POTENTIAL ANTICANCER ACTIVITY OF IN RHIZOMES OF GINGER

SPECIES (ZINGIBERACEAE FAMILY)

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university of tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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ACKNOWLEDGEMENTS

ABSTRACT

The aim of the work described in this thesis was initially to screen the ethanol extracts of eleven Indonesian ginger species (Zingiberaceae family) for anticancer activity. MCF-7 breast and HT-29 colon cancer cells were used for the investigations. Extracts of *Zingiber aromaticum* and *Boesenbergia pandurata* were found to be the most active species, similar to that of *Curcuma longa* which has been shown to possess anticancer activity *in vitro* and *in vivo* (Aruna and Sivaramakrishnan, 1992; Azuine and Bhide, 1992). These two active species were then further investigated. Bioactive compounds from the species were isolated and identified using various chromatography procedures and nuclear magnetic resonance (NMR) and their anticancer activities were further tested on MCF-7 breast and HT-29 colon cancer cells including cell cycle analysis and measurements of apoptosis. The ethanol extracts of these two active species were also investigated using the AOM-induced colon cancer model in rats. The antiinflammatory activity of the ethanol extract of *Z. aromaticum* was also investigated using dextran sulfate sodium (DSS) induced ulcerative colitis (UC) in rats.

The inhibitory activity of ethanol extracts of rhizomes of 11 ginger species was initially tested against MCF-7 breast and HT-29 colon cancer cells using colorimetric tetrazolium salt (MTT) assay. Ethanol extracts of eight species (*Amommum cardamomum, C. longa, C. mangga, C. xanthorrhiza, Boesenbergia pandurata, Zingiber aromaticum, Z. officinale, Z. cassumunar*) showed a strong inhibitory effect on the growth of the cancer cells with the IC_{50} concentrations between 10-100 µg/ml. The ethanol extract of *Curcuma aeruginosa* was less active (IC_{50} between 100-120 µg/ml) and extracts of *Kaempferia galangal* and *K. rotunda* had no effect on the growth of either cell lines at concentrations up to 250 µg/ml. Ethanol extract of *C. longa* was used as a comparison since curcumin, an active compound isolated from this species, has had demonstrated its anticancer activity *in vitro, in vivo* and is currently undergoing clinical trial against colon cancer (Greenwald, et al.,

2001; Sharma et al., 2001). Extracts of *Z. aromaticum* and *B. pandurata* had very strong inhibitory activity similar to the extract of *C. longa*. Curcumin was not detectable in either *Z. aromaticum* or *B. pandurata*. The ethanol extracts of the active species were not toxic on human skin fibroblast cells (SF 3169).

The ethanol extracts of *Z. aromaticum* and *B. pandurata* were further fractionated using two different solvents by reversed phase preparative HPLC. Fraction A was eluted with a mobile phase containing 5% v/v aqueous methanol containing 0.025% v/v trifluoroacetic acid (TFA) and fraction B was eluted with 100% methanol. The inhibitory activity of fractions was then investigated against HT-29 colon cancer cells and assayed using the MTT assay. Zerumbone, a sesquiterpenoid compound was isolated from fraction B of the extract of *Z. aromaticum* and a chalcone derivative, panduratin A was isolated from fraction B of the extract of *B. pandurata*. Curcumin was in fraction A of extract of *C. longa*.

The anticancer activity of zerumbone and panduratin A was investigated using MCF-7 breast, HT-29 and CaCo-2 colon cancer cells. The inhibitory activity of the active compounds was assessed using the MTT assay. The IC_{50} of zerumbone in each of the cell lines was about 10 μM and of curcumin on HT-29 cells was 25 μM. The IC_{50} of panduratin A in HT-29 cells was 16 μM and in MCF-7 cells was 9 μM. Zerumbone and panduratin A showed antiproliferative effects by alteration of the DNA distribution in the cell cycle and induction of apoptosis. HT-29 cells treated with zerumbone at concentrations of 10 – 25 μM or panduratin A at concentrations of 9 – 65 μM for 24 h were stained with propidium iodide (PI) to determine cell cycle distribution and analysed using FACScan flow cytometry. The proportion of cells in the S phase was reduced from 18.7% in untreated cells to 10.2% in HT-29 cells after treatment with zerumbone at 10 μM to 3.1% at 25 μM. Cells in the G2 phase increased from 18.5% at 10 μM to 40% at a concentration of 25 μM. Panduratin A increased the proportion of cells in the G0/G1 phase from 33% of untreated cells to 71% after treatment with 65 μM for 24 h. Panduratin A slightly reduced the proportion of cells in S phase and cells in G2/M phase also

decreased from 36.8% in untreated cells to 15.4% at 65 µM. Apoptosis was determined using double labelled (Annexin-V-Fluos and PI) and then evaluated using FACScan Flow Cytometry. Morphological features of apoptosis were also examined using DiffQuick stain and fluorescent Hoechst 3355 and 4,6-diamino-2-phenylindole (DAPI). Zerumbone induced apoptosis in HT-29 cells in a dose dependent manner. At 48 h, 2% of cells treated with 10 µM of zerumbone underwent apoptosis, which increased to 8% when treated with 50 µM. Panduratin A at 28 µM increased the number of cells undergoing apoptosis from 2.2% to 16.7% when treated with a concentration of 65 µM. The ethanolic extracts of *Z. aromaticum* and *B. pandurata* were also investigated using the azoxymethane (AOM) induced aberrant crypt foci (ACF) model of colon cancer in rats in a short and long term study. Ethanolic extracts of *C. longa* and curcumin were used as comparison. The basal diet used throughout all animal studies in this thesis was a semi-purified AIN-93 G diet (Reeves et al., 1993). ACF were induced by two doses (15 mg/kg BW) subcutaneously of AOM one week apart and ACF were visualised in the formalin fixed colon using methylene blue stain. The ACF study was run over a short (5 weeks) and long (13 weeks) experiments. Diets containing ethanol extracts prepared from the equivalent of 2% (w/w) dried rhizome of *Z. aromaticum, B. pandurate* or *C. longa* in a short term study did not affect the formation of ACF in rats compared to those in the control diet group. The ACF formation in a short term study was dominated by small numbers of aberrant crypts (1 or 2) per focus. It is suggested that large ACF (4 or more ACs/focus) are better predictors of colon cancer (Uchida et al., 1997; Jenab et al., 2001). Diets containing ethanol extracts of the equivalent of 4% by weight of dried rhizomes of *Z. aromaticum, B. pandurata, C. longa* were investigated over 13 week study. Total ACF were significantly reduced by *Z. aromaticum* extract (0.34%) in the diet (down 21%, p<0.05) relative to rats fed the control diet. A similar reduction was observed with *C. longa* extract (0.86%) in the diet (down 24%, p<0.01) and with 2000 ppm curcumin. There was no significant different in small ACFs (1-2 ACs/ focus) between dietary treatments. The number of foci containing 3-4 ACs/focus was significantly reduced (35%, p<0.001) in animals fed the *Z. aromaticum* extract and 34% (p<0.001) of

animals fed the *C. longa* extract. The total number of ACF containing 5 or more ACs per focus of animals fed 0.34% *Z. aromaticum* extract was 41% lower than control (p<0.05) and for 0.86 % *C. longa* extract was 22% (not significant). A diet containing extract (0.56%) of *B. pandurata* did not significantly affect the formation of ACF compared to the control AIN group. The concentration of zerumbone in the *Z.aromaticum* extract diet was assayed at 300 ppm, and of curcumin in the *C. longa* extract diet was also 300 ppm. The concentration of panduratin A was not assayed in the diet due to late identification of the active compound.

