was stored in cryopreservative vials (Nunc) and placed in a -70°C freezer overnight. The vials were then transferred into liquid nitrogen (-196°C) to make sure that cells were in good condition and always ready to be use.

3.2.3 Revival of cells

The cryovial of cells was removed from the liquid nitrogen and plunged into a beaker of ice. It was then, transferred to a 37°C water bath, keeping the top part of the vial above the surface of water to avoid any contamination. Occasionally, the vial was swirled to hasten thawing. When the cells were almost thawed, the outside of the vial was wiped with a hand towel imbibed with 70% ethanol, HCT-116 cells were transferred into 1 ml of 20% supplemented McCoy’s 5a in a polypropylene tube (Falcon) and HT29 cells were transferred into RPMI 1640 medium. Both were spun at 1000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 5 ml of fresh 20% supplemented McCoy’s 5a or RPMI 1640 medium and incubated in a 25 ml tissue culture flask (Falcon) at 37°C in a 5% CO₂ incubator (Shel Lab). The viability and density of the cells were checked using an inverted microscope (Olympus IMT-2).

3.2.4 Maintenance of cells

HCT-116 was maintained in McCoy’s 5a complete medium (CM) and HT29 was maintained in RPMI 1640 CM in 25 ml tissue culture flask. The cultures were incubated in a 5% CO₂ incubator (Shel Lab) at 37°C in a humidified atmosphere. The cultures were
subcultured every 2 or 3 days and routinely checked under an inverted microscope (Olympus IMT-2) for any contamination.

3.2.5 Subcultivation of cells

Adherent cells attached and formed a single layer in a culture flask. Confluent cells were washed twice using PBS. The cells were detached from the flask by incubating in 1 ml 0.25% trypsin-EDTA solution and 3 ml PBS solution for 5 minutes at 37°C, and then sharply tapped to detach the cells from the flask. The floating cells were transferred into a centrifuge tube and centrifuged for 5 min at 1000 rpm. The supernatant was removed and 2 ml of McCoy’s 5a CM was added to the HCT-116 pellet and 2 ml of RPMI 1640 CM was added to the HT29 pellet. The cells suspensions were transferred into 3 different flasks each containing 7 ml complete medium. The flask was then further incubated. Cells were grown to confluence.

3.3 Neutral Red Cytotoxic Activity Assay

Thirty crude petroleum ether, chloroform and methanol extracts of selected Zingiberaceae were evaluated for the cytotoxic activities in colon cancer cell lines, HCT-116 and HT29.

3.3.1 Neutral red cytotoxicity assay

The neutral red (NR) cytotoxicity assay is based on the initial protocol as described by Borenfreund and Puerner (1984) and it determines the accumulation of the neutral red dye in the lysosomes of viable and uninjured cells.
Cells were detached from the flask with 0.25% solution of trypsin in HANK’s Balanced Salt Solution and washed in culture medium. The cell pellet was obtained by centrifugation at 1000 rpm for 5 min and the density of the viable cells was counted by 0.4% of trypan blue exclusion in a haemocytometer.

Cells were then seeded in each of the flat-bottomed 96 microplate wells (Nunc), at the concentration of 30,000 cells / ml for HCT-116 and HT29 cells. The plate was incubated in a CO₂ incubator at 37°C for 24 h to allow the cells to adhere and achieve 60-70% confluence before addition of the test agents. After 24 h, the attached growing cells were treated with freshly prepared crude extract in complete medium at five different concentrations of 1, 10, 25, 50 and 100 μg/ml. Negative control is the well containing the untreated cells (without addition of any extract) (Plate 3.1). The plates were further incubated for 72 h.

At the end of the incubation period, 150 μl NR solution (Sigma) (50 μg/ml in McCoy’s 5a / RPMI 1640) were added to the plate. The plates were incubated for another 3 h to allow for uptake of the vital dye into the lysosomes of viable and uninjured cells. After the incubation period, the media were removed and cells were washed with the NR washing solution. Damaged or dead cells lose their ability to retain NR, which is then removed during this wash / fixation procedure. The dye was eluted from viable cells by adding 200 μl of NR resorb solution and incubated for 30 min at room temperature with rapid agitation on a microplate rotator (LT BioMax 500). The absorbance of the extracted dye was read on Multiskan MCC/340 spectrophotometer for microplates equipped with a 540 nm filter (Titertek). Results from triplicate samples were recorded as optical density units (OD) and averaged after subtraction of blank.
The percentage of inhibition of each of the test samples was calculated according to the following formula:

\[
\text{% of inhibition} = \frac{\text{OD negative control} - \text{OD sample}}{\text{OD negative control}} \times 100\%
\]

The optical density (OD) was calculated as the difference between the absorbance at the test wavelength and that at the reference wavelength. The IC\textsubscript{50} is the concentration of extract that causes 50\% cells inhibition or cell death. IC\textsubscript{50} for each extract was extrapolated from the graphs plotted using the percent of inhibition values. The extract that gave IC\textsubscript{50} of 20 μg/ml or less was considered cytotoxically active (Chiang \textit{et al.}, 2003; Geran \textit{et al.}, 1972)

Plate 3.1: Untreated (a) HCT-116 and (b) HT29 cell lines.

Experimental works including tissue culture techniques requires that all the equipment and reagent be scrupulously clean. Cabinet or hood with UV light is the best
and simplest way to kill the germicide and mutagen. However, overexposure can also harm the cells in human body if uncovered. Overexposure to UV light can cause serious retinal burns and other part of the body (Shechmeister, 1977). All the apparatus used in this study have been exposed to the UV light before starting the cytotoxicity assay.

The opening of the laminar hood should be as small as possible to maintain the sterility of the working environment. 70% ethanol is a common disinfectant by reason of effectiveness, readily vaporizes and is not toxic to most cells (Martin, 1994). Autoclave is another method in sterilizing equipments and reagents via high pressure steam (15 psi) combined with reduced temperature (~ 120°C). All of these methods and techniques have been applied in this study.

3.3.2 Treatment of cancer cell lines with Zingiberaceae extracts and 5-Fluorouracil (5-Fu)

Thirty thousand cells in 190 μl of complete medium were plated into flat-bottomed 96 microplate wells during exponential growth. Several concentrations of crude extracts and 5-Fu (Sigma) were added. 5-Fu alone was added to the wells at nine different concentrations ranging from 0.1 x 10^{-7} M to 1.0 M. Extracts which produced IC_{50} of 20 μg/ml or less during the NR cytotoxicity assay were further evaluated with 5-Fu in this assay. After treatment with extracts and 5-Fu for 72 h, cell viability was evaluated using the NR cytotoxicity assay as described before. As before, percentages of inhibition values were calculated. The values were plotted into a graph from which the IC_{50} values were extrapolated.
3.4 Effect of Zingiberaceae extracts on expression of Wt-p53 protein

Thirty crude petroleum ether, chloroform and methanol extracts of ten selected Zingiberaceae were evaluated for the effect on the expression of wild-type p53 protein in colon cancer cell lines, HCT-116 and HT29.

3.4.1 Treatment with Zingiberaceae extracts

Sub-confluent cells that formed a single layer in the culture flask were washed using PBS. The cells were detached from the flask by incubating in 1 ml 0.25% trypsin-EDTA solution and 3 ml PBS solution for 5 min at 37°C, and the flask was then sharply tapped. The cells suspension were transferred into a centrifuge tube and centrifuged for 5 min at 1,000 rpm. The supernatant was removed and 1 ml 10% complete medium was added to the pellet and then transferred into flask containing 1 ml of complete medium and 1 ml of Zingiberaceae extracts with known concentration. The cells were treated separately with each crude extract at five different concentrations ranging from 1 to 100 μg/ml and further incubated 72 h. Negative control is the flask containing untreated cells (without addition of any extract).

3.4.2 Slides preparation

The treated and untreated cells were harvested after 72 h of incubation with plant extract. The cells were trypsinized, transferred into centrifuge tubes and spun at 1000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 2 ml of
PBS. The cell suspension was then placed onto 12-wells teflon coated slides at 30 μl per well.

### 3.4.3 3-step Indirect Avidin-Biotin Immunoperoxidase Technique

Human colon cancer cell lines, HCT-116 and HT29 (untreated and treated with plant extracts) were immunocytochemically analysed using the 3-step indirect avidin-biotin immunoperoxidase technique for the presence of wild type p53 protein (wt-p53). The slides were dehydrated through a graded series of ethanol solution - 100%, 95%, 90% and 80%. Endogenous peroxidase activity was inhibited with the incubation with 0.3% hydrogen peroxide (H2O2) for 10 min.

The slides were rinsed with PBS, pH 7.6 for 5 min and treated with the primary antibody; DO-7 mouse anti-human p53 protein (DakoCytomation). The incubation with antibody was carried out for 90 minutes at 37°C. The surrounding areas of wells were blotted dry and 30 μl of diluted DO-7 mouse anti-human p53 protein (DakoCytomation), diluted 1:25 with sterile PBS pH 7.6 was added to four wells on one end of the slide. 30 μl of sterile PBS pH 7.6 was added to four wells on other end of the slide. These cells which were untreated with the DO-7 mouse anti-human p53 protein served as negative controls. The slides were incubated in a benchtop incubator (LT Biomax 500) at room temperature for 1 h. After that, slides were washed twice in PBS pH 7.6 for 15 min each. 30 μl of secondary biotinylated link anti-mouse immunoglobulin (Dako Cytomation) was subsequently added to all wells. The slides were incubated at 37°C for 10 min and then washed in PBS pH 7.6 for 5 min. 30 μl of streptavidin-horse radish peroxidase (Dako Cytomation) was added to each well followed by incubation at 37°C for 10 min. The
slides were washed in PBS pH 7.6 for 5 min. After which; the wells were then filled with 30 ml of 3, 3-diaminobenzidine (DAB) (Dako Cytomation) and incubated at 37°C for 10-20 min until the desired colour intensity was developed. The slides were rinsed in distilled water and then counterstained with Mayers Hematoxylin solution (Fluka) for 2 min. The slides were rinsed in distilled water again and then immersed into 30% ammonia solution (Systern) for 10 sec. After rinsing the slides with distilled water, the slides were mounted with pre-warmed glycergel at 53°C (Dako Cytomation) and were left to dry overnight in the dark. The slides stained were then visualized under the light microscope (Olympus). The expression was scored as weak (1+, 10-30% stained intensities), moderate (3+, 40-70% stained intensities) or strong (5+, >70% stained intensities. Example of the wt-p53 expressing scoring is showed in Appendix 1.
CHAPTER 4
RESULTS

4.1 Yield of crude extracts

Ten different species of Zingiberaceae family namely *Alpinia galanga, Boesenbergia rotunda, Curcuma aeruginosa, Curcuma domestica, Curcuma mangga, Curcuma xanthorriza, Kaempferia galanga, Zingiber montanum, Zingiber officinale* and *Zingiber zerumbet* were collected, dried and extracted for the present study. Table 4.1 shows the list of the selected Zingiberaceae species with the yields calculated for all 30 crude petroleum ether, chloroform and methanol extracts.

The total yield percentages ranged from 5.64% to 13.55% of dry weight. The rhizome of *Curcuma aeruginosa* showed the lowest total yield percentage while *Curcuma mangga* showed the highest. Overall, the yield percentages from the methanol Zingiberaceae extracts (ranged 1.45% to 8.26%) were found to be higher than that of the petroleum ether (0.68%-6.23%) and chloroform (0.82-3.24%) extracts.
Table 4.1: Yield percentages of selected species of Zingiberaceae

<table>
<thead>
<tr>
<th>No.</th>
<th>Zingiberaceae Species (local name)</th>
<th>Extraction Solvent</th>
<th>Weight of Dry Crude Extract (g)</th>
<th>Yield (%)</th>
<th>Total Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Alpinia galanga</em> (Lengkuas)</td>
<td>Petroleum ether</td>
<td>0.1357</td>
<td>0.68</td>
<td>9.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td>0.1640</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>1.6520</td>
<td>8.26</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>Boesenbergia rotunda</em> (Temu kunci)</td>
<td>Petroleum ether</td>
<td>0.2042</td>
<td>1.02</td>
<td>5.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td>0.6389</td>
<td>3.19</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>0.2905</td>
<td>1.45</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>Curcuma aeruginosa</em> (Temu hitam)</td>
<td>Petroleum ether</td>
<td>0.1386</td>
<td>0.69</td>
<td>5.64</td>
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<td></td>
<td></td>
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<td>0.4367</td>
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<td></td>
<td>Methanol</td>
<td>0.5545</td>
<td>2.77</td>
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<tr>
<td>4</td>
<td><em>Curcuma domestica</em> (Kunyit)</td>
<td>Petroleum ether</td>
<td>0.3309</td>
<td>1.65</td>
<td>8.08</td>
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<td></td>
<td></td>
<td>Chloroform</td>
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<td></td>
<td>Methanol</td>
<td>0.6516</td>
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</tr>
<tr>
<td>5</td>
<td><em>Curcuma mangga</em> (Temu pauh)</td>
<td>Petroleum ether</td>
<td>1.1015</td>
<td>5.51</td>
<td>13.55</td>
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<td></td>
<td>Chloroform</td>
<td>0.3888</td>
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<td>Methanol</td>
<td>1.2203</td>
<td>6.10</td>
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<tr>
<td>6</td>
<td><em>Curcuma xanthorrhiza</em> (Temu lawak)</td>
<td>Petroleum ether</td>
<td>1.2465</td>
<td>6.23</td>
<td>12.17</td>
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<td></td>
<td></td>
<td>Chloroform</td>
<td>0.6484</td>
<td>3.24</td>
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<td></td>
<td>Methanol</td>
<td>0.5401</td>
<td>2.70</td>
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<tr>
<td>7</td>
<td><em>Kaempferia galanga</em> (Cekur)</td>
<td>Petroleum ether</td>
<td>0.9000</td>
<td>4.50</td>
<td>8.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td>0.3287</td>
<td>1.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>0.4174</td>
<td>2.09</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td><em>Zingiber montanum</em> (Bonglai)</td>
<td>Petroleum ether</td>
<td>0.7050</td>
<td>3.53</td>
<td>7.89</td>
</tr>
<tr>
<td></td>
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<td>Chloroform</td>
<td>0.3374</td>
<td>1.69</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>0.5340</td>
<td>2.67</td>
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</tr>
<tr>
<td>9</td>
<td><em>Zingiber officinale</em> (Halit)</td>
<td>Petroleum ether</td>
<td>0.4623</td>
<td>2.31</td>
<td>6.54</td>
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<td></td>
<td>Chloroform</td>
<td>0.2545</td>
<td>1.27</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>0.5920</td>
<td>2.96</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td><em>Zingiber zerumbet</em> (Lempoyang)</td>
<td>Petroleum ether</td>
<td>0.3420</td>
<td>1.71</td>
<td>11.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td>0.4629</td>
<td>2.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>1.4203</td>
<td>7.10</td>
<td></td>
</tr>
</tbody>
</table>

4.2 *In vitro* cytotoxic activity against HCT-116 and HT29 cells
In the present study, 30 crude Zingiberaceae extracts were evaluated for their cytotoxic activities against two human colon cancer cell lines (HCT-116 and HT29) using the Neutral Red cytotoxicity assay. The positive controls consisted of cells treated with doxorubicin while the negative controls consisted of untreated cells. The cytotoxicity data obtained were expressed as killing percentages relative to negative controls and the IC\textsubscript{50} values were extrapolated from the dose-response curves plotted. The IC\textsubscript{50} value refers to the inhibition concentration of extracts in \( \mu \text{g/ml} \) that reduced 50\% of cell growth. Extracts having an IC\textsubscript{50} value equal to or less than 20 \( \mu \text{g/ml} \) are considered active for cytotoxicity assay against cells (Geran \textit{et al.}, 1972).