The antiinflammatory activity of ethanol extract of *Z. aromaticum* was investigated using dextran sulfate sodium (DSS) induced ulcerative colitis in rats. Sulfasalazine, a widely used compound to treat inflammatory bowel disease (IBD) in humans was used as the positive control. Diets containing ethanol extracts (0.34% and 0.68%) prepared from the equivalent of 4% and 8% by weight of dried rhizomes of *Z. aromaticum* were given to the animals throughout the experiment. On day three, rats were given 2% DSS in drinking water for 5 d and then just water for 3 d and then were killed. During the DSS treatment rats were maintained in metabolic cages, body weight, food and fluid intake and clinical symptoms such as consistency of stools and blood in faeces were recorded daily. There was slight but not significant reduction in the body weight of rats fed 0.68% extract of *Z. aromaticum* in the diet due to reduced food consumption. The extract of *Z. aromaticum* (0.34%) and sulfasalazine suppressed clinical signs of ulcerative colitis. Eleven percent of the controls were hemoccult positive on day 2 after DSS administration, which progressed further by day three with 67% being hemoccult positive and 100 % on day five. By comparison, blood appeared on day 3 of rats treated with diet containing 0.34% and 0.68% extract of *Z. aromaticum* and 0.05% sulfasalazine, and only 33%, 67% and 22%, of rats being hemoccult positive on day 5 respectively. The disease activity index (DAI) of rats fed diet containing 0.34% extract of *Z. aromaticum* was about 0.4 and similar to those which were fed with diet containing sulfasalazine. The DAI of untreated rats was 1.4. The crypt score of rats fed the extract of *Z. aromaticum* was slightly reduced but it was not significantly different

from those of untreated rats. Other histological scores were not significantly different between dietary treatments. Extract of *Z. aromaticum* significantly decreased the content of PGE-2 in colon tissue compared to that of untreated animals. There was a reduction of TXB-2 content in colonic tissue of rats fed with extracts of *Z. aromaticum* but this was not significant. The activity of myeloperoxidase (MPO) activity in the colonic tissue of rats fed with sulfasalazine was significantly lower than that of the untreated controls and those which fed with extracts of *Z. aromaticum*.

The results from the studies performed in this thesis showed that extract of *Z. aromaticum* which contains an active sesquiterpenoid zerumbone have anticancer and antiinflammatory activity suggesting that the extract may have benefits as a chemopreventative agent. However further studies are needed to elucidate their other pharmacological actions. Panduratin A showed potential anticancer activity in cell culture *in vitro*. However an extract of *B. pandurata* did not have effect on the AOM-induced colon cancer model. Different cancer models such as breast and prostate cancer could be used to further investigate the anticancer activity of extract of *B. pandurata* and panduratin A and to elucidate their mechanism.

PUBLICATIONS ARISING FROM THIS THESIS

Kirana, C., Record, I.R., McIntosh, G.H., and Jones, G.P. (2003). Screening for Antitumor activity of 11 species of Indonesian Zingiberaceae using MCF-7 and HT-29 cancer cells. Pharmaceutical Biology 41(4), 271-276.

Kirana. C., McIntosh, G.H., Record, I.R., and Jones, G.P. (2003).Antitumor activity of extract of *Zingiber aromaticum* and its bioactive sesquiterpeneoid zerumbone. Nutrition and Cancer 45(2), 218-225.

Kirana, C. McIntosh, G.H., Record, I.R., and Jones, G.P. (2002). Anticancer activity of zerumbone from Zingiber aromaticum in Azoxymethane (AOM) colon cancer model. Journal of Gastroenterology and Hepatology 17 (Suppl.) A 108

LIST OF ABBREVIATIONS

ACF	aberrant crypt foci
AIN	American Institute of Nutrition
AOM	azoxymethane
BW	body weight
CD	Crohn's disease
COX	cyclooxygenase
DMEM	dulbecco minimum essential medium
DNA	deoxyribonucleic acid
DSS	dextran sulfate sodium
EBV-EA	Epstein barr virus early antigen
FBS	foetal bovine serum
IBD	inflammatory bowel disease
IC	inhibition concentration
h	hours
g	grams
HEPES	nitrogen-2-hydroxyethylpiperazine-nitrogen'2-ethanesulfonic acid
HPLC	high performance liquid chromatography
LC	liquid chromatography
mg	milligrams
ml	millilitres
MPO	myeloperoxidase
MS	mass spectrometry
MTT	3-(4,5-dimethylthiasol-2-yl)-2,5-diphenyl tetrazolium bromide

NMR	nuclear magnetic resonance
μg	micrograms
μl	microlitres
PBS	phosphate buffered saline
PGE	prostaglandin E
PI	propidium iodide
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TPA	12-O-tetradecanoylphorbol 13- acetate
TXB	thromboxane
UC	ulcerative colitis

CHAPTER I

INTRODUCTION

1.1. Background

The use of herbs as medicines has played an important role in nearly every culture on earth, including Asia, Africa, Europe and the Americas. A recent survey suggested that the usage of alternative medicine especially for chronic or incurable diseases or acute illnesses has increased dramatically (Bernstein and Grasso, 2001). A study found that 83 % of 453 cancer patients had used at least one alternative medicine. Many of these supplements are herbal in nature (Elvin-Lewis, 2001).

Indonesia was known historically as the Spice Islands. It is therefore not surprising that most traditional dishes are highly spiced. In addition to their organoleptic use in cooking, herbs and spices also find general use in traditional medicines. *"Jamu"* is an Indonesian term for an indigenous medicine prepared from herbal and spice materials. *"Jamu"* is believed to be beneficial in a wide range of situations. However, scientific validation for the use of most traditional medicinal plants is almost non existent and therefore requires further elucidation.

Zingiberaceae species are members of a family of plants which have been used for centuries in cooking, cosmetic, and medicine especially in Asian regions. People in Indonesia use the leaves, roots, stems and rhizomes of the plants as preservatives, for flavouring and colouring in cooking. They also eat the leaves and flowers of certain species of the ginger family. The folk medicine *"Jamu"*, which is made up mainly from various rhizomes of Zingiberaceae has been consumed almost daily by people mainly women especially from the low and middle classes in Indonesia. These traditional medicines are used not only for curative purposes but also as a tonic. Drinking jamu as a beverage is also common amongst children. However, there has been no study on the effect of drinking jamu, especially of the extracts of Zingiberaceae on the health status of people in Indonesia.

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A number of herbs and spices including members of the Zingiberaceae family have been shown to have beneficial protection against degenerative diseases such as cancers in animal model studies (Milner et al., 2001; Surh, 2002). Some members of the family have been shown to possess antioxidant, antiinflammatory and anticancer activities (Rao et al., 1995; Lee et al., 1998, Surh et al., 1999). For example *Curcuma longa*, a yellow curry powder, is one of the ginger species which has been used worldwide. Its bioactive compound, curcumin, has been shown to have anticancer activity in *in vitro* and *in vivo* studies, and is currently undergoing phase II clinical trials against colon cancer in the US (Greenwald et al., 2001). However, of the large number of Zingiberaceae species, only a few members have been studied for their potential anticancer activities. Sixty three species of the family Zingiberaceae have been identified in Indonesia (Hyene, 1987) of which about 20 species are available in the market place.

1.2. Literature Review

1.2.1. Incidence of colon and breast cancers

Cancer is one of the leading causes of death in almost every country in the world. However the incidence and prevalence of cancers are different from country to country. For example, the incidence of breast cancer in Western industrialised countries can be up to five times greater then in Asian countries (Hin-Peng, 1998) and incidence of colon cancer is also higher in such societies (de Kok and van Maanen, 2000). Asian migrants have increased risk of cancers after residing in Western industrialised countries (Hin-Peng, 1998; Lawson et al., 2002), although the incidence of cancers in Asian countries is also increasing (Li et al., 2001) due to the adoption of Westernized lifestyles (Deapen et al., 2002).

Experimental and epidemiological evidence indicates that diet and nutrition are key factors in the development of breast and colorectal cancer. Walker (1999) reported that the incidence of colon cancer among Africans was very low, in contrast to the very high incidence among African-Americans

(Figure 1). He suggested that Africans eat more food plants, fermented food products but less animal foods compared to Western populations. In Asian populations, Indians have a very low rate of colon cancer compared to Chinese and Japanese (Walker, 1999). He also compared data on breast cancer incidence among Asian populations and Africans, which is relatively low compared to Westernised peoples (Figure 2). The former consume high fibre and low fat diets, which have been reported to be protective agents against breast cancer. However, he also discussed conflicting results with regard to various risk factors including diets which have been reported in the prevalence of this disease.

The incidence of colon cancer rate per 100,000 in different population
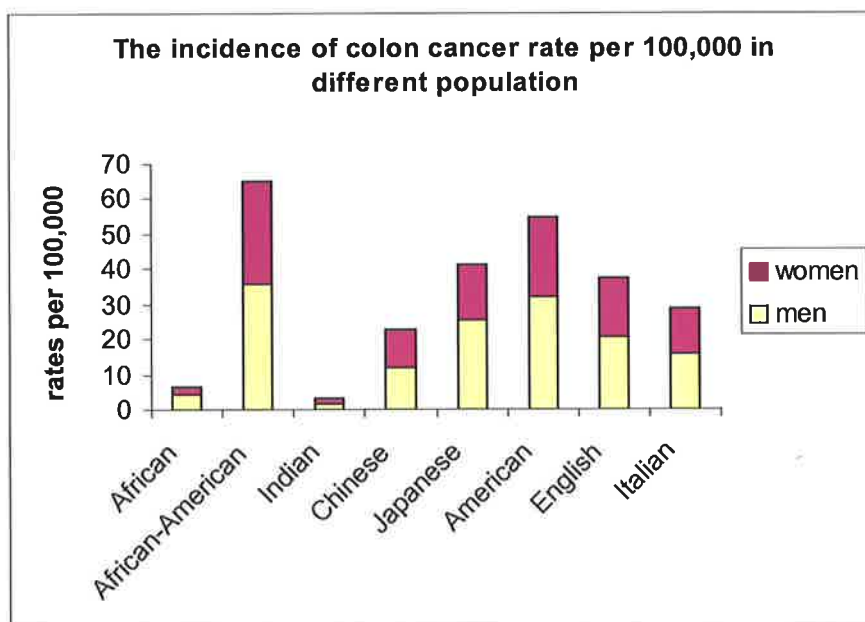
□Figure 1. The incidence of colon cancer rate per 100,000 in different populations (Walker, 1999).

In Indonesia, cancer ranks as sixth among the cause of death after infectious diseases, cardiovascular diseases, traffic accidents, nutritional deficiency and congenital diseases. However the exact incidence and prevalence of cancer in Indonesian society is not known. Based on the data in 1988-1991 the ten most frequent cancers in Indonesia were cervix, breast, lymph node, skin, nasopharynx, ovary, rectum, soft tissue, thyroid and colon (Tjindarbumi and Mangunkusumo, 2002). In Australia in 1996, the most fatal cause of death, after lung cancer and excluding non-melanocytic

skin cancer, was colorectal cancer for both men and women and followed by prostate cancer in men

and breast cancer in women (Burton, 2002).

Incidence of breast cancer rate per 100,000 in different populations
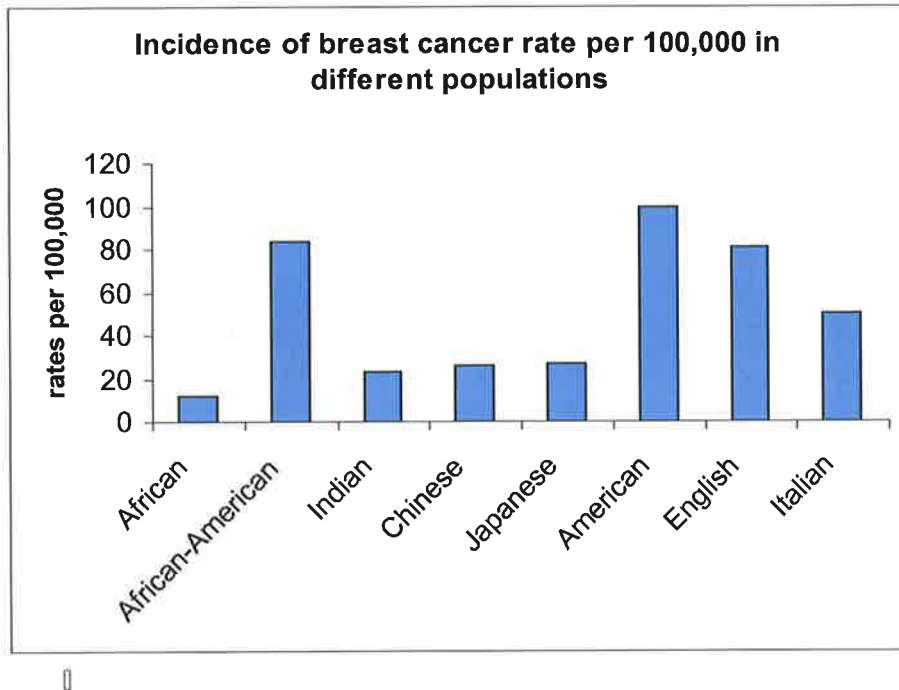
Figure 2. Incidence of breast cancer rate per 100,000 in different populations (Walker, 1999)

Kobayashi (1999) suggested that cancers were caused by interactions between internal host

factors and external environmental factors. Internal factors include ethnicity, sex, age and genetic

factors, while environmental factors are associated with lifestyle such as exposure to initiating

carcinogens and promotional or inhibitory nutritional factors.

According to Wattenberg (1985), inhibitors of carcinogenesis can be classified into three

categories according to their mechanisms of action. The first consists of compounds that prevent the

formation of carcinogens from precursor substances. The second are compounds that inhibit

carcinogenesis by preventing carcinogenic agents from reaching or reacting with critical target sites in

the tissues, ie "blocking agents". The third category is compounds which act subsequent to exposure

to carcinogenic agents and are called "suppressing agents". These compounds suppress the

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expression of neoplasia in cells previously exposed to doses of carcinogens that otherwise would cause cancer.

It is apparent that chemoprevention is primary prevention and is very effective and efficient in its impact. Such preventative agents have received a great deal of attention and are viewed as the very promising strategy for cancer prevention (Wattenberg, 1985).