4.2.1 \textit{In vitro} cytotoxic activity of doxorubicin

The human colon cancer cell lines, HCT-116 and HT29; and the normal lung cell line, MRC-5 were treated with doxorubicin as the positive control for the NR assay. Doxorubicin is the most commonly used cytotoxic drug in the treatment of lymphoma, osteosarcoma and other sarcomas, carcinomas and melanoma. The concentrations of doxorubicin used ranged from 0.01 to 1 \( \mu \text{g/ml} \). Doxorubicin demonstrated significant cytotoxic effect against the three cell lines tested (Figure 4.1). The IC\textsubscript{50} values of doxorubicin against HCT-116, HT29 and MRC-5 cell lines are shown in Table 4.2.

4.2.2 \textit{In vitro} cytotoxic activity of selected Zingiberaceae crude extracts

Thirty crude Zingiberaceae extracts were evaluated for their cytotoxicity activities against HCT-116 and HT29 cell lines. HCT-116 and HT29 cells grown in 10\% supplemented McCoy’s 5a and RPMI 1640 medium respectively, were incubated with
crude petroleum ether, chloroform and methanol extracts of selected Zingiberaceae at varying concentrations for 72 h in a 5% carbon dioxide (CO₂) water jacketed incubator at 37°C. Plate 4.1 shows the photomicrographs of HCT-116 and HT29 cells treated with plant extracts and those untreated with any extract. The negative controls exhibited normal proliferation rate and showed no signs of death at the end of incubation time of 72 h.

Figure 4.1: The in vitro growth inhibition of HCT-116, HT29 and MRC-5 cells at different concentrations of doxorubicin using the NR Cytotoxicity Assay. The IC₅₀ values were estimated from the graphical interpolation.

Table 4.2: The IC₅₀ values of doxorubicin tested against HCT-116, HT29 and MRC-5 cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC₅₀ value (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-116</td>
<td>0.36</td>
</tr>
<tr>
<td>HT29</td>
<td>0.79</td>
</tr>
<tr>
<td>MRC-5</td>
<td>0.40</td>
</tr>
</tbody>
</table>
4.2.3 *In vitro* cytotoxic activity of selected Zingiberaceae extracts against HCT-116 cells

HCT-116 cells were incubated with various extracts of the selected Zingiberaceae at concentrations of 1, 10, 25, 50 and 100 μg/ml to determine the cytotoxicity effect of...
these plants extracts. The controls which consisted of untreated HCT-116 (without addition of any extracts) showed no signs of cytotoxicity (Plate 4.1 (a)).

**Cytotoxic activity of crude petroleum ether extracts**

Generally, 60% of crude petroleum ether extracts of selected Zingiberaceae demonstrated active cytotoxic effect against HCT-116 cell line (Figures 4.2a and 4.2b). The petroleum ether extracts inhibited the proliferation of HCT-116 cells with the inhibition rates of 0-55% at 1 µg/ml, 1-90% at 10 µg/ml, 10-91% at 25 µg/ml, 31-92% at 50 µg/ml and 86-93% at 100 µg/ml.

The dose-dependent response can clearly be seen in HCT-116 cells treated with crude petroleum ether extracts of *Kaempferia galanga* (10-50 µg/ml), *Zingiber montanum* (1-50 µg/ml), *Curcuma aeruginosa* (10-50 µg/ml), *Boesenbergia rotunda* (1-50 µg/ml), *Zingiber officinale* (1-50 µg/ml), *Curcuma xanthorriza* (10-25 µg/ml) and *Curcuma domestica* (1-50 µg/ml). Dose-dependent responses were also seen in HCT-116 cells treated with 1-10 µg/ml of *Alpinia galanga* and *Zingiber zerumbet*. At higher concentrations of extracts tested here, the cytotoxic activity levelled off and became constant. This pattern however, was not seen in cells treated with petroleum ether extract of *Curcuma mangga* where the dose-dependent response remained even at higher concentrations.

Among all the crude petroleum ether extracts tested, *B. rotunda, Z. montanum, Z. zerumbet, C. xanthorriza, C. domestica* and *A. galanga* were considered active against HCT-116 cells with IC$_{50}$ values of 7.1 µg/ml, 7.6 µg/ml, 2.0 µg/ml, 16.8 µg/ml, 17.1
μg/ml and 5.5 μg/ml, respectively. *Z. zerumbet* was the most active extract with the lowest IC$_{50}$ value of 2.0 μg/ml (Table 4.3).
Figure 4.2(a): The *in vitro* growth inhibition of HCT-116 cells by crude petroleum ether extracts of selected Zingiberaceae species. The IC$_{50}$ values were estimated from the graphical interpolation.
Figure 4.2(b): The *in vitro* growth inhibition of HCT-116 cells by crude petroleum ether extracts of selected Zingiberaceae species. The IC$_{50}$ values were estimated from the graphical interpolation.
Cytotoxic activity of crude chloroform extracts

The cytotoxic effect on HCT-116 cells gradually increased with increasing concentration of certain crude chloroform extracts (Figures 4.2c and 4.2d). Crude chloroform extracts exhibited inhibition rates which ranged from 2 - 25% at 1 μg/ml, 8 - 89% at 10 μg/ml, 14 - 93% at 25 μg/ml, 28 - 94% at 50 μg/ml and 84 - 94% at 100 μg/ml. The IC$_{50}$ values determined from the graphs ranged between 6.00 μg/ml to 68.80 μg/ml (Table 4.3). Results show that active crude chloroform extracts (IC$_{50}$ < 20 μg/ml) against HCT-116 cells were derived from Boesenbergia rotunda, Zingiber montanum, Zingiber zerumbet, Curcuma xanthorrhiza, Curcuma domestica and Alpinia galanga with IC$_{50}$ values of 11.1 μg/ml, 14.4 μg/ml, 6.0 μg/ml, 15.0 μg/ml, 6.1 μg/ml and 6.0 μg/ml, respectively. The crude chloroform extracts of A. galanga and Z. zerumbet appeared to be the most active extracts with IC$_{50}$ values of 6 μg/ml.

In this study, an increasing of cytotoxic activity can be observed in HCT-116 cells treated with increasing concentrations of crude chloroform extracts of all selected Zingiberaceae. The crude chloroform extracts of Curcuma mangga and Curcuma aeruginosa still shows the dose-dependent response even at highest concentration.
Figure 4.2(c): The *in vitro* growth inhibition of HCT-116 cells by crude chloroform extracts of selected Zingiberaceae species. The IC$_{50}$ values were estimated from the graphical interpolation.
Figure 4.2(d): The *in vitro* growth inhibition of HCT-116 cells by crude chloroform extracts of selected Zingiberaceae species. The IC₅₀ values were estimated from the graphical interpolation.
Cytotoxic activity of crude methanol extracts

The inhibition rates of HCT-116 cells by the some crude methanol extract of Zingiberaceae species were low, 1 - 21% at 1 μg/ml, 3 - 47% at 10 μg/ml, 14 - 79% at 25 μg/ml, 15 - 87% at 50 μg/ml and 18 - 89% at 100 μg/ml (Figures 4.2e and 4.2f). The IC₅₀ values ranged from 16.70 μg/ml to more than 100 μg/ml (Table 4.3). The present study shows that most of the crude methanol extracts of Zingiberaceae species were inactive for cytotoxic activity against HCT-116 cells, with the exception for Curcuma domestica and Zingiber zerumbet which displayed IC₅₀ values of 16.7 μg/ml and 19.2 μg/ml, respectively.

In the graph plotted, it can clearly be seen that the crude methanol extracts of Kaempferia galanga, Zingiber montanum, Curcuma xanthorrhiza and Alpinia galanga still shows the dose-dependent manner up to 100 μg/ml. The two active extracts of Curcuma domestica and Zingiber zerumbet show the dose-dependent manner only up to 25 μg/ml and 50 μg/ml, respectively.
Figure 4.2(e): The \textit{in vitro} growth inhibition of HCT-116 cells by crude methanol extracts of selected Zingiberaceae species. The IC$_{50}$ values were estimated from the graphical interpolation.
Figure 4.2(f): The *in vitro* growth inhibition of HCT-116 cells by crude methanol extracts of selected Zingiberaceae species. The IC$_{50}$ values were estimated from the graphical interpolation.
Based on the cytotoxic activity against HCT-116 cell line, several Zingiberaceae extracts have been found to be active. Rhizome extracts of *Curcuma domestica* and *Zingiber zerumbet* in all three solvents were found to be the most active among the ten Zingiberaceae species tested, with IC₅₀ values of 17.1 μg/ml (petroleum ether), 6.1 μg/ml (chloroform) and 16.7 μg/ml (methanol) for *C. domestica*; and 2 μg/ml (petroleum ether), 6 μg/ml (chloroform) and 19.2 μg/ml (methanol) for *Z. zerumbet*. *Boesenbergia rotunda*, *Zingiber montanum*, *Curcuma xanthorriza* and *Alpinia galanga* crude extracts were considered less cytotoxic against HCT-116 cells where only crude petroleum ether and crude chloroform extracts of these rhizomes were active with IC₅₀ values less than 20 μg/ml. *Kaempferia galanga*, *Curcuma mangga*, *Curcuma aeruginosa* and *Zingiber officinale* were found to be inactive against HCT-116 cell line for all the three extracts extracted in different solvents.

The crude petroleum ether and crude chloroform extracts exhibited slightly active cytotoxicity than the corresponding crude methanol extracts. All crude petroleum ether and crude chloroform extracts exhibited an IC₅₀ values less than 100 μg/ml. This is followed by crude petroleum ether extract of *Alpinia galanga*, crude chloroform extracts of *Zingiber zerumbet*, *Alpinia galanga* and *Curcuma domestica*. Their IC₅₀ values were 5.5 μg/ml, 6.0 μg/ml, 6.0 μg/ml and 6.1 μg/ml, respectively.
Table 4.3: IC<sub>50</sub> values of crude extracts of selected Zingiberaceae species against HCT-116 cells

<table>
<thead>
<tr>
<th>No.</th>
<th>Zingiberaceae Species</th>
<th>Extraction Solvent</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; value (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Alpinia galanga</em> (Lengkuas)</td>
<td>Petroleum ether</td>
<td><strong>5.50</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td><strong>6.00</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>69.20</td>
</tr>
<tr>
<td>2</td>
<td><em>Boesenbergia rotunda</em> (Temu kunci)</td>
<td>Petroleum ether</td>
<td><strong>7.10</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td><strong>11.12</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>27.00</td>
</tr>
<tr>
<td>3</td>
<td><em>Curcuma aeruginosa</em> (Temu hitam)</td>
<td>Petroleum ether</td>
<td>34.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>87.00</td>
</tr>
<tr>
<td>4</td>
<td><em>Curcuma domestica</em> (Kunyit)</td>
<td>Petroleum ether</td>
<td><strong>17.10</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td><strong>6.10</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td><strong>16.70</strong></td>
</tr>
<tr>
<td>5</td>
<td><em>Curcuma mangga</em> (Temu pauh)</td>
<td>Petroleum ether</td>
<td>66.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>&gt;100</td>
</tr>
<tr>
<td>6</td>
<td><em>Curcuma xanthorrhiza</em> (Temu lawak)</td>
<td>Petroleum ether</td>
<td><strong>16.80</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td><strong>15.00</strong></td>
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<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>37.80</td>
</tr>
<tr>
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<td><em>Kaempferia galanga</em> (Cekur)</td>
<td>Petroleum ether</td>
<td>22.50</td>
</tr>
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<td></td>
<td></td>
<td>Chloroform</td>
<td>27.10</td>
</tr>
<tr>
<td></td>
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<td>Methanol</td>
<td>73.77</td>
</tr>
<tr>
<td>8</td>
<td><em>Zingiber montanum</em> (Bonglai)</td>
<td>Petroleum ether</td>
<td><strong>7.60</strong></td>
</tr>
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<td></td>
<td></td>
<td>Chloroform</td>
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<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>76.80</td>
</tr>
<tr>
<td>9</td>
<td><em>Zingiber officinale</em> (Halia)</td>
<td>Petroleum ether</td>
<td>22.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>&gt;100</td>
</tr>
<tr>
<td>10</td>
<td><em>Zingiber zerumbet</em> (Lempoyang)</td>
<td>Petroleum ether</td>
<td><strong>2.00</strong></td>
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<td></td>
<td>Chloroform</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td><strong>19.20</strong></td>
</tr>
</tbody>
</table>

**Bold** – IC<sub>50</sub> of ≤ 20 μg/ml is considered active (WHO, 1972; Geran *et al*., 1972; Cordell *et al*., 1993; Chiang *et al*., 2003)
4.2.4 *In vitro* cytotoxic activity of selected Zingiberaceae against HT29 cells

The cytotoxic activities of crude extracts of selected Zingiberaceae species against HT29 cells at various concentrations were evaluated and the dose-response curves are shown in Figures 4.3(a-f).

**Cytotoxic activity of crude petroleum ether extracts**

Results demonstrated that certain crude petroleum ether extracts of Zingiberaceae possessed the ability to suppress the proliferation of HT29 cells in a dose-dependent manner up to certain concentrations (Figures 4.3a and 4.3b). The inhibition activities observed ranged from 3 - 45% at 1 µg/ml, 9 - 87% at 10 µg/ml, 12 - 89% at 25 µg/ml, 49 - 89% at 50 µg/ml and 63 - 90% at 100 µg/ml. IC$_{50}$ values were extrapolated from the dose-response curves. Crude extracts from the rhizomes of *Boesenbergia rotunda*, *Zingiber officinale*, *Alpinia galanga* and *Zingiber zerumbet* exhibited IC$_{50}$ values of 17.5 µg/ml, 13.0 µg/ml, 5.8 µg/ml and 2.8 µg/ml, respectively (Table 4.4). The crude petroleum ether extract of *Z. zerumbet* was the most active.

The crude petroleum ether extracts of *A. galanga*, *B. rotunda*, *Z. zerumbet*, *Curcuma domestica*, *C. xanthorrhiza* and *Z. officinale* show the dose-dependent response at lower concentration in HT29 cells up to concentration of 20 µg/ml after which the cytotoxic activity remained constant.
Figure 4.3(a): The *in vitro* growth inhibition of HT29 cells by crude petroleum ether extracts of selected Zingiberaceae species. The IC$_{50}$ values were estimated from the graphical interpolation.
Figure 4.3(b): The *in vitro* growth inhibition of HT29 cells by crude petroleum ether extracts of selected Zingiberaceae species. The IC$_{50}$ values were estimated from the graphical interpolation.
Cytotoxic activity of crude chloroform extracts

The cytotoxic effect of crude chloroform extracts of Zingiberaceae in HT29 cells increased with increasing concentrations of the extracts up to certain concentration. The inhibition rates ranged from 0 - 48% at 1 μg/ml, 12 - 88% at 10 μg/ml, 19 - 88% at 25 μg/ml, 32 - 90% at 50 μg/ml and 64 - 90% at 100 μg/ml (Figures 4.3c and 4.3d). The IC₅₀ values for crude chloroform extracts ranged from 2.30 μg/ml to 77.10 μg/ml. Six of the extracts were considered actively cytotoxic towards HT29 cells, namely Boesenbergia rotunda, Zingiber montanum, Curcuma aeruginosa, Curcuma domestica, Alpinia galanga and Zingiber zerumbet with the IC₅₀ values of 18.0 μg/ml, 6.8 μg/ml, 2.3 μg/ml, 7.7 μg/ml, 5.0 μg/ml and 7.7 μg/ml, respectively (Table 4.4).

In the graph plotted, it can clearly be seen that the crude chloroform extracts of Kaempferia galanga, Curcuma mangga, Curcuma aeruginosa, and Zingiber officinale still shows the dose-dependent manner up to 100 μg/ml. The three extracts of Zingiber montanum, Alpinia galanga and Curcuma domestica show the dose-dependent manner only up to 10 μg/ml while the extracts of Curcuma xanthorrhiza and Boesenbergia rotunda show the dose-dependent manner up to 25 μg/ml.
Figure 4.3(c): The *in vitro* growth inhibition of HT29 cells by crude chloroform extracts of selected Zingiberaceae species. The IC$_{50}$ values were estimated from the graphical interpolation.
Figure 4.3(d): The *in vitro* growth inhibition of HT29 cells by crude chloroform extracts of selected Zingiberaceae species. The IC$_{50}$ values were estimated from the graphical interpolation.
Cytotoxic activity of crude methanol extracts

Results show that crude methanol extracts of Zingiberaceae have low ability to retard the growth of HT29 cells with percentage of inhibition range from 0 - 19% at 1 μg/ml, 0 - 51% at 10 μg/ml, 8 - 81% at 25 μg/ml, 8 - 83% at 50 μg/ml and 24 - 88% at 100 μg/ml (Figures 4.3e and 4.3f). Among the ten Zingiberaceae species studied, only crude extract obtained from the *Z. zerumbet* rhizome showed IC<sub>50</sub> value less than 20 μg/ml (Table 4.4). The IC<sub>50</sub> value was 10.00 μg/ml. On the other hand, the IC<sub>50</sub> values of four of the crude extracts could not be determined from dose-response curves because of the percentage of inhibition was less than 50% at all concentrations tested. The crude extracts of *Curcuma mangga*, *Boesenbergia rotunda*, *Curcuma aeruginosa* and *Zingiber officinale* were considered not cytotoxic against HT29 cells.

In the graph plotted, it can clearly be seen that the crude methanol extracts of *Kaempferia galanga*, *Zingiber montanum*, *Curcuma mangga*, *Curcuma aeruginosa*, *Boesenbergia rotunda*, *Zingiber officinale*, *Alpinia galanga* and *Curcuma xanthorriza* still shows the dose-dependent manner up to 100 μg/ml. The extracts of *Zingiber zerumbet* and *Curcuma domestica* show the dose-dependent manner only up to 25 μg/ml.
Figure 4.3(e): The *in vitro* growth inhibition of HT29 cells by crude methanol extracts of selected Zingiberaceae species. The IC\(_{50}\) values were estimated from the graphical interpolation.
Figure 4.3(f): The *in vitro* growth inhibition of HT29 cells by crude methanol extracts of selected Zingiberaceae species.
In the present study, it was found that the crude chloroform extracts of Zingiberaceae were more cytotoxic against HT29 cells as compared to crude petroleum ether and crude methanol extracts. Overall results indicated that 11 out of 30 crude petroleum ether, chloroform and methanol Zingiberaceae extracts were found to be actively cytotoxic against HT29 cells with IC$_{50}$ values less than 20 μg/ml (Table 4.4). The most active extract against HT29 cells in the present study was Zingiber zerumbet showing IC$_{50}$ values less than 20 μg/ml in all three solvents system used, 2.8 μg/ml (petroleum ether), 7.7 μg/ml (chloroform) and 10.0 μg/ml (methanol).

Other extracts which inhibit 50% of the growth of HT29 cells with the concentration less than 20 μg/ml were the crude petroleum ether and crude chloroform extracts of Boesenbergia rotunda, Alpinia galanga and Zingiber zerumbet. The Zingiber montanum, Curcuma aeruginosa and Curcuma domestica exerted a much less cytotoxic effect against HT29 cells where only crude chloroform extracts was found to be active with the IC$_{50}$ values of 6.8 μg/ml, 2.3 μg/ml and 7.7 μg/ml, respectively. In contrast, none of the Kaempferia galanga crude extracts showed cytotoxic effect on HT29 cells at all concentrations tested.
Table 4.4: IC\textsubscript{50} values of crude extracts of selected Zingiberaceae species against HT29 cells

<table>
<thead>
<tr>
<th>No.</th>
<th>Zingiberaceae Species</th>
<th>Extraction Solvent</th>
<th>IC\textsubscript{50} value (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Alpinia galanga</em> (Lengkuas)</td>
<td>Petroleum ether</td>
<td>5.80, 5.00</td>
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<tr>
<td></td>
<td></td>
<td>Chloroform</td>
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<td></td>
<td></td>
<td>Methanol</td>
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</tr>
<tr>
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<td><em>Boesenbergia rotunda</em> (Temu kunci)</td>
<td>Petroleum ether</td>
<td>17.50</td>
</tr>
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<td></td>
<td></td>
<td>Chloroform</td>
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</tr>
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<td></td>
<td></td>
<td>Methanol</td>
<td>&gt;100</td>
</tr>
<tr>
<td>3</td>
<td><em>Curcuma aeruginosa</em> (Temu hitam)</td>
<td>Petroleum ether</td>
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</tr>
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<td>Methanol</td>
<td>&gt;100</td>
</tr>
<tr>
<td>4</td>
<td><em>Curcuma domestica</em> (Kunyit)</td>
<td>Petroleum ether</td>
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<td>Chloroform</td>
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<td></td>
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<td>Methanol</td>
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<td></td>
<td>Methanol</td>
<td>&gt;100</td>
</tr>
<tr>
<td>6</td>
<td><em>Curcuma xanthorriza</em> (Temu lawak)</td>
<td>Petroleum ether</td>
<td>22.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td>22.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>80.00</td>
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<tr>
<td>7</td>
<td><em>Kaempferia galanga</em> (Cekur)</td>
<td>Petroleum ether</td>
<td>53.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td>77.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>81.00</td>
</tr>
<tr>
<td>8</td>
<td><em>Zingiber montanum</em> (Bonglai)</td>
<td>Petroleum ether</td>
<td>21.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td>6.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>70.00</td>
</tr>
<tr>
<td>9</td>
<td><em>Zingiber officinale</em> (Haliala)</td>
<td>Petroleum ether</td>
<td>13.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td>30.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>&gt;100</td>
</tr>
<tr>
<td>10</td>
<td><em>Zingiber zerumbet</em> (Lempoyang)</td>
<td>Petroleum ether</td>
<td>2.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td>7.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol</td>
<td>10.00</td>
</tr>
</tbody>
</table>

**Bold** – IC\textsubscript{50} of \(\leq 20\) μg/ml is considered active (WHO, 1972; Geran \textit{et al.}, 1972; Cordell \textit{et al.}, 1993; Chiang \textit{et al.}, 2003).
4.2.5 Comparison of cytotoxic activities of selected Zingiberaceae crude extracts against 2 colon cancer cell lines

Of all the Zingiberaceae extracts studied, the petroleum ether and chloroform extracts showed better cytotoxic activities than the methanol extracts. The crude petroleum ether extracts which showed active cytotoxicity against both HCT-116 and HT29 cell lines were the extracts of *Alpinia galanga*, *Boesenbergia rotunda* and *Zingiber zerumbet*. On the other hand, the petroleum ether extracts of *Curcuma domestica*, *Curcuma xanthorriza* and *Zingiber montanum* only showed active cytotoxicity against HCT-116 cells while the petroleum ether extracts of *Zingiber officinale* showed active cytotoxic activity against HT29 cells.

Active cytotoxic activities against both cell lines were shown by crude chloroform extracts of *A. galanga*, *B. rotunda*, *C. domestica*, *Z. montanum* and *Z. zerumbet*. The crude chloroform extract of *Curcuma aeruginosa* only showed active cytotoxic activity in HT29 whereas the crude chloroform extract of *C. xanthorriza* was active against HCT-116 cell lines.

Only methanol extract of *Zingiber zerumbet* exerted active cytotoxicity against both cell lines tested. The crude methanol extract of *C. domestica* were actively cytotoxic against HT29 cells only. The rest of the methanol extracts of selected Zingiberaceae species were found inactive in inhibiting the proliferation of HCT-116 and HT29 cell lines (Table 4.5).
Table 4.5: IC\textsubscript{50} values of crude extracts of selected Zingiberaceae species against HCT-116 and HT29 cells

<table>
<thead>
<tr>
<th>Zingiberaceae Species</th>
<th>Extraction Solvent</th>
<th>IC\textsubscript{50} value (μg/ml) HCT-116</th>
<th>IC\textsubscript{50} value (μg/ml) HT29</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Petroleum ether</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Alpinia galanga</em> (Lengkuas)</td>
<td>Chloroform</td>
<td>5.50</td>
<td>5.80</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>6.00</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>69.20</td>
<td>44.10</td>
</tr>
<tr>
<td><em>Boesenbergia rotunda</em> (Temu kunci)</td>
<td>Petroleum ether</td>
<td>7.10</td>
<td>17.50</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>11.12</td>
<td>18.00</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>27.00</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Curcuma aeruginosa</em> (Temu hitam)</td>
<td>Petroleum ether</td>
<td>34.50</td>
<td>24.00</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>67.30</td>
<td>2.30</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>87.00</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Curcuma domestica</em> (Kunyit)</td>
<td>Petroleum ether</td>
<td>17.10</td>
<td>22.00</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>6.10</td>
<td>7.70</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>16.70</td>
<td>20.90</td>
</tr>
<tr>
<td><em>Curcuma mangga</em> (Temu pauh)</td>
<td>Petroleum ether</td>
<td>66.00</td>
<td>48.30</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>68.80</td>
<td>72.00</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Curcuma xanthorriza</em> (Temu lawak)</td>
<td>Petroleum ether</td>
<td>16.80</td>
<td>22.20</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>15.00</td>
<td>22.00</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>37.80</td>
<td>80.00</td>
</tr>
<tr>
<td><em>Kaempferia galanga</em> (Cekur)</td>
<td>Petroleum ether</td>
<td>22.50</td>
<td>53.28</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>27.10</td>
<td>77.10</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>73.77</td>
<td>81.00</td>
</tr>
<tr>
<td><em>Zingiber montanum</em> (Bonglai)</td>
<td>Petroleum ether</td>
<td>7.60</td>
<td>21.00</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>14.40</td>
<td>6.80</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>76.80</td>
<td>70.00</td>
</tr>
<tr>
<td><em>Zingiber officinale</em> (Halia)</td>
<td>Petroleum ether</td>
<td>22.10</td>
<td>13.00</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>38.50</td>
<td>30.50</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Zingiber zerumbet</em> (Lempoyang)</td>
<td>Petroleum ether</td>
<td>2.00</td>
<td>2.80</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>6.00</td>
<td>7.70</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>19.20</td>
<td>10.00</td>
</tr>
</tbody>
</table>

**Bold** – IC\textsubscript{50} of ≤ 20 μg/ml is considered active (WHO, 1972; Geran *et al.*, 1972; Cordell *et al.*, 1993; Chiang *et al.*, 2003).
4.3  **Cytotoxic activity of 5-Fluorouracil (5-Fu)**

5-fluorouracil (5-Fu) is one of the oldest chemotherapy drugs. In the present study, the effect of selected Zingiberaceae extracts on the toxicity of 5-Fu against HCT-116 and HT29 cells was investigated.

4.3.1  **Cytotoxicity of 5-Fu against HCT-116 and HT29 cells**

HCT-116 and HT29 cells were incubated with nine different concentrations of 5-Fu ranging from $0.1 \times 10^{-7}$ M to 1.0 M. 5-Fu alone exhibited inhibition rates more than 70% against both cell lines when tested at the concentration of 1 M (Table 4.6). The controls which consisted of untreated HCT-116 and HT29 cells (without addition of 5-Fu and any extracts) showed no signs of cytotoxicity (Plate 4.1).

Figure 4.4 shows the dose-response curve of 5-Fu against HCT-116 and HT29 cells. Different pattern of the cytotoxicity against both cell lines can clearly be seen. At lower concentrations of 5-Fu ($0.1 \times 10^{-7}$ - $0.1 \times 10^{-3}$ M), better cytotoxic effect can be seen against HT29 cells but at higher concentrations ranging from $0.1 \times 10^{-2}$ M to 1.0 M, a better cytotoxic effect was seen in HCT-116. The IC$_{50}$ values were extrapolated from the curves and are shown in Table 4.7.
Table 4.6: Percentage of growth inhibition of HCT-116 and HT29 cells by 5-Fluorouracil (5-Fu) using the NR cytotoxicity assay

<table>
<thead>
<tr>
<th>Concentration of 5-Fu (M)</th>
<th>Percentage of inhibition (%)</th>
<th>HCT-116</th>
<th>HT29</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 x 10^{-7}</td>
<td></td>
<td>7.07</td>
<td>16.31</td>
</tr>
<tr>
<td>0.1 x 10^{-6}</td>
<td></td>
<td>10.55</td>
<td>17.67</td>
</tr>
<tr>
<td>0.1 x 10^{-5}</td>
<td></td>
<td>11.07</td>
<td>18.46</td>
</tr>
<tr>
<td>0.1 x 10^{-4}</td>
<td></td>
<td>14.82</td>
<td>23.73</td>
</tr>
<tr>
<td>0.1 x 10^{-3}</td>
<td></td>
<td>19.81</td>
<td>27.75</td>
</tr>
<tr>
<td>0.1 x 10^{-2}</td>
<td></td>
<td>47.31</td>
<td>34.28</td>
</tr>
<tr>
<td>0.1 x 10^{-1}</td>
<td></td>
<td>49.20</td>
<td>38.69</td>
</tr>
<tr>
<td>0.1</td>
<td></td>
<td>68.76</td>
<td>48.56</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>87.15</td>
<td>77.59</td>
</tr>
</tbody>
</table>

Figure 4.4: The *in vitro* growth inhibition of HCT-116 and HT29 cells by nine different concentrations of 5-Fluorouracil (5-Fu) using the NR cytotoxicity assay. The IC$_{50}$ values were estimated from the graphical interpolation.

Table 4.7: The IC$_{50}$ values of 5-Fu against HCT-116 and HT29 cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC$_{50}$ value (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-116</td>
<td>0.02</td>
</tr>
<tr>
<td>HT29</td>
<td>0.15</td>
</tr>
</tbody>
</table>
4.3.2 Cytotoxicity of 5-Fu and Zingiberaceae extracts

Based on the results previously obtained from the cytotoxicity assay, several active Zingiberaceae extracts were selected to be further tested with 5-Fu. The extracts that gave the IC₅₀ values lower than 20 μg/ml were tested again on HCT-116 and HT29 cell lines with the addition of 5-Fu. Table 4.8 shows the selected extracts and their IC₅₀ values. Several crude petroleum ether and chloroform extracts were chosen. Since none of the methanol Zingiberaceae extracts were active when evaluated previously in the cytotoxicity assay, they were not further tested together with 5-Fu. The concentration of extracts used here were the same concentration which gave 50% inhibition of colon cancer cell (IC₅₀) in the previous cytotoxicity assay (Table 4.5).

Table 4.8: IC₅₀ values of Zingiberaceae extracts tested on HCT-116 and HT29 cell lines chosen for the 5-Fu study

<table>
<thead>
<tr>
<th>Zingiberaceae Species</th>
<th>HCT-116</th>
<th>HT29</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ value (μg/ml)</td>
<td>IC₅₀ value (μg/ml)</td>
</tr>
<tr>
<td><strong>Petroleum ether</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpinia galanga</td>
<td>5.50</td>
<td>5.80</td>
</tr>
<tr>
<td>Boesenbergia rotunda</td>
<td>7.10</td>
<td>17.50</td>
</tr>
<tr>
<td>Zingiber montanum</td>
<td>7.60</td>
<td>13.00</td>
</tr>
<tr>
<td>Zingiber zerumbet</td>
<td>2.00</td>
<td>2.80</td>
</tr>
<tr>
<td><strong>Chloroform</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Curcuma domestica</td>
<td>6.10</td>
<td>5.00</td>
</tr>
<tr>
<td>Curcuma xanthorrhiza</td>
<td>15.00</td>
<td>2.30</td>
</tr>
</tbody>
</table>

112
i) HCT-116

The cytotoxic activities of 5-Fu at various concentrations ranging from $0.1 \times 10^{-7}$ M to 1 M and selected Zingiberaceae extracts on HCT-116 cells were evaluated and the dose-response curves are shown in Figure 4.5 (a-b). The IC$_{50}$ values for 5-Fu plus extracts were extrapolated from the curves.

Results demonstrated that all selected Zingiberaceae extracts possessed the ability to increase the capability of 5-Fu in suppressing the proliferation of HCT-116 cells. The range of inhibition percentages by 5-Fu and selected crude petroleum ether and crude chloroform extracts of Zingiberaceae are summarized in Table 4.9.