Morse and Stoner (1993) explained that chemoprevention is process of inhibiting, delaying or reversing the process of carcinogenesis by the administration of one or more naturally-occuring and/or synthetic compounds. Kobayashi (1999) described chemoprevention as an important approach for cancer prevention. He defined chemoprevention as the use of certain chemicals to inhibit cancer development at the precancerous stage.

As the principle of cancer prevention could reside in a person's daily lifestyle, chemopreventive agents found in foods could be one of the most desirable and applicable preventative strategies against cancer.

1.2.2. Roles of diet and food components in prevention for cancers

Although studies have suggested that nutrients and non-nutrients have an important role on the development of colon cancer, little is known about the precise mechanisms of action and how diet interferes with the development and progression of this disease. It is therefore of interest for scientists to examine the habit and lifestyle of certain societies associated with the prevalence of cancers. Human epidemiological data and laboratory studies suggest a strong relationship between different types of diets and risk of several cancers including breast, colon and prostate cancers. However, some epidemiological data on the effect of dietary factors and cancers have given inconsistent results (Trock et al, 1990).

Dietary factors can influence the risk of cancer by inhibiting or enhancing carcinogenesis through diverse mechanisms of action. The identification and elucidation of active components in diets

as been focus of nutrition and cancer research for decades. High fat, low fibre and low calcium, known as the Western diet, have been linked with increasing risk of cancers (Burton, 2002). On the other hand, consumption of fruits and vegetables and diet with low fat, high fibre and high calcium have been linked with low risk of cancer (Lipkin et al., 1999). Scientists have also associated non-nutrient involvement in daily life as a precaution of low incidence of cancers in some countries for example drinking tea (Kuroda and Hara, 1999), consumption of soybean-based products (Brouns, 2002) and certain herbs and spices (Surh, 2002).

Plant foods have been reported to have benefits in cancer prevention due to the presence of phytochemicals, non-nutritive compounds that possess "health protective effects" (Craig, 1997). Such plant foods include fruits, vegetables, nuts, soybean, herbs and spices. *In vitro* and *in vivo* studies have shown that some vegetables and, to a lesser extent, fruits contain minor non-nutrient constituents capable of inhibiting the growth of cancers. Epidemiological studies have shown that diets containing large quantities of vegetables and fruits are associated with reduced risk of certain types of cancers (Williams, 1993) and low intake of fruits and vegetables are strongly associated with an increased prevalence of stomach cancer, due to inadequate levels of micronutrients and antioxidants. The occurrence of cancers can be prevented by a large number of those compounds in plants (Wattenberg, 1993).

1.2.3. <u>Biological activity of extracts of members of Zingiberaceae</u>

Like other food components such as soybean and tea which may have benefit to reduce risk of certain cancers, people from some Asian regions also include lots of herbs and spice in their food intake. The use of medicinal plants, or their crude extracts, in the prevention and/or treatment of several chronic diseases has also been traditionally practiced in Asian ethnic societies. The Zingiberaceae family is one of the family plants which has been used for colouring, preserving, as

spices and medicine. Members of Zingiberaceae have been shown to possess antioxidant, antiinflammatory and anticancer activities (Surh, 2002).

Studies of the anticancer activities of some members of Zingiberaceae have been conducted, especially *Z. officinale*, *C. longa*, *Alpinia oxyphylla* and more recently on *Z. zerumbet* and are summarised in table 1.

Table 1: Anticancer-related studies of Zingiberaceae species of and their

Name of Species	Specific extract/compound	Conclusion of Studies	References
Curcuma longa	Turmeric powder	At 160 mg/g diet reduced the incidence of B[a]P-induced neoplasia in mice and 3'MeDAB-induced hepatomas in rat by 50%	Aruna and Sivaramakrishnan (1992)
C. longa	Ethanolic extract	2% turmeric diet reduced the incidence of DMBA-induced skin tumorigenesis in mice by 74% and 5% turmeric decreased the incidence of BP-induced fore-stomach tumors by 40%	Azuine and Bhide (1992)
C. longa	curcumin	2000 ppm curcumin inhibited AOM-induced ACF formation in rats	Rao et al. (1993)
C. longa	Commercial grade curcumin	0.5-2.0% curcumin inhibited BP-induced forestomach, AOM-induced colon and ENNG-induced duodenal tumorigenesis	Huang et al. (1994)
C. longa	curcumin	10 µg/ml curcumin reduced lung tumor nodules in B16F10 melanoma-induced mice by 89.3%	Menon et al. (1999)
C. longa	curcumin	0.2% curcumin in the diet reduced tumor multiplicity up to 81% and reduced incidence of DEN-induced hepatocarcinogenesis in	Chuang et al. (2000)

		murine by 61%	
C. longa	curcumin	2% curcumin in the diet suppressed tumor growth in initiation and progression stages of prostate cancer model using nude mice	Dorai et al. (2001)
Zingiber officinale	Ethanol extract	Topical application of extract at 4 mg/mouse inhibited TPA-induced skin tumorigenesis by 77%	Katiyar et al. (1996)
Z. officinale	[6]-gingerol	Topical application of [6] gingerol at 4 μmol/μl reduced tumor incidence and tumor burden in DMBA-induced skin carcinogenesis	Park et al. (1998)
Alpinia oxyphylla	Methanol extract	Topical application of extract at reduced incidence and multiplicity of skin papillomas by 60%	Lee at al. (1998)
Zingiber zerumbet	zerumbone	0.05% zerumbone in the diet reduced AOM-induced ACF	Tanaka et al. (2001)

It is not surprising that much research has been conducted on the extracts of *C. longa* and its active compound, curcumin, since this plant has been used worldwide as a spice, preservative and colouring agent. The findings strongly indicate that extracts of *C. longa* show chemopreventive activity *in vitro* and in animal cancer model studies against various types of cancers. However, many members of the ginger family, which have been used as traditional medicines in certain regions of Asia have not been extensively studied. Vimala et al. (1999) screened anticancer activity of extracts of eleven species of Zingiberaceae using 12-*O* tetradecanoylphorbol 13-acetate (TPA)-induced Epstein-Barr virus early antigen (EBV-EA) in Raji cells. They found that extracts of *C. longa*, *Z. cassumunar* and *Z. zerumbet* possessed strong inhibitory activity. Extracts of *C. mangga*, *C. xantorrhiza*, *K. pandurata* and *C. aeruginosa* have been found to show a strong toxicity in Raji cells at concentrations of 20 – 640 g/ml. However, the concentrations of active components in each species were not stated.

Different species of Zingiberaceae have different active compounds. *C. longa* contains three active curcuminoids: curcumin, demethoxy-curcumin and bisdemethoxycurcumin. Curcumin is the major component which has been extensively studied. However, Simon et al. (1998) reported that demethoxycurcumin was the most active compound found in *C. longa*, followed by curcumin and bisdemethoxycurcumin. These curcuminoids have the same number of hydoxyl groups but different numbers of methoxyl groups. Their individual structures do not show any consistency with their activity. Simon et al (1998) suggested that diketone systems in curcuminoids may play an important role in anticancer activity. The chemical structure of these curcuminoids and other bioactive compounds isolated from the ginger family are shown in figure 3. Surh (1999) reported that *C. zedoaria*, which is used as medicine also contains curcuminoids.

The active compounds found in *Z. officinale* are pungent compounds: 6-gingerol (1-[4'-hydroxy-3'-methoxyphenyl]-5-hydroxy-3-decanone) and 6-paradol (1-[4-hydroxy-3-methoxyphenyl]-3-decanone) (Surh, 1999).

Diarylheptanoids structurally-related to curcumin, which are present in some species of the ginger family such as yakuchinone A and B in *Alpinia oxyphilla* (Flynn and Rafferty, 1986) and non phenolic diarylheptanoids in *C. xanthorrhiza* (Claeson et al, 1996) have been identified as antiinflammatory agents.

Members of Zingiberaceae are also known to be rich sources of essential oils (terpenes) most of which show some pharmacological activity. It is therefore of interest to isolate and identify the active compounds, especially from those species used in normal nutrition.

I ($R_1 = R_2 = OH$, $R_3 = R_4 = OCH_3$)
II ($R_1 = R_2 = OH$, $R_3 = OCH_3$, $R_4 = H$)
III ($R_1 = R_2 = OH$, $R_3 = R_4 = H$)
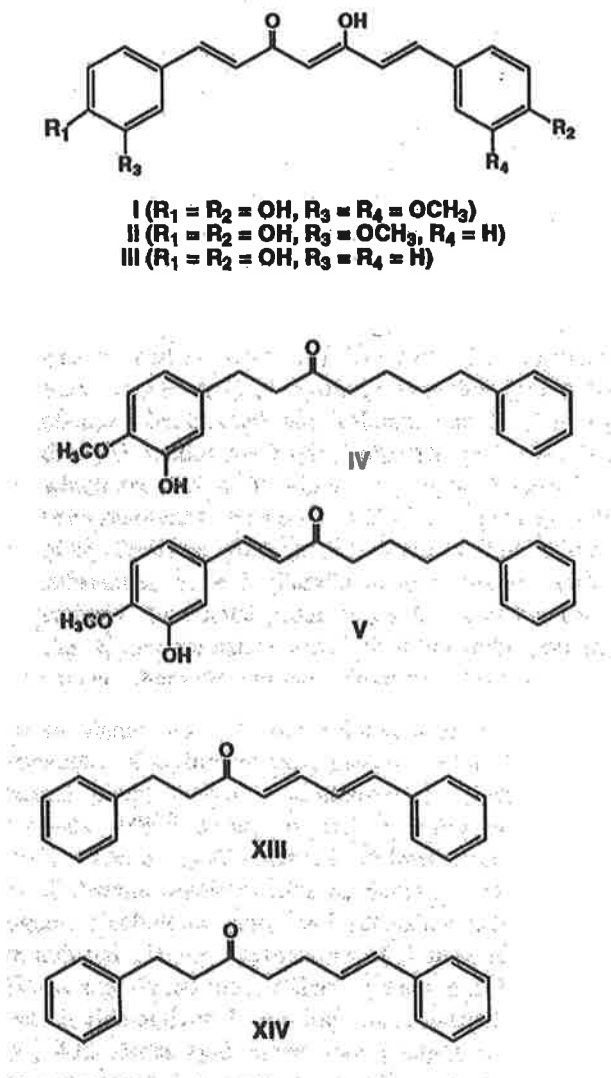
Figure 3.
Chemical structures of some naturally occurring diarylheptanoids isolated from plants of Zingiberaceae. Curcumin (I) and its demethoxy (II) and bis demethoxy derivatives found in *C. longa*. Yakuchinone A (IV) and yakuchinone B (V) found in *A. oxyphilla* and non-phenolic diarylheptanoids (XIII and XIV) isolated from *C. xanthorrhiza*.

1. 2.4. Cancer aetiology

Cancers are diseases initiated mainly by changes in genes. They are caused by an accumulation of gene alterations including activated oncogenes and/or inactivated suppressor genes (Bale and Li, 1997). Carcinogenesis is a multistage process which involves initiation, promotion and progression of transformed accelerated "uncontrolled cell" growth (Tokudome, 1999).

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There is a very close association between oxidative stress, inflammation and tumor promotion. In the intracellular system of every aerobic organism, a balance between oxidant and antioxidant is vital for physiological processes. Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide are initially produced as a normal host-defense mechanism. However due to their high reactivity, ROS are prone to cause damage to normal tissues and are thereby potentially toxic, mutagenic or carcinogenic to epithelium and connective tissues (Fitzpatrick, 2001). Antioxidants serve to protect cells and organisms from the lethal effects of excessive ROS formation by eliminating the unpaired electron or acting as a free radical scavenger (Lopaczynski and Zeisel, 2001).

Inflammation is a complex process involving many different cell types, signalling factors, tissues and oxidants which destroy invading organisms and damage normal tissues. Inflammation is produced by a necessary response of the host to counteract the threat of infectious agents and other foreign bodies. The inflammatory response is coordinated by the number of immune cells such as macrophages, B and T lymphocytes, basophils, eosinophils and mast cells. A large number of mediators produced by these cells play a key role in the inflammatory response including prostaglandins (PGs) and leukotrienes (LT) (Prescott and Fitzpatrick, 2000). Thus inhibition of those mediators by some agents is an effective mechanism of chemoprotection against inflammation.

1.2.5. Colorectal Cancer

Colorectal carcinogenesis is a complex multistage process involving both genetic and environmental factors and is thought to result from an accumulation of multiple genetic changes which resulting in a transformed phenotype and eventual progression of cells to cancer (Fearon and Vogelstein, 1990). Therefore agents capable of causing DNA damage may be potentially carcinogenic. A model of colorectal development and the potential target therapy is depicted in figure 4. There are two main processes by which a cell becomes an invasive cancer cell, initiation and promotion. Initiation occurs as a result of DNA damage and mutations that are likely to proceed along

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the multistage pathway of carcinogenesis. A number of initiating agents have been identified to cause

colon cancer including chemical mutagens (such as heterocyclic amines), dietary contaminants,

irradiation, pathogenic bacteria and viruses (Migliore and Coppede, 2002).
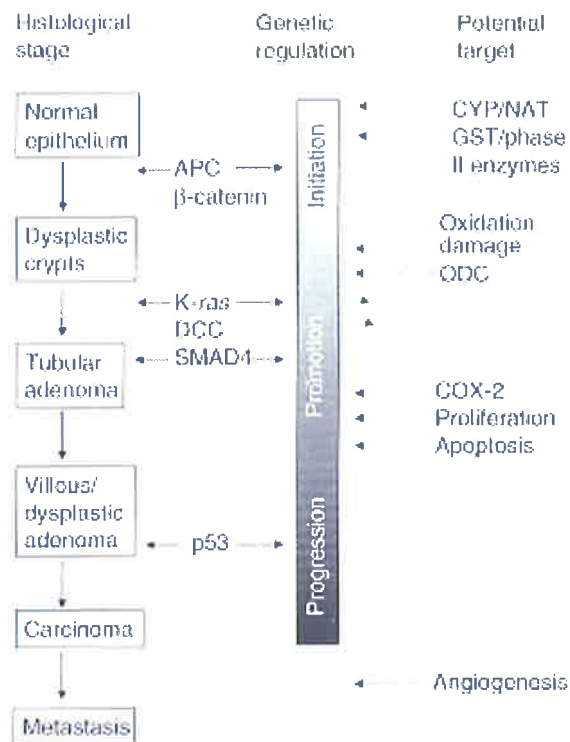
Figure 4. Multistep model of carcinogenesis with targets for chemoprevention. Significant genes involved in regulation at sequential stages in carcinogenesis include adenomatous pholyposis coli (APC), K-ras, DCC and p53. A number of inducible enzymes are involved at critical stages in promotion and/or protection. COX-2, cyclooxygenase; CYP, cytochromes P450; DCC, deleted in colorectal cancer; GST, glutathione S-transferase; NAT, N-acetyl transferases; ODC, ornithine decarboxylase (Sharma et al., 2001)