Graphical analysis revealed additive effect of Zingiberaceae extracts on 5-Fu-induced growth inhibition of HCT-116 (Figure 4.5 (a-b)). It shows that even at the lowest concentration of 5-Fu used, the inhibition has been increased.

The combination of 5-Fu with petroleum ether extracts of *Alpinia galanga*, *Boesenbergia rotunda*, *Zingiber montanum* and *Zingiber zerumbet* and with the chloroform extracts of *Curcuma domestica* and *Curcuma xanthorriza* gave lower IC$_{50}$ values (Table 4.11) as compared to 5-Fu alone (Table 4.7).
Table 4.9: The range of inhibition percentages of HCT-116 cells by selected Zingiberaceae and 5-Fluorouracil (5-Fu) using the NR cytotoxicity assay

<table>
<thead>
<tr>
<th>Concentration of 5-Fu (M)</th>
<th>Range of inhibition (%) for 5-Fu alone</th>
<th>Range of inhibition (%) for 5-Fu + extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 x 10^{-7}</td>
<td>7.07</td>
<td>8 – 44</td>
</tr>
<tr>
<td>0.1 x 10^{-6}</td>
<td>10.55</td>
<td>15 – 49</td>
</tr>
<tr>
<td>0.1 x 10^{-5}</td>
<td>11.07</td>
<td>18 – 56</td>
</tr>
<tr>
<td>0.1 x 10^{-4}</td>
<td>14.82</td>
<td>20 – 57</td>
</tr>
<tr>
<td>0.1 x 10^{-3}</td>
<td>19.81</td>
<td>22 – 61</td>
</tr>
<tr>
<td>0.1 x 10^{-2}</td>
<td>47.31</td>
<td>28 – 67</td>
</tr>
<tr>
<td>0.1 x 10^{-1}</td>
<td>49.20</td>
<td>43 – 77</td>
</tr>
<tr>
<td>0.1</td>
<td>68.76</td>
<td>68 – 90</td>
</tr>
<tr>
<td>1.0</td>
<td>87.15</td>
<td>90 – 91</td>
</tr>
</tbody>
</table>
Figure 4.5 (a): The *in vitro* growth inhibition of HCT-116 cells by 5-Fu in combination with 5.5 μg/ml crude petroleum ether extracts of *A. galanga*, 7.1 μg/ml crude petroleum ether extracts of *B. rotunda*, 7.6 μg/ml crude petroleum ether extracts of *Z. montanum*, and 2.0 μg/ml crude petroleum ether extracts of *Z. zerumbet*. The IC₅₀ values were estimated from the graphical interpolation.
Figure 4.5 (b): The \textit{in vitro} growth inhibition of HCT-116 cells by 5-Fu in combination with 6.1 \( \mu \)g/ml crude chloroform extracts of \textit{C. domestica}, and 15.0 \( \mu \)g/ml crude chloroform extracts of \textit{C. xanthorrhiza}. The IC\textsubscript{50} values were estimated from the graphical interpolation.
ii) HT29

The cytotoxic effects of nine different concentrations of 5-Fu and crude petroleum ether extracts of *Alpinia galanga*, *Boesenbergia rotunda*, *Zingiber officinale* and *Zingiber zerumbet*; and chloroform extracts of *A. galanga*, *Curcuma aeruginosa*, *Curcuma domestica* and *Zingiber montanum* (Table 4.8) tested on HT29 cells were evaluated and the dose-response curves are presented in Figures 4.6 (a-b). The IC\(_{50}\) values are summarized in Table 4.11.

It was found that the petroleum ether and chloroform extracts of selected Zingiberaceae enhanced the cytotoxic capacity of 5-Fu against HT29. The cytotoxic effect of 5-Fu combined with all the petroleum ether and chloroform extracts of selected Zingiberaceae against HT29 cells increased with all increasing concentrations of 5-Fu (Table 4.10).

**Table 4.10: The range of inhibition percentages of HT29 by selected Zingiberaceae and 5-Fluorouracil (5-Fu) using the NR cytotoxicity assay**

<table>
<thead>
<tr>
<th>Concentration of 5-Fu (M)</th>
<th>Range of inhibition (%) for 5-Fu alone</th>
<th>5-Fu + extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 x 10^-7</td>
<td>7.07</td>
<td>6 – 47</td>
</tr>
<tr>
<td>0.1 x 10^-6</td>
<td>10.55</td>
<td>21 – 56</td>
</tr>
<tr>
<td>0.1 x 10^-5</td>
<td>11.07</td>
<td>25 – 61</td>
</tr>
<tr>
<td>0.1 x 10^-4</td>
<td>14.82</td>
<td>27 – 69</td>
</tr>
<tr>
<td>0.1 x 10^-3</td>
<td>19.81</td>
<td>29 – 70</td>
</tr>
<tr>
<td>0.1 x 10^-2</td>
<td>47.31</td>
<td>38 – 73</td>
</tr>
<tr>
<td>0.1 x 10^-1</td>
<td>49.20</td>
<td>41 – 75</td>
</tr>
<tr>
<td>0.1</td>
<td>68.76</td>
<td>77 – 90</td>
</tr>
<tr>
<td>1.0</td>
<td>87.15</td>
<td>88 – 93</td>
</tr>
</tbody>
</table>
Figure 4.6 (a): The *in vitro* growth inhibition of HT29 cells by 5-Fu in combination with 5.8 μg/ml crude petroleum ether extracts of *A. galanga*, 17.5 μg/ml crude petroleum ether extracts of *B. rotunda*, 13.0 μg/ml crude petroleum ether extracts of *Z. officinale*, and 2.8 μg/ml crude petroleum ether extracts of *Z. zerumbet*. The IC\textsubscript{50} values were estimated from the graphical interpolation.
Figure 4.6 (b): The *in vitro* growth inhibition of HT29 cells by 5-Fu in combination with 5.0 μg/ml crude chloroform extracts of *A. galanga*, 2.3 μg/ml crude chloroform extracts of *C. aeruginosa*, 7.7 μg/ml crude chloroform extracts of *C. domestica*, and 6.8 μg/ml crude chloroform extracts of *Z. montanum*. The IC\(_{50}\) values were estimated from the graphical interpolation.
Table 4.11: IC$_{50}$ values of 5-Fu combined with selected Zingiberaceae extracts against HCT-116 and HT29 cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>HCT-116</th>
<th>HT29</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zingiberaceae species</td>
<td>IC$_{50}$ value (M)</td>
</tr>
<tr>
<td>Petroleum ether</td>
<td><em>Alpinia galanga</em></td>
<td>2.5 x 10^{-9}</td>
</tr>
<tr>
<td></td>
<td><em>Boesenbergia rotunda</em></td>
<td>0.3 x 10^{-3}</td>
</tr>
<tr>
<td></td>
<td><em>Zingiber montanum</em></td>
<td>2.8 x 10^{-3}</td>
</tr>
<tr>
<td></td>
<td><em>Zingiber zerumbet</em></td>
<td>3.2 x 10^{-2}</td>
</tr>
<tr>
<td>Chloroform</td>
<td><em>Curcuma domestica</em></td>
<td>7.2 x 10^{-4}</td>
</tr>
<tr>
<td></td>
<td><em>Curcuma xanthorrhiza</em></td>
<td>1.4 x 10^{-7}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-Fu alone</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>
4.4 Wild type-p53 Promoting Activity of Zingiberaceae

In the present study, a total of 30 crude petroleum ether, chloroform and methanol extracts of selected Zingiberaceae species were analyzed qualitatively for wild type p53 (wt-p53) promoting activity in colon cancer cell lines, HCT-116 and HT29. The cells were treated with Zingiberaceae extracts at varying concentrations of 1, 10, 25, 50 and 100 μg/ml.

The cells were then evaluated using an immunocytochemistry technique - 3 step indirect avidin-biotin immunoperoxidase with the primary antibody, DO-7 mouse anti-human p53 protein. The expression of wt-p53 protein in treated and untreated HCT-116 and HT29 cell lines were evaluated. The presence of wt-p53 protein was recorded with the appearance of reddish-brown stain either in the nuclear and/or cytoplasmic regions of HCT-116 and HT29 cells. The staining intensity was classified as: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+) as illustrated in Figure 4.7. The higher the intensity of the stain, the higher the amount of wt-p53 protein in the cells, indicating better wt-p53-promoting ability by the plant extracts.
Two types of negative controls used in this study were i) HCT-116 and HT29 cells incubated without the extract but with monoclonal mouse anti-human p53 protein antibody during immunocytochemistry, and; ii) HCT-116 and HT29 cells incubated without the extract and without anti-human p53 protein antibody during immunocytochemistry. The staining results for untreated HCT-116 and HT29 cells, with and without anti-human p53 protein antibody are shown in Figures 4.8 and 4.9. All untreated HCT-116 and HT29 cells appeared to be morphologically intact. The negative controls incubated with anti-human p53 protein antibody exerted very weak reddish-
brown stain (+) due to the low expression of wt-p53 while no stain (-) was observed for untreated HCT-116 and HT29 cells without incubation with anti-human p53 protein.

Figure 4.8: (a) HCT-116 cells not treated with Zingiberaceae extracts without incubation with anti-human p53 protein antibody. No staining (0-) was observed. (b) HCT-116 cells not treated with Zingiberaceae extracts but incubated with anti-human p53 protein antibody. Cells showed very weak staining (1+) in the cytoplasmic regions. (400x)

Figure 4.9: (a) HT29 cells not treated with Zingiberaceae extracts without incubation with anti-human p53 protein antibody. No staining (0-) was observed. (b) HT29 cells not treated with Zingiberaceae extracts but incubated with anti-human p53 protein antibody. Cells showed very weak staining (1+) in the cytoplasmic regions. (400x)

The resulting staining intensities (reflecting the expression of wt-p53) in the HCT-116 and HT29 cells treated with different Zingiberaceae extracts at various concentrations were compared with each other and with the negative controls. The morphology and the expression of wt-p53 in treated HCT-116 cells was analysed and summarised in Figures 4.10-4.19. The morphology and the expression of wt-p53 in treated HT29 cells was analysed and summarised in Figures 4.20-4.29.
4.4.1 Wt-p53 promoting activities of Zingiberaceae extracts in HCT-116 cell line

i) *Alpinia galanga*

The staining results illustrated in Figure 4.10 shows an increment pattern of the reddish-brown stain in HCT-116 cells treated with increasing concentrations of petroleum ether and chloroform extracts of *A. galanga*. This indicates that the expression of wt-p53 in HCT-116 increased with increasing concentrations of *A. galanga*. However, for HCT-116 cells treated with methanol extract of *A. galanga*, the expression of wt-p53 was high after treatment with 1 μg/ml of the extract but decreased at 50 μg/ml and became weaker at 100 μg/ml. The HCT-116 cells treated with petroleum ether and chloroform extracts of *A. galanga* start to lyse at the concentration of 25 μg/ml while those treated with methanol extract started to lyse at 1 μg/ml. This result shows that methanol extract of *A. galanga* has better promoting activity in wt-p53 at lower concentration but the activity decreased with the increasing concentrations of the extracts.
Figure 4.10: Wt-p53 expression shown as staining intensities of HCT-116 cells treated with *Alpinia galanga*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

ii) *Boesenbergia rotunda*

The results illustrated in Figure 4.11 shows that the *Boesenbergia rotunda* extracts increased the expression of wt-p53 in HCT-116 cell lines. The increment pattern of the reddish-brown stain can only be seen in HCT-116 cells treated with increasing concentrations of petroleum ether extracts of *B. rotunda*. At concentration of 1 μg/ml, the chloroform and methanol extracts showed better expression of wt-p53 while the staining intensities were better in cells treated with higher concentrations i.e. 25 μg/ml to 100 μg/ml of petroleum ether extracts.

This shows that the petroleum ether extract of *B. rotunda* enhanced the expression of wt-p53 with increasing concentrations. On the other hand, the chloroform and methanol induced strong staining at 1 μg/ml but became weaker at higher concentrations evaluated here. Overall results indicated that the petroleum ether extract of *B. rotunda* was more effective in boosting the expression wt-p53 at higher concentrations while the chloroform and methanol extracts were good at low concentration i.e. 1 μg/ml.
iii) *Curcuma aeruginosa*

The crude petroleum ether, chloroform and methanol extracts of *Curcuma aeruginosa* increased the expression of wt-p53 in the HCT-116 cell lines. As denoted by the induction of reddish-brown stain in HCT-116 cells, the amount of wt-p53 in HCT-116 cells treated with petroleum ether extract of *C. aeruginosa* very strong at 1 μg/ml and 10 μg/ml of petroleum ether extract and become less with the increasing concentrations of the extracts (Figure 4.12). Meanwhile, both chloroform and methanol extracts of *C. aeruginosa* induced strong staining at all concentrations.

Overall results indicated that all of the *C. aeruginosa* extracts were excellent in enhancing the activities of wt-p53. The cells started to lyse at 50 μg/ml in cells treated with petroleum ether extract and at 10 μg/ml in cells treated with both chloroform and methanol extracts of *C. aeruginosa*. 

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**Figure 4.11:** Wt-p53 expression shown as staining intensities of HCT-116 cells treated with *Boesenbergia rotunda*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+).
iv) \textit{Curcuma domestica}

As compared to cells not treated with the extracts, cells treated with \textit{Curcuma domestica} exhibited increased wt-p53 expression. As seen in Figure 4.13, the intensities of reddish-brown stain indicating expression of wt-p53 were moderate to strong even at the lowest concentrations. In cells treated with petroleum ether extract of \textit{C. domestica}, the expression of wt-p53 in HCT-116 cells was strong at 1 \(\mu\)g/ml and 10 \(\mu\)g/ml but became moderate at concentration 25 \(\mu\)g/ml of the extract.

Meanwhile, for chloroform extracts, the moderate expression of wt-p53 was seen in cells treated with concentrations of 1 \(\mu\)g/ml to 50 \(\mu\)g/ml and further increased at 100 \(\mu\)g/ml. Cells treated with methanol extract of \textit{C. domestica} induced moderate wt-p53

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**Figure 4.12: Wt-p53 expression shown as staining intensities of HCT-116 cells treated with \textit{Curcuma aeruginosa}. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)**
expression at all concentrations tested. The reddish-brown stain was clearly seen in the cytoplasmic regions of treated HCT-116 cells.

![Graph showing staining intensity of HCT-116 cells treated with different extract concentrations of Curcuma domestica.](image)

Figure 4.13: Wt-p53 expression shown as staining intensities of HCT-116 cells treated with *Curcuma domestica*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

v) *Curcuma mangga*

The crude petroleum ether, chloroform and methanol extracts of *Curcuma mangga* increased the expression of wt-p53 in the HCT-116 cell lines. As denoted by the induction of reddish-brown stain in HCT-116 cells, the expression of wt-p53 in HCT-116 cells treated with petroleum ether extract of *C. mangga* was strong at 1 µg/ml and 25 µg/ml and very strong at 50 µg/ml and 100 µg/ml (Figure 4.14). The intensity of reddish-brown stains in HCT-116 cells treated with chloroform extracts derived from...
C. mangga was strong at 1 μg/ml and 10 μg/ml and became very strong at 25, 50 and 100 μg/ml.

The methanol extract induce moderate expression of wt-p53 at 1 μg/ml and 10 μg/ml. However, at 25 μg/ml and 50 μg/ml of methanol extract, the staining intensity became strong indicating higher expression of wt-p53. The staining intensity decreased to moderate again in cells treated with 100 μg/ml of methanol extracts. This indicates that petroleum ether and chloroform extracts of C. mangga were successful in inducing the expression of wt-p53 in a dose-dependent manner where higher extract concentration induced greater expression of wt-p53.