The increasing incidence of colon cancer in the Western industrialised world suggests that

environmental factors and energy excess and inadequacy promote the development of the disease. *In*

vitro, in vivo and epidemiological studies have demonstrated a risk reduction for colon cancer with

certain drugs such as non-steroidal antiinflammatory drugs, and dietary constituents for example

calcium and antioxidants (Krishnan et al., 2000). Moreover preclinical evidence showed that certain

non-cytotoxic drugs may alter proliferation rate of potential precancerous colonic epithelial cells and

programmed cell death (apoptosis). Chemopreventive strategies are therefore feasible for colon cancer. Animal models of the disease allow for preventative and therapeutic intervention to be carried out in a controlled manner as well as the development of early marker or therapies for colon carcinogenesis using chemical carcinogens such as azoxymethane (AOM), methoxymethane (MAM) and 1,2-dimethylhydrazine (DMH) (Weisburger et.al., 1977).

1.2.6. Biomarkers of colon cancer

Biomarkers are increasingly used for screening, chemoprevention and chemotherapy programs. There are many types of preneoplastic biomarkers to identify colorectal cancer: pathological for example tumour histology and aberrant crypt foci (ACF), cellular (cell proliferation and apoptosis), biochemical (polyamine, arachidonic acids, other enzymes), molecular (cell cycle and DNA adducts) and genetic markers (Sharma et al., 2001). In this study, apoptosis and cell cycle assay were analysed in a cell culture study and ACF were used as biomarkers in the carcinogen induced colon cancer model.

1.2.6.1. Cell Cycle and Proliferation

Cell proliferation has long been suspected of enhancing the frequency of tumor initiation and is considered to play an important role in the tumor promotion. Control of cell proliferation is therefore important for cancer prevention and effective agents are expected to suppress cell proliferation and inhibit the occurrence of malignant lesions (Mori et al., 1999). Cancer cells have a diverse set of phenotypic abnormalities such as lack of control response to cell-cell interaction, resulting in loss of regulatory cell growth signal or dysregulation of cell cycle control. These cells will proliferate faster than normal cells and undergo dedifferentiation. During cancer development, the cancer cells have increased motility or invasiveness and also may have decreased sensitivity to drugs (Kastan, 1997).

Chemopreventive agents might alter one or more of these abnormal processes in cell cycle and therefore result in inhibition of cell proliferation.

Cell proliferation and regulation of the cell cycle seem intimately associated with apoptosis, such that dysregulation of the cell cycle may directly affect sensitivity to apoptotic stimuli. The tumor suppressor gene p53 has an essential role in surveillance of DNA damage, regulating the cell cycle and apoptosis. Current evidence suggest that p53 functions to detect DNA damage and subsequently arrest cells in the G1 phase of the cell cycle to allow for repair. However if the damage cannot be repaired then apoptotic cell death is triggered (Mendoza-rodriguez and Cerbon, 2001).

1.2.6.2. Apoptosis

Apoptosis or programmed cell death occurrs in physiological conditions, for example organogenesis, adult tissue homeostasis and immune system development or in response to external stimuli such as DNA damaging agents, growth factor deprivation or death receptor binding. It is also an important component in the aetiology and pathophysiology of some human diseases including Alzheimer's syndrome, autoimmune disease, cancer and AIDS (Thompson, 1995). Apoptosis is considered to have an important role in carcinogenesis to remove damaged precancerous cells and therefore is a valuable strategy for the management of cancer (Lowe and Lin, 2000). Apoptosis is initially described by its morphological characteristics, including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (180-200 bp DNA fragment) and formation of apoptotic bodies (Kerr et al., 1972). Biochemical features of apoptosis are characterized chronologically by the activation of ICE-related proteases (caspases), mitochondrial permeability transition leading to the disruption of the mitochondrial transmembrane potential, exposure of phosphatidylserine (PS) to the outer leaflet of plasma membrane and DNA cleavage (Van England et.al, 1998). It is suggested that apoptosis is induced through p 53-dependent or independent

pathways and both pathways may interfere the mitochondrial membrane and facilitate cytochrome c

release and then lead to cascade of caspases (Gao et al., 2001) as depicted in figure 5.
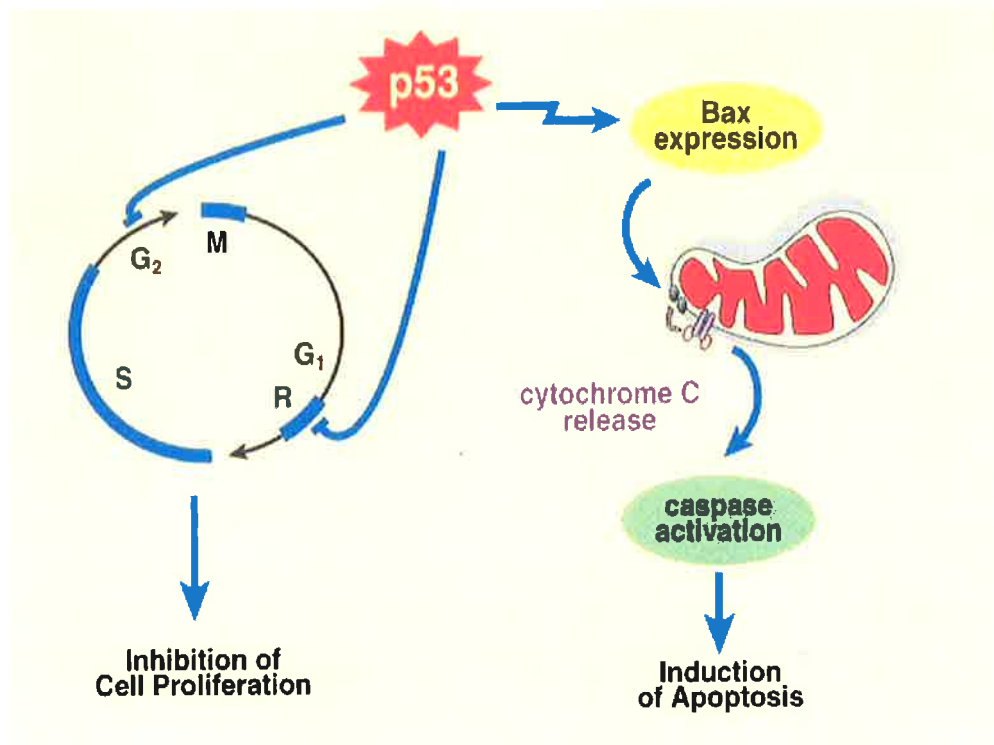
Figure 5. The role of a gene suppressor, p 53, in inducing apoptosis and influenced cell cycle arrest to inhibit cell proliferation. p 53 reacts to DNA damage by activating transcription-dependent and – independent pathways that lead to cell cycle arrest or apoptosis thereby preventing proliferation of cells with a damaged genome.