![Staining Intensity Graph](image)

**Figure 4.14:** Wt-p53 expression shown as staining intensities of HCT-116 cells treated with *Curcuma mangga*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

vi) *Curcuma xanthorriza*
The resulting staining intensity of HCT-116 cells treated with *Curcuma xanthorriza* extracts were shown in Figure 4.15. The unsteady pattern was observed in HCT-116 cells treated with increasing concentrations of petroleum ether, chloroform and methanol extracts of this plant. As denoted by the induction of reddish-brown stain in HCT-116 cells, the expression of wt-p53 in HCT-116 cells treated with petroleum ether and chloroform extracts of *C. xanthorriza* were very strong even at 1 μg/ml. Then, the staining decreased slightly in cells treated with 25 μg/ml of petroleum ether extract but increased again at 50 μg/ml only and decreased slightly at 100 μg/ml.

The staining intensity appeared to be moderate in cells treated with 10 μg/ml and 25 μg/ml for chloroform and became very strong again at concentrations of 50 and 100 μg/ml. Meanwhile, in HCT-116 cells treated with methanol extracts of *C. xanthorriza*, the staining intensities were strong at concentrations of 1 and 50 μg/ml, weak at 10 and 25 μg/ml and became very strong at 100 μg/ml.
Figure 4.15: Wt-p53 expression shown as staining intensities of HCT-116 cells treated with *Curcuma xanthorriza*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

vii) *Kaempferia galanga*

Based on the staining results shown in Figure 4.16, all of the *Kaempferia galanga* extracts were found to moderately increase the expression of wt-p53 where the reddish brown stain were clearly seen in the cytoplasmic region of treated HCT-116 cells. Overall results showed moderate expression of wt-p53 by *K. galanga* extracts even at the lowest concentration of extracts used.

The crude petroleum ether, chloroform and methanol extracts of *K. galanga* increased the expression of wt-p53 in the HCT-116 cell lines. As indicated by the induction of reddish-brown stain in HCT-116 cells, the amount of wt-p53 in HCT-116 cells treated with petroleum ether and chloroform extracts of *K. galanga* were moderate at 1 μg/ml until 50 μg/ml and become weaker with the highest concentration of the extracts. The intensity of reddish-brown stains in HCT-116 cells treated with 1 mg/ml of methanol extracts derived from *K. galanga* was weak but increased moderate intensity at 10, 25, and 50 μg/ml. However the staining intensity became weaker at 100 μg/ml.

This indicates that petroleum ether, chloroform and methanol extracts of *K. galanga* were successful in induce the expression of wt-p53 in concentrations ranging from 1 to 50 μg/ml but became weaker at 100 μg/ml.
Figure 4.16: Wt-p53 expression shown as staining intensities of HCT-116 cells treated with *Kaempferia galanga*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

viii) *Zingiber montanum*

The resulting staining intensity denoting wt-p53 expression in HCT-116 cells treated with *Zingiber montanum* extracts are shown in Figure 4.17. The increase in staining intensity was observed in HCT-116 cells treated with increasing concentrations of methanol extract. In cells treated with 1 μg/ml and 10 μg/ml of the methanol extracts of *Z. montanum* moderate stains were observed which became very strong at higher concentrations. The petroleum ether extract induced weak expression of wt-p53 at 1 μg/ml and 100 μg/ml and moderate expression of wt-p53 at concentrations 10 μg/ml, 25 μg/ml and 50 μg/ml. The chloroform extract induced strong expression of wt-p53 at 10 μg/ml, 50 μg/ml and 100 μg/ml.
The crude petroleum ether extracts of *Z. montanum* increased the expression of wt-p53 in the HCT-116 cell lines at concentrations of 1 until 50 μg/ml but became weaker at 100 μg/ml. However, the chloroform extracts of *Z. montanum* performed unsteady intensity of the staining where it became moderate at 1 μg/ml, strong at 10 μg/ml, moderate again at 25 μg/ml and finally became strong at 50 and 100 μg/ml.

![Graph showing staining intensity of HCT-116 cells treated with Zingiber montanum extracts.](image)

**Figure 4.17:** Wt-p53 expression shown as staining intensities of HCT-116 cells treated with *Zingiber montanum*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+).

**ix) Zingiber officinale**

The resulting staining intensity of the HCT-116 cells treated with *Zingiber officinale* extracts were shown in Figure 4.18.
All HCT-116 cells treated with *Z. officinale* extracts demonstrated strong staining intensity of reddish-brown at the very beginning of the concentrations - 1 μg/ml. For cells treated with petroleum ether extract, very strong staining intensity was observed at the concentrations 25 μg/ml, 50 μg/ml and 100 μg/ml. In cells treated with chloroform extracts, the staining intensity became moderate at 10 μg/ml and 25 μg/ml but became strong again at 50 μg/ml and very strong at 100 μg/ml. The methanol extract of *Z. officinale* induced strong staining intensity at concentrations 1, 10, 25 and 50 μg/ml and very strong staining at 100 μg/ml of the extract.

![Graph](image)

**Figure 4.18**: Wt-p53 expression shown as staining intensities of HCT-116 cells treated with *Zingiber officinale*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

x) *Zingiber zerumbet*
As revealed in Figure 4.19, the *Zingiber zerumbet* extracts induced increased wt-p53 expression in HCT-116 cells even at very low concentration of 1 μg/ml. The reddish-brown stain can be clearly seen in the nucleus and cytoplasmic region in HCT-116 cells treated with petroleum ether and chloroform extracts of *Z. zerumbet*.

The resulting staining intensity of the HCT-116 cells treated with *Z. zerumbet* extracts are shown in Figure 4.19. The increase in staining intensity was observed in HCT-116 cells treated with increasing concentrations of chloroform extract. In cells treated with 1 μg/ml and 100 μg/ml of the petroleum ether extracts of *Z. zerumbet*, very strong stains were observed which became weaker at concentrations of 10, 25 and 50 μg/ml. The methanol extract induced moderate expression of wt-p53 at 1 μg/ml and 10 μg/ml but became weak at 25 μg/ml. However, the staining intensity increased again and became strong at 50 μg/ml and very strong at 100 μg/ml.

![Figure 4.19: Wt-p53 expression shown as staining intensities of HCT-116 cells treated with Zingiber zerumbet. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)](image-url)
4.4.2 Wt-p53 promoting activities of Zingiberaceae extracts in HT29 cell line

i) *Alpinia galanga*

The resulting staining intensity of the HT29 cells treated with *Alpinia galanga* extracts are shown in Figure 4.20. The increase in staining intensity was observed in HT29 cells treated with petroleum ether, chloroform and methanol extracts. This indicates that the *A. galanga* extracts increase the expression of wt-p53.

In cells treated with petroleum ether extract of *A. galanga*, the expression of wt-p53 were weak at 1 μg/ml, became moderate at 10 μg/ml and very strong at 25, 50 and 100 μg/ml. The higher the amount of wt-p53 protein in cells indicated by the higher intensity of the stain, showed better wt-p53 promoting ability by the plant extracts.

The increase in staining intensity was observed in HT29 cells treated with increasing concentrations of chloroform extract. In cells treated with 1 μg/ml until 25 μg/ml of the chloroform extracts of *A. galanga* moderate stains were observed which became strong and very strong at concentrations of 50 μg/ml and 100 μg/ml. Meanwhile, the methanol extracts showed moderate expression of wt-p53 at all concentrations tested.
Figure 4.20: Wt-p53 expression shown as staining intensities of HT29 cells treated with *Alpinia galanga*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

ii) *Boesenbergia rotunda*

Petroleum ether, chloroform and methanol extracts of *Boesenbergia rotunda* were tested on HT29 cells to evaluate the wt-p53-promoting ability. Stains of moderate intensity was observed in cells treated with as low as 1 μg/ml of extracts (Figure 4.21). This shows that enhancement of the expression of wt-p53 by extracts of *B. rotunda*.

The unsteady expression of wt-p53 can be seen in cells treated with chloroform extract of *B. rotunda*. In cells treated with methanol extract of *B. rotunda*, the staining intensity remained moderate at all concentrations tested here.

The increase in staining intensity was observed in HT29 cells treated with increasing concentrations of petroleum ether extract. In cells treated with 1 μg/ml until 25 μg/ml of the petroleum ether extracts of *B. rotunda*, stains of moderate intensity were observed which became very strong at 50 and 100 μg/ml. The chloroform extract
showed strong expression of wt-p53 at 1 μg/ml but decreased into moderate at 10 and 25 μg/ml. However, the expression became strong again at 50 μg/ml and very strong at 100 μg/ml.

In general, HT29 cells treated with *B. rotunda* extracts remained morphologically intact except for those treated with petroleum ether extract at 50 μg/ml and 100 μg/ml; and chloroform extract at 100 μg/ml.

**Figure 4.21**: Wt-p53 expression shown as staining intensities of HT29 cells treated with *Boesenbergia rotunda*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+).

iii) **Curcuma aeruginosa**

As compared to cells not treated with the extracts, cells treated with *Curcuma aeruginosa* extracts exhibited increased wt-p53 expression even at 1 μg/ml of the extracts (Figure 4.22). In cells treated with petroleum ether, chloroform and methanol extracts of
*C. aeruginosa*, the moderate expression of wt-p53 in HT29 cells remain unchanged at all concentrations tested.

![Bar chart showing wt-p53 expression intensities of HT29 cells treated with *Curcuma aeruginosa*](chart)

**Figure 4.22**: Wt-p53 expression shown as staining intensities of HT29 cells treated with *Curcuma aeruginosa*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

iv) *Curcuma domestica*

Very strong staining intensity indicating strong expression of wt-p53 was observed in HT29 cells treated with 1 μg/ml petroleum ether extract of *Curcuma domestica* (Figure 4.23). The wt-p53 expression however decreased to moderate at 10, 25, 50 and 100 μg/ml. Meanwhile chloroform and methanol extracts of this plant induced moderate staining indicating moderate wt-p53 expression at 1 μg/ml and 10 μg/ml in HT29. An unsteady pattern was seen in cells treated by chloroform extract whereby strong cells staining intensities was seen at 25 μg/ml and again at 100 μg/ml but moderate at 50 μg/ml. The staining intensity appeared to be moderate in cells treated
with 1, 10 and 25 μg/ml of methanol extracts of *C. domestica* and became strong at 50 μg/ml and finally very strong at 100 μg/ml.

![Figure 4.23: Wt-p53 expression shown as staining intensities of HT29 cells treated with *Curcuma domestica*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)](image)

v) *Curcuma mangga*

The crude petroleum ether, chloroform and methanol extracts of *Curcuma mangga* increased the expression of wt-p53 in the HT29 cell lines. Figure 4.24, shows moderate staining intensities indicating moderate expression of wt-p53 in cells treated with as low as 1 μg/ml of the *C. mangga* extracts. The moderate expression of wt-p53 remained with all concentrations of petroleum ether and chloroform extracts of *C. mangga* evaluated here.
As compared to the other two, the methanol extract of *C. mangga* was the most active in inducing the expression of wt-p53. The methanol extract induced strong expression of wt-p53 in HT29 cells at 50 μg/ml and 100 μg/ml.

![Figure 4.24: Wt-p53 expression shown as staining intensities of HT29 cells treated with *Curcuma mangga*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)](image)

vi) *Curcuma xanthorriza*

The crude petroleum ether, chloroform and methanol extracts of *Curcuma xanthorriza* increased the expression of wt-p53 in the HT29 cell lines. As denoted by the induction of reddish-brown stain in HT29 cells, the expression of wt-p53 in HT29 cells treated with petroleum ether extract of *C. xanthorriza* was moderate at 1 μg/ml and 25 μg/ml and become very strong at concentrations of 50 and 100 mg/ml (Figure 4.25). The intensity of reddish-brown stains in HT29 cells treated with chloroform extracts derived from *C. xanthorriza* increased from moderate at 1, 10 and 25 μg/ml to strong at 50 μg/ml.
and very strong at 100 μg/ml. On the other hand, the staining intensity of methanol extract in HT29 remained moderate at all concentrations used.

The treated HT29 cells remain morphologically intact except for those treated with the petroleum ether and chloroform extracts at concentrations of 50 μg/ml and 100 μg/ml, respectively where the cells lysed.

Figure 4.25: Wt-p53 expression shown as staining intensities of HT29 cells treated with *Curcuma xanthorriza*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

vii) *Kaempferia galanga*

As illustrated in Figure 4.26, all *Kaempferia galanga* extracts increased the expression of wt-p53 in HT29 cells. All of the extracts induced moderate staining intensities indicating moderate expression of wt-p53 in HT29 cells at 1, 10 and 25 μg/ml. In cells treated with chloroform and methanol extracts, the intensities of the reddish-brown stain remained moderately expressed at higher concentrations of 50 μg/ml and 100
μg/ml. This trend was not seen in cells treated with petroleum ether extracts of *K. galanga* where the expression of wt-p53 became weak at 50 μg/ml and strong again at 100 μg/ml.

![Staining Intensity Graph](image)

**Figure 4.26:** Wt-p53 expression shown as staining intensities of HT29 cells treated with *Kaempferia galanga*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

viii) *Zingiber montanum*

As shown in Figure 4.27, petroleum ether and chloroform extracts of *Zingiber montanum* were found to stimulate increased expression wt-p53 in HT29 cells. The reddish-brown stains were clearly seen in the nucleus and cytoplasmic regions of treated HT29 cells.

The crude petroleum ether extract of *Z. montanum* increased the expression of wt-p53 in the HT29 cell lines. It shows moderate staining intensities indicating moderate expression of wt-p53 in cells treated with as low as 1 μg/ml and 10 μg/ml of the *Z.*
*Z. montanum* extracts. The expression of wt-p53 became strong at concentrations of 25 μg/ml and 50 μg/ml but decreased to moderate level at 100 μg/ml.

The chloroform extracts maintained the expression of wt-p53 at moderate staining intensity at 1 μg/ml, 10 μg/ml and 25 μg/ml. However, the expression of wt-p53 became very strong at 50 μg/ml but decreased again to moderate level at 100 μg/ml. As compared to the other two, the methanol extract of *Z. montanum* at concentration of 1 μg/ml was the least active in inducing the expression of wt-p53. The methanol extracts induced weak expression of wt-p53 at 1 μg/ml and moderate expression of wt-p53 in HT29 cells at 10 μg/ml, 25 μg/ml, 50 μg/ml and 100 μg/ml.

![ bar graph showing staining intensity of HT29 cells treated with *Z. montanum* extracts](image)

**Figure 4.27**: Wt-p53 expression shown as staining intensities of HT29 cells treated with *Zingiber montanum*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

ix) *Zingiber officinale*

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The petroleum ether, chloroform and methanol extracts of *Zingiber officinale* were tested on HT29 cells to analyze the wt-p53-promoting ability qualitatively. The staining intensities in HT29 cells treated with various concentrations of the extracts are shown in Figure 4.28. In general, the extracts of *Z. officinale* increased the expression of wt-p53 in the HT29 cell line. Except for petroleum ether, the chloroform and methanol extracts of *Z. officinale* induced moderate expression of wt-p53 at the concentration as low as 1 μg/ml. The expression remains unchanged at 10 μg/ml, 25 μg/ml, 50 μg/ml and 100 μg/ml.

In cells treated with 1 μg/ml of petroleum ether extract, strong staining intensity indicating strong expression of wt-p53 was seen in the nucleus and cytoplasmic regions of HT29 cells. However, the intensities of the staining become moderate at the higher concentrations of the extract.

![Figure 4.28: Wt-p53 expression shown as staining intensities of HT29 cells treated with Zingiber officinale. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)](image-url)
x) **Zingiber zerumbet**

Figure 4.29 shows the staining intensities in HT29 cells treated with various concentrations of petroleum ether, chloroform and methanol extracts of *Zingiber zerumbet*. All of the extracts induced moderate staining intensity, indicating moderate expression of wt-p53 at the lowest concentration of 1 μg/ml. The staining intensities became strong for HT29 cells treated with petroleum ether extracts at concentrations of 25, 50 and 100 μg/ml. Meanwhile, in chloroform extracts, the staining intensities remained moderate at concentrations of 1, 10, 25 and 50 μg/ml and became strong at concentration of 100 μg/ml.

For cells treated with methanol extract, the pattern of staining intensities remains unchanged from 1 μg/ml to 100 μg/ml indicating consistent moderate expression of wt-p53, at all concentrations of *Z. zerumbet* extracts tested.

![Graph showing staining intensities of HT29 cells treated with Zingiber zerumbet extracts](image_url)

**Figure 4.29:** Wt-p53 expression shown as staining intensities of HT29 cells treated with *Zingiber zerumbet*. Classification for the staining intensity: no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)
CHAPTER 5

DISCUSSION

In the current study, crude petroleum ether, crude chloroform and crude methanol extracts from ten selected Zingiberaceae species were prepared. The extracts were then analysed for their potential cytotoxic and wt-p53 promoting activities in human colorectal cancer-derived cell lines, HCT-116 and HT29.

5.3 Preparation of crude extract

Ten Zingiberaceae rhizomes were extracted using petroleum ether, chloroform and methanol. Each of the solvents used has different polarity ranging from low (petroleum ether), medium (chloroform) to high (methanol). The extraction procedure generated three crude extracts from each Zingiberaceae rhizome.

Drying which is the commonest method of preservation, remove presence of water in the rhizome specimen. This was achieved by leaving the material in warm and dry air.

Infusions were carried out by soaking the rhizome powder in the solvent for 24 hours with concomitant shaking, followed by filtration to separate away the plant debris. Filtration removes solid particles from a liquid in which they are suspended. The size of the pores determined the size of particles that are retained. The pore size of the filter that used in this study was 0.2 μm. The commonly used filters in cell culture have pore size of 0.2 μm because the contaminants such as bacteria and fungi are usually larger than this (Martin, 1994).
The filtration materials used was paper but the disadvantage was the cellulose fibres that paper contain, swell in the presence of water and this will decrease in pore size and cause reduction in flow rate. The amount of extract will be lost due to the aqueous absorption by the fibres but it can be overcome by thoroughly washing the paper with the solvent used (Houghton and Raman, 1998). To overcome this problem, a blunt spatula has been used by careful disturbance with the flat end and by stirring it continuously.

After filtration, the extracts were placed in a round-bottomed flask and a rotary evaporator was used to remove most of the solvent. Using this method the boiling point of the liquid is reduced and decomposition is minimized to increase the rate of evaporation. The solvent collected in a vessel can be recycled if it was a single solvent.

In spite of the advantages, the rotary evaporator method also has limitations. The heat applied may cause a loss of volatile compound from the extract. Contamination might happen if the apparatus was not thoroughly cleanse from trace residues of other extracts for which it has been used. Some of the liquid might be lost from the flask due to the sudden boiling. This can be prevented by adding together some anti-bumping granules to the solution (Houghton and Raman, 1998).

Overall, the percentage of crude methanol extracts of Zingiberaceae was found to be higher than their petroleum ether and chloroform counterparts. The least polar solvent petroleum ether is used to extract phytochemical compounds such as waxes, fats, fixed and volatile oils from samples. Chloroform is normally used as one of the medium polarity solvent to extract compounds such as alkaloids, aglycones and volatile oils while the high polarity methanol is approved to remove chemicals such as sugars, amino acids and glycosides from the samples (Houghton and Raman, 1998).
The crude extracts obtained have been dissolved in DMSO due to its poor solubility in aqueous solution. The final concentration of DMSO used was below 1% v/v to ensure the growth of cultured cells is not affected in the cytotoxicity assay (Riddell et al., 1986).

5.2 In vitro cytotoxic activity of Zingiberaceae crude extracts

There are many viability dyes used to determine membrane integrity, such as tryphan blue, eosin Y, naphthalene black, nigrosin, eryhtrosin B and fast green. However, staining for viability assessment is more suited to suspension cultures than to monolayers, because dead cells detach from the monolayer and are therefore lost from the assay (Wilson, 2000).

In the present study, thirty Zingiberaceae crude extracts were evaluated for their cytotoxic activity – with or without 5-Fu, against human colon cancer-derived cell lines, HCT-116 and HT29 using the neutral red (NR) cytotoxicity assay. This assay is based on the uptake and subsequent lysosomal accumulation of the supravital dye, NR by viable cells. It quantifies the number of viable, uninjured cells after their exposure to toxicants. Thus it is possible to distinguish between the viable, damaged or dead cells (Babich and Borenfreund, 1987).

NR has been used previously for the identification of vital cells in cultures (DeRenzis and Schechtman, 1973). Quantification of the dye extracted from the cells has been revealed to be linear with cell numbers, both by direct cell counts and by protein determinations of cell populations (Borenfreund and Puerner, 1985 and 1986). The
NR assay when use in combination with other test, is a useful tool to distinguish between cytotoxicity and organelle damage (Fotakis et al., 2005).

One advantage of assays dealing with cell cultures is that all potential mechanisms concerning cellular proliferation are simultaneously monitored. It is recognized that cytotoxicity is neither necessary nor sufficient for anti-tumour activity. Therefore, it should be of no surprise that isolates derived from cytotoxicity-based assay procedures are often toxic toward mammalian species and do not demonstrate therapeutic efficacy. However, cytotoxicity is an activity that is consistent with anti-tumour activity, and virtually every known naturally occurring anti-tumour agent demonstrates a positive response in a cell culture system (Cordell et al., 1993). Although these test procedures have proven to be effective for the isolation of cytotoxic compounds that may be of novel structure, it is often the case that compounds identified as active with certain cells are not active with in vivo tumour models. In many instances the isolates are simply toxic (Cordell et al., 1993).

The cytotoxicity results obtained were expressed as IC\textsubscript{50} (equals to ED\textsubscript{50}). IC\textsubscript{50} is the concentration required to inhibit cell growth by 50%. The IC\textsubscript{50} values were established using five different concentrations of each extract ranging from 1 \(\mu\text{g/ml}\) to 100 \(\mu\text{g/ml}\). All of them were then tested against two colon cancer cell lines, HCT-116 and HT29. As established by National Cancer Institute (US), the IC\textsubscript{50} values to consider an extract as active is less or equal to 20 \(\mu\text{g/ml}\) for extracts and less or equal to 4 \(\mu\text{g/ml}\) for pure compound (WHO, 1972; Geran et al., 1972; Cordell et al., 1993; Chiang et al., 2003).
5.2.1 *Alpinia galanga*

*Alpinia galanga* is locally known as *lengkuas*. In South East Asian countries, it has been extensively used as one of the main ingredients in preparation for cooking. In traditional medicines, *A. galanga* is important in the treatment of post-partum protective medicine, stomach-ache, dysentery, diarrhoea and dealing with sinus problem (Halijah and Ahmad, 1988).

*A. galanga* demonstrated outstanding cytotoxic effect against both human colon cancer cell lines in the current study. The crude petroleum ether and crude chloroform extracts of *A. galanga* were found active in inhibiting the growth of HCT-116 and HT29 cells, with IC$_{50}$ values less than 20 $\mu$g/ml. On the other hand, the crude methanol extract of rhizome was found weakly effective in suppressing the growth of both cell lines. From the present studies, it is obvious that almost all the active compounds in *A. galanga* which exhibit cytotoxic affect, were mostly extracted into the non-polar petroleum ether and followed by the medium polarity chloroform solvent.

This finding is supported by the research done by Leow (2006), which found that only crude petroleum ether and crude chloroform extracts of *A. galanga* actively inhibited the growth of HeLa, CaSki and HT29 cell lines. Crude methanol extract was found to be only effective in suppressing the proliferation of HeLa cells. Murakami *et al.* (2000) revealed that 1-acetoxychavicol acetate (ACA) in *A. galanga* is the active constituent of strongly active plants in the tumour promoter-induced-EBV activation test. It exhibited significant inhibitory activity toward EBV activation. The IC$_{50}$ value of ACA is ten times lower compared to $\beta$-carotene. As has been proved in this study, the petroleum ether and chloroform extracts of *A. galanga* had a tremendous effect in
cytotoxicity assay by inhibiting the proliferation of HCT-116 and HT29 cell lines. These two extracts also showed a good promoting wt-p53 activity by increasing the stains intensities with increasing concentrations of the extracts.

5.2.2 Boesenbergia rotunda

Boesenbergia rotunda or locally known as temu kunci is used as a treatment for rheumatism and post-partum protective medicine for women after childbirth in South East Asia (Halijah and Ahmad, 1988). The present study found crude petroleum ether and crude chloroform extracts of B. rotunda to be cytotoxic against HCT-116 and HT29 cells. Similar to other Zingiberaceae extracts, the crude methanol extract of B. rotunda was not active against HT29 cell line at all concentrations tested.

Based on these findings, it can be concluded that the active phytochemical(s) in the B. rotunda which exhibit cytotoxic effect were mostly extracted by the non-polar petroleum ether and followed by the medium polarity solvent i.e. chloroform. The present findings are consistent with previous study by Leow (2006), which reported that crude petroleum ether extract of B. rotunda possessed strong inhibition activity against three cancer cell lines, HeLa, CaSki and HT29. It was most active, followed by crude chloroform extract. The high inhibition activities observed from this study correlate well with previous findings on B. rotunda crude extracts, also by Leow (2006) and suggest that this plant may be an effective chemopreventive agent for the treatment of colon cancer.
5.2.3 *Curcuma aeruginosa*

*Curcuma aeruginosa* is locally recognized as *temu hitam*. Although rarely available at the market, it is commonly used as a treatment for asthma (Halijah and Ahmad, 1988). In this study, it was found that only crude chloroform extract of *C. aeruginosa* was active in inhibiting the proliferation of HT29 cell line with an IC$_{50}$ value of 2.30 $\mu$g/ml. The crude petroleum ether and crude methanol extracts of *C. aeruginosa* on the other hand were inactive against HCT-116 and HT29 cell lines. In a different study *C. aeruginosa* extracts has been reported to be inactive for Epstein-Barr virus activation induced by TPA in Raji cell lines (Vimala et al., 1999).

5.2.4 *Curcuma domestica*

*Curcuma domestica* which is commonly known as turmeric or locally known as *kunyit*, has been used as a spice and one of the ingredients in the preparation of traditional medicines worldwide. In the current study, crude extracts of *C. domestica* demonstrated good inhibition activity on HCT-116 cells. The crude chloroform extract of *C. domestica* showed excellent inhibition activity on both human colon cancer cell lines, HCT-116 and HT29 where the IC$_{50}$ values were below than 20 $\mu$g/ml. The crude petroleum ether and crude methanol extracts of *C. domestica* were found to actively inhibit the proliferation of the HCT-116 cells but not HT29 cells. The results showed that the active cytotoxic compounds present in the rhizome of *C. domestica* are mainly extracted from the high polarity solvent, chloroform. This findings support the finding of previous study. Leow (2006) reported that the crude
chloroform extract of *C. domestica* actively inhibited the growth of human cervical cancer cells, HeLa and CaSki human colon cancer cell, HT29.

The well known bioactive compound derived from the rhizome of *C. domestica* was curcumin. The anticancer properties of curcumin have been reported in *in vitro* studies and in *in vivo* studies done by researchers all over the world. Chauhan (1997) reported that curcumin inhibits lipooxygenase activity and is a specific inhibitor of cyclooxygenase-2 expression. It inhibits the initiation of carcinogenesis by inhibiting the cytochrome P-450 enzyme activity and increasing the levels of glutathione-S-transferase. Curcumin inhibits the growth of DNA mismatch repair defective colon cancer cells thus it may have value as a safe chemotherapeutic agent for the treatment of tumours exhibiting DNA mismatch repair deficient and microsatellite instable phenotype.

Rao *et al.* (1995) indicated that dietary administration of curcumin significantly inhibited incidence of colon adenocarcinomas and the multiplicity of invasive, noninvasive and total adenocarcinomas in rats. Studies done by Goel *et al.* (2001) also showed that curcumin inhibited the growth of HT29 cells in a concentration and time-dependent manner and it inhibited the mRNA and protein expression of COX-2.

Curcumin has also been found to inhibit the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced nuclear factor κB (NF-κB) activation by preventing the degradation of the inhibitory protein IκBα and the subsequent translocation of the p65 subunit in cultured human promyelocytic leukemia (HL-60) cells (Han *et al.*, 2002). *In vitro* study by Brouet *et al.* (1995) demonstrated that low concentrations of curcumin incubated with activated macrophages resulted in decrease in mRNA levels and nitric
oxide synthase activity which is an element in inflammation and possibly in the process of carcinogenesis.

Studies done in animal demonstrated that 40-85% of an oral dose of curcumin passes through the gastrointestinal tract unchanged with most of the absorbed flavonoid being metabolized in the intestinal mucosal and liver (Wahlstrom et al., 1978; Ravindranath et al., 1980).

### 5.2.5 *Curcuma mangga*

*Curcuma mangga* or *temu pauh* is commonly sold in the market as a seasoning for food and used medicinally as a stomachic (Burkill, 1966). Its application is less frequent in Malaysia compared to other species in Zingiberaceae family.

Among the ten Zingiberaceae species tested in the cytotoxicity assay, it was found that *C. mangga* was not active against both colon cancer cell lines HCT-116 and HT29. Crude petroleum ether, crude chloroform and crude methanol extracts only weakly suppressed the proliferation of the colon cancer cells. In another study by Leow (2006), only crude chloroform extract of *C. mangga* were active against HeLa and CaSki cells, giving an active IC$_{50}$ value which were $<1.00$ μg/ml and 14.40 μg/ml, respectively. Vimala *et al*., (1999) reported that *C. mangga* extracts showed no inhibition activity towards Epstein-Barr virus activation induced by TPA in Raji cells. On the other hand, studies by Faridah *et al*., (2005) showed that *C. mangga* do have antioxidant activity, with moderate free radical-scavenging activities.

### 5.2.6 *Curcuma xanthorriza*
Curcuma xanthorriza or locally known as temu lawak has been used widely as traditional medicine to treat rheumatism, indigestions and abdominal pain by the local village folk (Halijah and Ahmad, 1988). The present findings demonstrated that crude petroleum ether, crude chloroform and crude methanol extracts of C. xanthorriza showed good cytotoxic activity against HCT-116. IC$_{50}$ values indicate that the overall cytotoxic effect of C. xanthorriza was better against HCT-116 cells as compared to that against HT29 cells.

The findings obtained from this study show a good correlation with the studies done by Leow (2006) who disclosed that the extracts of C. xanthorriza demonstrated cell type specificity with better cytotoxic activity against HeLa rather than CaSki cells. Vimala et al. (1999) suggested C. xanthorriza as a possible chemopreventive agent with high inhibitory activity against Epstein-Barr virus early antigen induced by TPA in Raji cell lines.

5.2.7 Kaempferia galanga

Data obtained showed that the crude petroleum ether, crude chloroform and crude methanol extracts of K. galanga were not active in suppressing the proliferation rate of HCT-116 and HT29 cells.

Leow (2006) had also reported that the extracts of K. galanga were inactive in inhibiting the growth of human cervical cancer derived cell lines- HeLa and CaSki. In another study, the rhizome extract of K. galanga showed EBV activation inhibitory when screened for anti-tumour promoter activity in Raji cells using the short-term assay of
inhibition of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced EBV early antigen (Vimala et al., 1999).

5.2.8 Zingiber montanum

Zingiber montanum or known as bonglai is cultivated all over Malaysia and can be found in the form of tonics and jamu, often used to treat fever, rheumatism and gonorrhea (Halijah and Ahmad, 1988).

In the present study, it was found that the crude chloroform extract of *Z. montanum* exhibited strong cytotoxic activity against colon cancer cell lines (HCT-116 and HT29), with IC\(_{50}\) values less than 20 \(\mu\)g/ml. The crude petroleum ether extract of *Z. montanum* was found only active against HCT-116 cells while the crude methanol extract was not cytotoxic against both colon cancer cell lines. This suggests that the crude chloroform extract of this plant contained more active compounds with cytotoxic activity as compared to crude petroleum ether and crude methanol extracts. Similar results were obtained by Leow (2006), where the crude petroleum ether and crude chloroform extracts of *Z. montanum* demonstrated high inhibition against human cervical derived-HeLa cells. In another study, Vimala et al. (1999) reported that the rhizome of *Z. montanum* proved strong inhibitory activity against TPA-induced Epstein-Barr virus early antigen in Raji cell line.