Apoptotic cell death can be distinguished from necrotic cell death. Necrotic cells are a

pathological form of cell death resulting from acute cellular injury, which is typified by rapid cell

swelling and lysis (Thompson, 1995). Apoptosis is believed to protect against carcinogenesis by

removal of mutated cells. Cells undergoing apoptosis will rapidly be phagocytosed by macrophages

and neighbouring cells without showing inflammation. Therefore, defects in apoptosis regulating

genes for example p 53 are important for progression of human cancer (Thompson, 1995).

1.2.6.3. Aberrant crypt foci (ACF)

The ACF is considered to be a precursor lesion of colonic adenomas and carcinomas and is often used as an intermediate biomarker for colorectal tumorigenesis. It was first identified by Bird (1987) in the colons of carcinogen treated rodents and has been shown to be present in humans who have at greater risk of developing colon cancer (Pretlow et al., 1992).

Aberrant crypts can easily be distinguished from surrounding normal crypts under a light microscope after staining with methylene blue. They are a number of changes, such as enlarged crypt diameter, darker staining appearance, thicker epithelial lining and slit like lumens (figure 6).
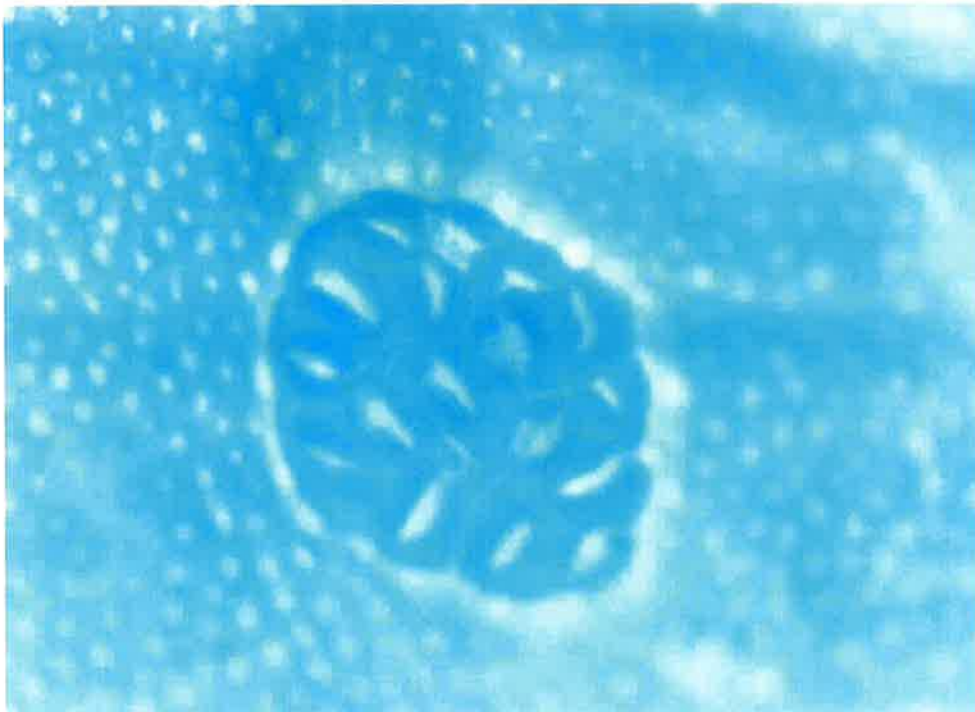
Figure 6. Colon tissue stained with methylene blue and observed under light microscopy using 10X magnification. ACF with 17 AC per focus surrounding by normal colonocytes (from an experiment in chapter 5).

By modifying the diet before, during and/or after exposure to the carcinogen it is possible to assess the chemopreventive/promotive influence of the diet using ACF expression in the colons of these animals. The advantage of this assay is that it is possible to examine the carcinogenic process during the precancerous stage, thereby enabling a shorter experimented time than a tumour study,

fewer animals and therefore the study is less costly (Corpet and Tache, 2002). ACFs appear within two weeks after injection and initially appear as single abnormal crypt. As time progresses, a single ACF may expand by crypt branching or multiplication (Bird and Good, 2000). ACFs predicted the tumor outcome in several rodent studies and were correlated with colon cancer risk and adenoma size and number (Corpet and Tache, 2002). However it must be acknowledged that not all ACF will progress to adenoma or carcinoma. It is suggested by some groups that the larger ACF ($\geq$ 4 aberrant crypt per focus) are more predictive (Uchida, 1997).

1.2.6.4. Prostaglandins and cyclooxygenase

Prostaglandins are produced by the action of the prostaglandin endoperoxidase, which is also called cyclooxygenase (COX), on arachidonic acid (AA). There are two forms of COX, a constitutive enzyme (COX-1) present in most cells and tissues and an inducible isoenzyme (COX-2) expressed in response to cytokines, growth factors and other stimuli (Simon, 1999). COX-1 is involved in the regulation of normal homeostatic functions, while COX-2 is responsible for many pathological processes such as inflammation and tumor development. COX-2 is upregulated in various tumors including colon (Reddy et al., 1996) and breast cancers (Hwang et al., 1998). Inhibitors of COX-2 suppress proliferation and differentiation of human leukaemia cell lines (Nakanishi et al, 2001). Lipoxygenase (LOX), which generates bioactive prostanoids from arachidonic acid, is also found to be very potent in supporting tumor progression. LOX metabolites such as 12(S) hydroxy eicosatetraenoic acid (HETE) are produced during hyperproliferation and tumor development (Nie and Honn, 2002).

1.2.7. Inflammation and colorectal cancer

A chronic inflammatory response to damage caused by chemicals or biological toxins is associated with increase ambient tissue oxidative stress which in turn has been linked with carcinogenesis. Rudolf Virchow who first described the inflammatory disease process noted

leucocytes in neoplastic tissue and made a connection between inflammation and cancer in 1863 (Balkwill and Mantovani, 2001). Epidemiological data showed that persistent inflammation elevated the risk of cancer in involved organs (Prescott and Fitzpatrick, 2000). It is possible therefore that compounds with antioxidant and antiinflammatory properties may have anticancer activity. Clinical studies showed that the risk of having colorectal cancer from a patient who has had inflammatory bowel disease (ulcerative colitis) for 15-20 years increased by 1 %. After 20 years of the disease, the cumulative risk of colorectal cancer increased by 7-10 % (Rudy and Zdon, 2000). Ekbom (1998) summarised the situation that colorectal cancer was a major cause of the increased morbidity and mortality in patients with ulcerative colitis.

Macrophages are a major component of the infiltrate of most, if not all, tumors   and   other inflammatory cells and cytokines found in tumors are more likely to contribute to tumor growth, progression and immunosuppression (Balkwill and Mantovani, 2001). The hypothesis of mechanisms for colon cancer development as a consequence of inflammation is depicted on figure 7 (Rhodes and Campbell, 2002). Obyrne and Dalgleish (2001) suggested that inflammatory process with upregulation of COX-2, to the production of inflammatory cytokines and prostaglandins may suppress cell mediated immune responses and promote angiogenesis. This provides the prerequisite environment for the development of malignancy. These factors may also impact on cell growth and survival signalling pathways resulting in induction of cell proliferation and inhibition of apoptosis. Apoptosis has a vital role in the deletion of cells with potentially carcinogenic mutations. Glycosylation abnormalities, which occur in IBD may affect intracellular, cell surface and secreted glycoconjugates such as shortening of O-linked oligosaccharides (e.g. sialyl Tn (sialyl 2,6, N-acetylgalactosamine α-)). Sialyl Tn expression has been shown to be a marker of high risk for cancer development (Rhodes and Campbell, 2002).