Studies by Murakami et al. (1994) showed that curcumin in *Z. montanum* is the active constituent in the tumour promoter-induced-EBV activation test. They found a strong inhibitory activity of curcumin from the methanol extract of its rhizome. Habsah et
al. (2000) reported that dichloromethane and methanol extracts of Z. montanum showed strong antioxidant activity compared to α-tocopherol when tested with antioxidant assay.

5.2.9 Zingiber officinale

Zingiber officinale is locally known as halia or ginger worldwide. In the present study, crude chloroform and methanol extracts of Z. officinale were found to be non-cytotoxic against human colon cancer-derived cell lines, HCT-116 and HT29. This is in contrast to crude petroleum ether extract of Z. officinale which produced an IC₅₀ value of 13.0 μg/ml.

Previous study done by Leow (2006) showed that almost all crude petroleum ether, crude chloroform and crude methanol extracts of Z. officinale were not active when tested for its cytotoxicity against HeLa, Caski and HT29. In contrast, other researchers have shown that specific pure compounds isolated from Z. officinale are cytotoxic to human cancer cells. Kim et al. (2005) stated that [6]-gingerol, a pungent ingredient in ginger, has potent anti-angiogenic activity in vitro and in vivo in preventing cancers from becoming malignant. Lee and Surh (1998); and Keum et al. (2002) have shown the in vitro anti-tumour activity of [6]-gingerol and [6]-paradol against different cell lineages. The pharmacologic activity is thought to lie in the pungent principles (gingerols and shogaols) as well as volatile oils (sesquiterpenes and monoterpenes).

5.2.10 Zingiber zerumbet
*Zingiber zerumbet* is locally known as *lempoyang*. It is often used in the treatment for post-partum protective medicine, stomachache, diarrhea, dysentery, vermifuge and swellings (Halijah and Ahmad, 1988). In present study, all of the crude petroleum ether, crude chloroform and crude methanol extracts of *Z. zerumbet* showed strong cytotoxic effects against both human colon cancer cell lines, HCT-116 and HT29. The IC$_{50}$ values of all crude extracts were below 20 μg/ml. This result revealed that the actively cytotoxic compound(s) in *Z. zerumbet* were extracted by the three solvents with different polarities. This is in agreement with a study done by Leow (2006) in which the crude petroleum ether and crude chloroform extracts of *Z. zerumbet* exhibited strong inhibition activity against HeLa, CaSki and HT29 cells. In another study, Murakami *et al.* (1999) reported that zerumbone in *Z. zerumbet* is a distinct suppressor of tumour promoter 12-0-tetradecanoylphorbol-13-acetate (TPA)-induced EBV activation in Raji cells.

### 5.3 Comparison of *in vitro* cytotoxic activity among crude extracts of selected Zingiberaceae species

In the recent study, it was found that the Zingiberaceae crude extracts effectively inhibited the growth of human colon cancer derived- HCT-116 and HT29 cell lines where 14 crude extracts were active against HCT-116 cells and 11 crude extracts were active against HT29 cells. The crude petroleum ether and crude chloroform extracts of Zingiberaceae appeared to be more effective compared to crude methanol extracts of Zingiberaceae in restraining the propagation of HCT-116 and HT29 cells. The present findings are comparable with previous study which reported that the methanol extracts of local *ulam* were the least toxic to Raji and KB cells (Norhanom *et al.*, 1999). 50% of
crude petroleum ether Zingiberaceae extracts demonstrated good inhibition activity against HCT-116 and HT29 cells whereas 60% (12 out of 20) of crude chloroform Zingiberaceae extracts showed good inhibition against both cell lines. On the other hand only 15% (3 out of 20) of crude methanol Zingiberaceae extracts showed good inhibition activity on HCT-116 and HT29 cells.

The crude petroleum ether, crude chloroform and crude methanol extracts of *Z. zerumbet* were active against both human colon cancer cell lines HCT-116 and HT29 while all three crude extracts of *C. domestica* were actively inhibited the growth of HT29 cells. The results from this study disclosed that all the Zingiberaceae crude extracts inhibited the growth of HCT-116 and HT29 cells at varying levels. Among ten Zingiberaceae species being studied, the *Zingiber zerumbet* emerged as the most promising sample with very strong cytotoxic effect against both human colon cancer cell lines followed by *Alpinia galanga, Curcuma domestica, Boesenbergia rotunda, Zingiber montanum, Curcuma xanthorriza, Zingiber officinale, Curcuma aeruginosa, Kaempferia galanga* and *Curcuma mangga* in a decreasing order.

In the present study it was found that the different genus of Zingiberaceae samples has exerted different effect towards both cell lines, HCT-116 and HT29. It was found that the genus Zingiber was the most effective in inhibiting the proliferation of the cell lines followed by the genus Curcuma, Alpinia, Boesenbergia and Kaempferia. According to Malaysian Herbal Monograph (1999), among chemicals that contained in the genus Zingiber were acetaldehyde, acetone, hexanol, zingiberol, curcumere, α-curcumene and gingerol. These constituents give the synergistic effects by inhibiting the proliferation of both cell lines.
Leow (2006) has studied the cytotoxic effect of Zingiberaceae extracts against three cancer cells, HeLa and CaSki from cervical cancer; and HT29 from colon cancer. The results from her study stated that Zingiberaceae species did inhibit the proliferation of three cancer cells stated above and these findings supported the findings in the current study. Therefore it is necessary to evaluate the cytotoxic effect of these Zingiberaceae extracts against other types of human cancer. The cytotoxic compound(s) contained in each Zingiberaceae samples used should be further investigated. The physicians and scientists believe that if a plant has any medicinal value at all, it is because different components of the plant act synergistically (Cupp, 2000).

5.4 Cytotoxic activity of 5-Fu and selected Zingiberaceae extracts against HCT-116 and HT29 cell lines

5-Fluorouracil (5-Fu) is an antimetabolic agent that is often used as adjuvant chemotherapy in colon cancer. The study done by Advanced Colorectal Cancer Meta-Analysis Project in 1992 found that biochemical modulation of bolus regimens of 5-Fu with either folinic acid or methotrexate improved the activity of 5-Fu compared with 5-Fu alone. Whilst combination chemotherapy has been widely used in a number of different tumour types, it was not possible to apply this approach in colorectal cancer because colorectal cancer showed primary resistance to the majority of chemotherapeutic agents tested. However, in recent years evidence has merged for the efficacy of mitomycin C, irinotecan (CPT11) and oxaliplatin in advanced colorectal cancer (Tebbutt et al., 2002). In 1980s, Goldie and Coldman predicted that the results of chemotherapy
would be improved by the use of concurrent or alternating drug schedules provided that the individual agents were independently active and non-cross-resistant.

Buyse and colleagues (1988) has reported that 5-Fu treatment can decrease the risk of death for colorectal cancer. On the other hand, Sargent et al. (2001) has showed that the combination of 5-Fu with levamisole proved more toxic than the combination of 5-Fu with folinic acid, leading to increased frequency of grade 3 or greater leucopenia to patients older than 70 years in one meta-analysis. Beretta et al. (2004) found that treatment with 5-Fu plus levamisole was associated with a reduction in the recurrence rate by 40% and a reduction in the death rate by 33%, while levamisole alone failed to show benefit in the Dukes C patients. Combination chemotherapy with 5-Fu and irinotecan or oxaliplatin has improved survival for patients with advanced disease.

In the current study, six crude extracts of Zingiberaceae samples were chosen for testing against HCT-116 cells together with 5-Fu. Four of them were the crude petroleum ether extracts and the other two were crude chloroform extracts. The samples were selected based on the results obtained from the cytotoxicity assays using HCT-116 done previously. The petroleum ether extracts selected to be tested together with 5-Fu were from *A. galanga, B. rotunda, Z. montanum* and *Z. zerumbet*; and the selected chloroform extracts were from *C. domestica* and *C. xanthorrhiza*. Results revealed that all selected Zingiberaceae samples possessed the ability to increase the capability of 5-Fu in suppressing the proliferation of HCT-116 cells. The IC$_{50}$ of 5-Fu decreased when tested with the selected extracts. Importantly, synergy with Zingiberaceae extracts was seen at low 5-Fu concentrations. For example, incubation of HCT-116 cells with selected
concentration petroleum ether extract of *A. galanga* reduced the IC₅₀ for 5-Fu from 0.02 M to 2.5 x 10⁻⁹ M.

Eight crude extracts of Zingiberaceae samples were tested with 5-Fu against HT29 cells using the NR assay. As before the extracts were chosen based on the results from cytotox assays using HT29 cells done earlier. The selected crude petroleum ether extracts were from *B. rotunda*, *Z. officinale*, *A. galanga* and *Z. zerumbet*; and the selected crude chloroform extracts were from *Z. montanum*, *C. aeruginosa*, *C. domestica* and *A. galanga*. Each one of them was tested with nine different concentrations of 5-Fu varying from 0.1 x 10⁻⁷ M to 1.0 M against colon cancer derived- HT29 cells. The combination of 5-Fu with all the extracts exhibited good cytotoxic effects and gave the better values of IC₅₀ as compared to 5-Fu alone. Synergy with Zingiberaceae extracts was also seen at low 5-Fu concentrations. For instance, incubation of HT29 cells with selected concentration petroleum ether extract of *B. rotunda* reduced the IC₅₀ for 5-Fu from 0.15 M to 1.5 x 10⁻⁷ M.

The results of the present study agree with that of earlier findings by Ross (1997), Van Cutsem (2000) and Tebbutt (2002). The use of mitomycin-C (MMC) alone in patients who were resistant to 5-Fu appeared less active compared to combination of 5-Fu and MMC, indicating a requirement for synergism between 5-Fu and MMC (Ross *et al*., 1997; Van Cutsem *et al*., 2000; Tebbutt *et al*., 2002).

Two randomised trials done by Rougier *et al.* (1998) and Cunningham *et al.* (1999) has led to the use of combinations of CPT11 (irinotecan) and 5-Fu as initial therapy for metastatic colorectal cancer. Both demonstrated improved response rate,
prolongation of time to progression, and improvement in overall survival for the combination treatment compared with 5-Fu alone.

Russo et al., (2002) reported that RPR-115135 (a farnesyltransferase inhibitor) has the capability to enhance the antiproliferative effects of 5-Fu against the human colon cancer HCT-116 isogenic cell system. The results indicated that RPR-115135 enhanced 5-Fu induced apoptosis only when wt-p53 is present. In p53 mutated cell lines, subtoxic concentrations of RPR-115135 in combination with 5-Fu were antagonist or synergistic, but did not influence the cell cycle and did not encourage apoptosis.

Tebbutt et al. (2002) explained that the randomized trials gave an overall survival benefit for the initial use of combination treatment using CPT11 and 5-Fu, but not for the combination of oxaliplatin and 5-Fu. Tracking studies in the United States suggest that around 30% of patients who receive 5-Fu monotherapy will not receive second-line chemotherapy. These may well represent patients whose tumours are 5-Fu-resistant and who develop tumour progression with 5-Fu monotherapy, thereby becoming too unwell to receive second-line treatment (Tebbutt et al., 2002).

Bloemendaal et al. (2005) demonstrated a significant increase in median survival of patients who completed their chemotherapy as compared to those who received minimal or no chemotherapy. Taeib et al. (2005) and Tournigand et al. (2004) showed a median survival of more than 20 months in patients receiving modern chemotherapeutic regimens like combinations of oxaliplatin, irinotecan and 5-Fu/Leucovorin, both without combined with surgery. Cost and concerns regarding toxicity may, however, mean that it is not always feasible to offer combination therapy to all patients as initial treatment. Alternate treatments such as oxaliplatin and CPT11 can be used instead of 5-Fu for those
patients who are likely to be resistant to 5-Fu (Tebbutt et al., 2002). Randomised phase III trials of 5-Fu and folinic acid versus follow-up in the adjuvant setting have consistently shown a reduction in the risk of dying from colorectal cancer (Francini et al., 1994; O’Connell et al., 1998).

5.5 Screening of wt-p53 activity in Zingiberaceae using immunocytochemistry technique

P53 is a nuclear phosphoprotein with a molecular mass of 53 kDa. As a transcription factor, wt-p53 works as a modulator which can turn crucial genes either on or off. In transfection assays, while mutant p53 performs as a dominant transforming oncogene, the wt-p53 behaves as a tumour suppressor (Vojtesek et al., 1992; Nieder et al., 2001).

Ross et al. (2006) has proposed that over-expression of an activated oncogene such as c-myc in the presence of high levels of the wt-p53 tumour suppressor protein drives the tumour cells towards apoptosis and thus better survival. The absence of p53 protein facilitates increased proliferation and tumour progression.

Two models have been proposed to explain the role of p53 as a tumour suppressor; 1) p53 participates in the cellular response to DNA damage by delaying cell cycle progression at a G1 checkpoint- to provide time for repair of damaged DNA; 2) p53 has been shown to initiate apoptosis in response to agents that cause DNA strand breakage- failure to eliminate cells that have undergone genetic alterations following
DNA damage could lead to the appearance of transformed clones (Gangopadhyay et al., 1997).

Diverse types of human cancer are caused by p53 mutations (Hollstein et al., 1991). It has been shown that hormone-inducible wild-type p53 gene could inhibit the growth of human brain cancer cells (Mercer et al., 1990). P53 is found in very low levels in normal cells. However, in a variety of transformed cell lines, it is expressed in high amounts, and believed to contribute to transformation and malignancy. Mutants of p53 which frequently occur in a number of different human cancers fail to bind the consensus DNA binding site, and hence cause the loss of tumour suppressor activity.

*In vitro* introduction of wt-p53 into p53-deficient cells has been shown to cause rapid death of cancer cells or prevention of further division. The rationale for developing therapeutics targeting p53 is that ‘the most effective way of destroying a network is to attack its most connected nodes’. It is believed that restoring of p53 function in tumour cells may block tumour expansion and may sensitize cells to cytotoxic killing, thus improving therapeutic response in curing many cancers (Bouchet et al., 2006).

Mutations in p53 and in the p53 pathway can produce multi-drug resistance *in vitro* and *in vivo*, and reintroduction of wt-p53 into p53 null tumour cells can re-establish chemosensitivity. P53 status is not a universal of treatment response, in part because not all drugs absolutely require p53 for their apoptotic function and in some settings; p53 loss can enhance drug-induced apoptotic cell death (Ravizza et al., 2004).

Evaluation of cell cycle perturbations after irradiation in a number of human tumour cell lines revealed that cell lines with wild-type p53 genes were arrested at both $G_1$ and $G_2$ phases of the cell cycle after irradiation, whereas tumour cell lines with
mutant p53 alleles were only arrested at the G₂ phase of the cell cycle after irradiation (Canman \textit{et al}., 1994).

The mutant p53 protein, which appears necessary for maintenance of malignant phenotype, has longer half-life than the wild type protein. The wild type is usually undetectable in normal cells, while the mutant protein is over expressed by 5 to 100 fold in transformed cells and tumour cell lines (Levine \textit{et al}., 1991; Hollstein \textit{et al}., 1991; de Fromentel \textit{et al}., 1991).

In the present study, petroleum ether, chloroform and methanol extracts of 10 selected Zingiberaceae species were screened for wt-p53 promoting activities in colon cancer derived- HCT-116 and HT29 cells. The method used in this study was the 3-step indirect avidin-biotin immunoperoxidase immunocytochemistry technique. The immunocytochemistry technique is easy to handle and is able to generate consistent, sensitive and reliable results. The reagents are readily obtainable and only basic equipment is required to perform the staining procedure (Gay and Docherty, 1986).

The negative control in this study was HCT-116 and HT29 cells not incubated with any Zingiberaceae extracts. PBS was substituted for primary antibodies. Control slide incubated with primary antibody, DO-7 mouse anti-human p53 protein showed the presence of reddish-brownish staining in cytoplasm region. The negative control without wt-p53 antibody did not demonstrate any reddish-brown stain. This correlates well with the principle of immunocytochemistry staining where the absent specific primary antibody caused failure in detecting any antigen and therefore no complex (reddish-brown precipitate) being formed at the end of the staining process. The negative controls with no anti-p53 antibody were used as internal control in order to
assess the sensitivity of the immunocytochemistry method. Estimate of the percentage of tumour cells with positively labeled cytoplasm and nuclei were made, and a score of no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+) was assigned.

In the present study, the HCT-116 and HT29 cells were treated with Zingiberaceae extracts at varying concentrations (1 μg/ml, 10 μg/ml, 25 μg/ml, 50 μg/ml and 100 μg/ml). The staining results for HCT-116 and HT29 cells treated with Zingiberaceae extracts were interpreted relative to the negative controls and among the various concentrations of the same extract. The various appearance of reddish-brownish stain indicates the presence of various level of wt-p53 in both colon cancer cell lines. The intensity of reddish-brownish stain directly correlated with the expression of wt-p53 protein; the stronger stain indicates higher expression of wt-p53 protein.

Plaumann et al. (1996) has reported previously that flavonoids increase the cell content of wild-type p53, leading to enhance rates of apoptosis. Studies done by Soleas et al. (2001) demonstrated that trans-resveratrol (at certain concentration) dramatically reduced p53 content to around 10% of the controls, but many cells showed severe morphologic damage under microscopy and were strongly stained by tryphan blue, suggesting cytotoxic effects. The studies show little or no association between p53 protein levels and stimulation of cells by wine polyphenols.

### 5.5.1 Wt-p53 activity of the petroleum ether Zingiberaceae extracts

The expression of wt-p53 in HCT-116 and HT29 cells tested by crude petroleum ether, crude chloroform and crude methanol Zingiberaceae extracts were evaluated in
current study. Generally, all the petroleum ether extracts enhanced wt-p53 expression in both cell lines.

Based on the staining intensities being observed in HCT-116, five of the petroleum ether extracts from *A. galanga*, *B. rotunda*, *C. mangga* and *Z. officinale* did enhance the expression of wt-p53 steadily. Fluctuated expression of wt-p53 can be seen clearly in HCT-116 cells treated by petroleum ether extracts of *C. xanthorriza*, *Z. montanum* and *Z. zerumbet*. On the other hand, in *C. aeruginosa*, *C. domestica* and *K. galanga*, the expression of wt-p53 was very strong, strong and moderate, respectively, at concentration of 1 μg/ml but became weak and decreased into moderate for *C. aeruginosa* and *C. domestica*, and very weak for *K. galanga* at concentration of 100 μg/ml.

As for HT29, petroleum ether extracts of *A. galanga*, *B. rotunda*, *C. xanthorriza* and *Z. zerumbet* improved the expression of wt-p53 with the increasing concentrations of the extracts. The fluctuated of wt-p53 expression can also be seen in HT29 cells treated with *K. galanga* and *Z. montanum*. At the same time, the expressions of wt-p53 remain unchanged in HT29 cells treated by *C. aeruginosa* and *C. mangga*. Though in HT29 cells treated with *C. domestica* and *Z. officinale*, the expression of wt-p53 were very strong and strong at 1 μg/ml and decreased with rising concentrations of the extracts and became moderate at 100 μg/ml.

Results in the present study showed that petroleum ether extracts of all selected Zingiberaceae extracts increased the expression of wt-p53 in both colon cancer derived-HCT-116 and HT29 cell lines even at the lowest concentration of the extracts. Besides, results also revealed that 40% (4 out of 10) of the petroleum ether extracts were
successful in inducing the expression of wt-p53 in both HCT-116 and HT29 cells, in a dose-dependent manner where higher concentration exhibited greater stimulative effect of wt-p53 expression.

5.5.2 Wt-p53 activity of the chloroform Zingiberaceae extracts

The wt-p53 promoting activities of ten chloroform Zingiberaceae extracts were evaluated in the present study using HCT-116 and HT29 cells. From the staining intensities being observed, the expressions of wt-p53 in HCT-116 cells were enhanced to a higher level by the extracts as compared to HT29 cells. The extracts of *A. galanga*, *C. domestica*, *C. mangga*, and *Z. zerumbet* have increased the expression of wt-p53 in HCT-116 cells. Chloroform extract of *C. aeruginosa* showed moderate expression of wt-p53 at all concentrations tested while the chloroform extract of *C. xanthorriza*, *Z. montanum* and *Z. officinale* gave the unsteady expression of wt-p53 with increasing concentrations of the extract. Only two chloroform extracts showed strong and moderate expression of wt-p53 at the beginning of study, became weaker with the increasing concentrations of the extracts in HCT-116 cells, which were from *B. rotunda* and *K. galanga*, respectively.

For HT29 cells, all of the chloroform extracts increased the expression of wt-p53 even at the lowest concentration - 1 μg/ml. The expression of wt-p53 became stronger with the increasing concentrations of *A. galanga*, *C. xanthorriza*, and *Z. zerumbet* extracts. Meanwhile, the unsteady expressions of wt-p53 were produced by the chloroform extracts of *B. rotunda*, *C. domestica* and *Z. montanum* in HT29 cells. Overall, in HT29 cells, staining results showed that four out of ten chloroform extracts exhibited
moderate expression of wt-p53 at all concentrations tested. The four extracts were *C. aeruginosa*, *C. mangga*, *K. galanga*, and *Z. officinale*.

### 5.5.3 Wt-p53 activity of the methanol Zingiberaceae extracts

The methanol extracts from Zingiberaceae species exhibited mixture activity against wt-p53 expression in HCT-116 and HT29 cells. Among ten methanol extracts being investigated in this study, *Z. officinale* stood out as the most prominent methanol extract showing remarkable promoting activity of wt-p53 in HCT-116 cells with the increasing concentrations. This was followed by *Z. montanum*. However, the methanol extracts of *C. mangga*, *C. xanthorriza*, *K. galanga*, and *Z. zerumbet* showed unstable wt-p53 promoting activity in HCT-116 cells with the increasing concentrations. The extracts that maintained the expression of wt-p53 in a strong and moderate level throughout the assay were the extracts of *C. aeruginosa* and *C. domestica*, respectively. However, the expression of wt-p53 became weaker in HCT-116 cells treated with higher concentrations of *A. galanga* and *B. rotunda* though the expressions were moderate and strong at lower concentration tested.

The methanol extracts of *C. mangga*, *C. domestica* and *Z. montanum* increased the expression of wt-p53 with the increasing concentration throughout the assay used. The rest of methanol extracts from *A. galanga*, *B. rotunda*, *C. aeruginosa*, *C. xanthorriza*, *K. galanga*, *Z. officinale*, and *Z. zerumbet* showed moderate staining intensity of wt-p53 in HT29 cells.

From the results obtained, it can be concluded that all of the methanol extracts were promote the wt-p53 activity in both colon cancer cell lines, HCT-116 and HT29.
Most of the methanol extracts gave influence on the activity of wt-p53 even at the lowest concentration.

5.5.4 Comparison of wt-p53 activity among petroleum ether, chloroform and methanol Zingiberaceae extracts

Results obtained demonstrated that there were different wt-p53 promoting activity shown by the petroleum ether, chloroform and methanol extracts of selected Zingiberaceae species. In general, the petroleum ether Zingiberaceae extracts were more effective in promoting the activity of wt-p53 in both colon cancer cell lines compared to their chloroform and methanol extracts. The difference polarity of the solvents used extracted different compounds. This can have an effect on the wt-p53 activity and that resulted in the different staining intensities.

The Zingiberaceae extracts exhibited average wt-p53 promoting activity among their petroleum ether, chloroform and methanol extracts. For example, petroleum ether and chloroform extracts of A. galanga showed outstanding wt-p53 promoting activity in both colon cancer cell lines, HCT-116 and HT29, while petroleum ether, chloroform and methanol extracts of Z. zerumbet promotes very strong expression of wt-p53 for HCT-116 cells. Petroleum ether and chloroform extracts of Z. zerumbet induced strong wt-p53 staining intensity in HT29 cells tested while methanol extract of Z. zerumbet moderate intensity at all concentrations tested.
CHAPTER 6

CONCLUSION

In the current study, the in vitro NR cytotoxicity assay and 3-step indirect avidin-biotin immunoperoxidase immunocytochemistry technique have been successfully applied to screen the cytotoxic (with and without 5-Fu) and wt-p53 promoting activities in ten selected Zingiberaceae species. Based on the results obtained from this study, it was found that most of the Zingiberaceae rhizome tested exhibited cytotoxic activity and all exhibited wt-p53 promoting activity.

The overall results showed that petroleum ether and chloroform Zingiberaceae extracts possessed greater cytotoxicity effect against colon cancer cell lines and were also effective in promoting the expression of wt-p53. The selected extracts tested together with 5-Fu gave promising results, where the cytotoxicity capability of 5-Fu was enhanced dramatically by the extracts. The combination therapy between the natural products namely Zingiberaceae and 5-Fu opened a new possibility in finding a new way to decrease the risk of cancer. 5-Fu based schedules remain the standard treatment in this setting, however combination therapy can be considered in patients at high risk.

Synthetic anti-cancer drugs cause nonspecific killing of cells. On the other hand, natural products offer protective and therapeutic actions to all cells with low cytotoxicity. Results indicated that the petroleum ether and chloroform extracts of Zingiber zerumbet gave the remarkable cytotoxic effect against HCT-116 and HT29 cells when treated with or without 5-Fu. The expression of wt-p53 in both cell lines was best enhanced by the petroleum ether and chloroform extracts of Alpinia galanga.
Since the Zingiberaceae showed signs of selectivity for colon cancer-derived, HCT-116 and HT29 cells, it is therefore essential to also evaluate the cytotoxic effect of these Zingiberaceae extracts on other type of human cancer cells. Further studies should be initiated to isolate and identify the specific bioactive compound(s) from the active Zingiberaceae extract which demonstrated significant cytotoxic or wt-p53 promoting activities.

Clinical cancer chemoprevention research has recently made significant advances. However, a large number of potential chemopreventive compounds in foods and plants remain to be studied. Interactions between nutrients and genes are important in carcinogenesis as well cancer prevention. Understanding the nutritional modulation of genetic and epigenetic pathways of carcinogenesis will help develop foundations for future clinical chemoprevention trials with nutritional agents and botanicals. Chemopreventive agents must have low toxicities compared with chemotherapeutic agents used in cancer patients.

The family of ginger has been widely used food preservatives, colouring agents, cooking ingredients and as one of the main ingredients in traditional medicine preparation. Investigation of its wt-p53 promoting activity property together with the mechanism of action involved is crucial in order to exploit a potential food-based anti-cancer agent. Hopefully, by increasing the wt-p53 level, the carcinogenesis or progression of colon cancer will be slowed, if not stop the progress.

In conclusion, cancer chemoprevention is a field of great promise. Although it is not reasonable to assume cancer chemopreventive agents will safeguard humans from
known carcinogenic risks such as cigarette smoking, it is reasonable to anticipate that these agents will play an increasing role in cancer prevention strategies.
BIBLIOGRAPHY


Surh, Y J (2002). Anti-tumour promoting potential of selected spice ingredients with antioxidative and anti-inflammatory activities: a short review. *Food and Chemical Toxicology*, 40; 1091-1097.


APPENDIX

Appendix 1
Appearance of HCT-116 cells after treatment with petroleum ether extract of *Alpinia galanga*

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>i) Petroleum ether</th>
<th>Intensity</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1" alt="Image" /></td>
<td>2+</td>
<td>-the expression of p53 increased with increasing concentration of the extracts -cytoplasms were stained</td>
</tr>
<tr>
<td>10</td>
<td><img src="image2" alt="Image" /></td>
<td>2+</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td><img src="image3" alt="Image" /></td>
<td>3+</td>
<td>-cells lysis begin</td>
</tr>
<tr>
<td>50</td>
<td><img src="image4" alt="Image" /></td>
<td>4+</td>
<td>-cytoplasms and nucleus were stained</td>
</tr>
<tr>
<td>100</td>
<td><img src="image5" alt="Image" /></td>
<td>5+</td>
<td></td>
</tr>
</tbody>
</table>

**HCT-116 cells treated with petroleum ether extract of *Alpinia galanga* (400x)**

Note: Classification for the intensity of staining as no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)
## Appendix 2

Appearance of HCT-116 cells after treatment with methanol extract of *Boesenbergia rotunda*

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>iii) Methanol</th>
<th>Intensity</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4+</td>
<td>-the expression of p53 decreased with increasing concentration of the extracts -cells lysis begin</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2+</td>
<td>-cytoplasms were stained</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>2+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>2+</td>
<td>-cytoplasms were stained</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**HCT-116 cells treated with methanol extract of *Boesenbergia rotunda* (400x)**

Note: Classification for the intensity of staining as no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)
### Appendix 3
Appearance of HCT-116 cells after treatment with methanol extract of *Curcuma aeruginosa*

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>iii) Methanol</th>
<th>Intensity</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
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</tr>
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</tr>
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<td>-cells lysis begin</td>
</tr>
<tr>
<td>50</td>
<td><img src="Image193x244.png" alt="Image" /></td>
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<td>-cytoplasms and nucleus were stained</td>
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</table>

**HCT-116 cells treated with methanol extract of *Curcuma aeruginosa* (400x)**

Note: Classification for the intensity of staining as no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)
### Appendix 4
Appearance of HT29 cells after treatment with methanol extract of *Curcuma xanthorriza*

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>iii) Methanol</th>
<th>Intensity</th>
<th>Comment</th>
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</thead>
<tbody>
<tr>
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<td><img src="image1" alt="Image" /></td>
<td>3+</td>
<td>-the expression of p53 increased with increasing concentration of the extracts -cytoplasms were stained</td>
</tr>
<tr>
<td>10</td>
<td><img src="image2" alt="Image" /></td>
<td>3+</td>
<td></td>
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<td>25</td>
<td><img src="image3" alt="Image" /></td>
<td>3+</td>
<td>-cells lysis begin</td>
</tr>
<tr>
<td>50</td>
<td><img src="image4" alt="Image" /></td>
<td>3+</td>
<td>-cytoplasms and nucleus were stained</td>
</tr>
<tr>
<td>100</td>
<td><img src="image5" alt="Image" /></td>
<td>3+</td>
<td></td>
</tr>
</tbody>
</table>

**HT29 cells treated with methanol extract of *Curcuma xanthorriza* (400x)**

Note: Classification for the intensity of staining as no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)
Appendix 5
Appearance of HT29 cells after treatment with methanol extract of *Boesenbergia rotunda*

<table>
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<th>Concentration (μg/ml)</th>
<th>iii) Methanol</th>
<th>Intensity</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>3+</td>
<td>-the expression of p53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>increased with increasing</td>
</tr>
<tr>
<td>Concentration (μg/ml)</td>
<td>iii) Methanol</td>
<td>Intensity</td>
<td>Comment</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------</td>
<td>-----------</td>
<td>---------</td>
</tr>
<tr>
<td>1</td>
<td>3+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>3+</td>
<td>-cells lysis begin</td>
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</tr>
<tr>
<td>50</td>
<td>3+</td>
<td>-cytoplasms and nucleus were stained</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>3+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**HT29 cells treated with methanol extract of Boesenbergia rotunda (400x)**
Note: Classification for the intensity of staining as no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

**Appendix 6**
Appearance of HT29 cells after treatment with methanol extract of Curcuma xanthorriza
<table>
<thead>
<tr>
<th>concentration of the extracts</th>
<th>cytoplasms were stained</th>
<th>3+</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>3+</td>
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<tr>
<td>cells lysis begin</td>
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<tr>
<td>50</td>
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<tr>
<td>cytoplasms and nucleus were stained</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>3+</td>
</tr>
</tbody>
</table>

**HT29 cells treated with methanol extract of *Curcuma xanthorrhiza* (400x)**

Note: Classification for the intensity of staining as no stain (0-), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)