The common precursor lesion of colorectal cancer (CRC) in UC is epithelial dysplasia which is defined as an unequivocal neoplastic change in the colonic epithelium (Ridell et al., 1983). The development of colorectal adenocarcinoma arising via the dysplasia-carcinoma morphological

sequence was shown using a DSS-induced model of UC, after long-term administration in animal model (Kullmann et al., 2001; Serill et al., 2002).
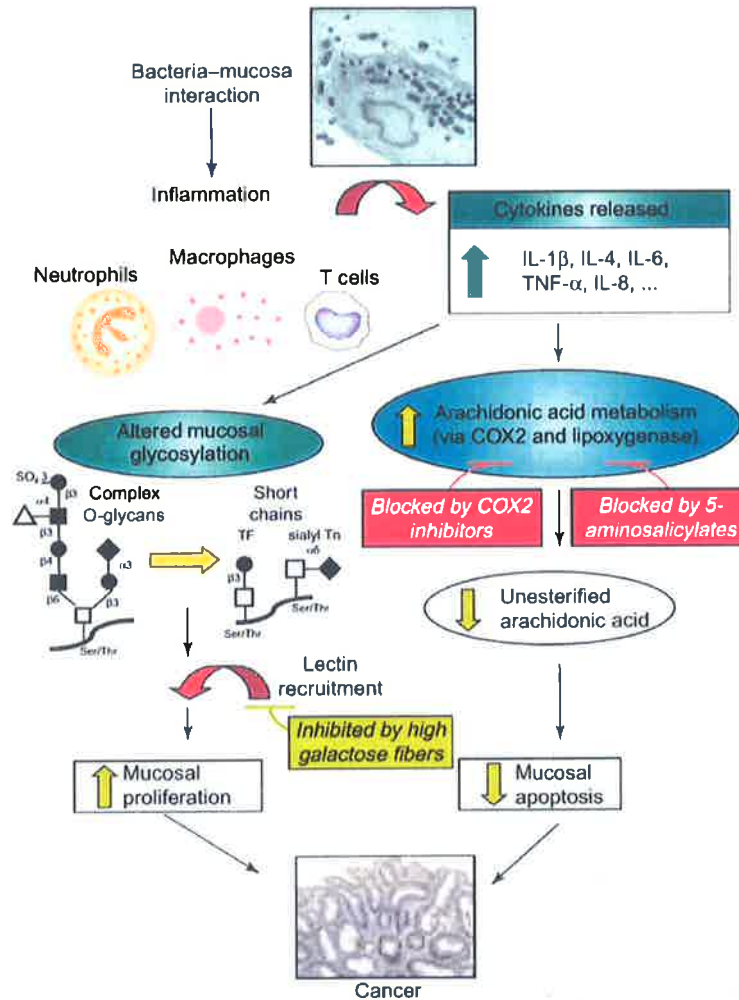
Figure 7. Mechanisms for colon cancer development a consequences of inflammation. Glycosylation abnormalities and prostaglandins metabolism in inflamed colon mucosa may indirectly inhibit apoptosis and increase proliferation which lead to colon carcinogenesis (Rhodes and Campbell, 2002).

1.2.8. Inflammatory Bowel Disease

The inflammatory bowel diseases (IBD), which include Crohn's disease (CD) and ulcerative colitis (UC), are chronic, spontaneous multifactorial diseases of unknown aetiology. Research on the causative factors for these diseases is very important in order to provide a rationale for therapeutic

intervention and an understanding of the complications of these diseases (Guarner et al., 2002). These diseases are suggested to be immunologically mediated and to have genetic and environmental influences on their expression. The incidence of IBD is much higher in westernised countries and is associated with high sucrose consumption and high intakes of animal fat (Reif et al, 1997; Corrao et al., 1988). Rhodes has proposed that hereditary factors alter surface glycoprotein and mucin glycolsylation patterns in the intestine which are responsible for increased risk for both UC and CD (Rhodes, 1996).

It is suggested that UC and CD share similar genetic or immunoregulatory abnormalities but are separate entities responding to different immunological stimuli (Sartor, 1995). Fundamental differences in disease location, histology and immunological responses are listed in table 2. A trigger in UC, most likely an antigen, activates T-lymphocytes that release cytokines, thereby recruiting large numbers of neutrophils and mononuclear cells in to the colon mucosa. Subsequent activation of these cells causes a self-augmenting cycle of cytokine production, cell recruitment and inflammation. In addition to cytokines, leukotrienes, thromboxane, platelet-activating factor, nitric oxide and reactive oxygen species are released from activated mucosal cells-predominantly from neutrophils and macrophages. The increased influx of neutrophils, macrophages and increased production of inflammatory mediators are major components of active lesion UC. The large numbers of those cytokines pass out of the circulation and enter the inflamed mucosa and submucosa leading to overproduction of oxygen free radicals (Ogawa et al. 2002).

Myeloperoxidase is an enzyme found in neutrophils and at a much lower concentration in monocytes and macrophages. The enzyme catalyses the oxidation of electron donors (for example halides) by hydrogen peroxide. The level of MPO activity in a suspension of neutrophils is directly proportional to the number of neutrophils present over a wide range of neutrophil concentrations. Myeloperoxidase is used as a biomarker for UC (Krawisz et al., 1984).

Table 2. Clinical, immunologic and genetic differences between ulcerative colitis and chrohn's disease (Sartor, 1995)

	Ulcerative Colitis	Crohn's Diseases
Disease location	Colon only	All regions of gastro-intestinal tract
Inflammatory response	Mucosal	Transmural, granulomatous
Lymphokine profile	IL-4, IL-10 (TH$_2$)	IL-2, IFN-$_\gamma$ (TH$_1$)
Immunoglobulin profile	IgG$_1$	IgG$_2$
Epithelial IgG and complement disposisiton	Yes	No
Association with smoking	Negative	Positive
Autoantibodies	Yes	No
HLA haplotype	DR-2	DR-1, DQw5

Clinical symptoms of patients with UC include weight loss, blood in the faeces and diarrhoea (Murthy et al., 1993). Diarrhoea in UC is a multifactorial event, influenced by exudation of blood, plasma and interstitial fluid from the ulcerated colon mucosa, subsequently decrease absorption due to inhibition of Na$^+$ and Cl$^-$ absorption by inflammatory mediators, immature and poorly functioning epithelial cells, loss of surface area and bacterial growth and therefore enhance electrolyte secretion and cause rapid transit due to alteration of smooth muscle contraction (Sartor, 1995).

The medical treatment of ulcerative colitis is mainly by the use of antiinflammatory drugs, corticosteroids, sulfasalazine or salicylates. However therapeutic failures and relapses are common and have been associated with a relatively high incidence of adverse side-effects (Guslandi, 1998). The usefulness of potential immunomodulators agent such as azathioprine, 6-mercaptopurine and cyclosporin, are considered to be too toxic at a high dose for patients with severe active UC (Farrel and Peppercorn, 2002). Current research showed that probiotics and prebiotics (such as inulin) appear as the most promising of several experimental and traditional agents so far investigated and are currently undergoing clinical trial (Guarner et al., 2002). Salicylate-containing plants have been utilized in several cultures for centuries to relieve the signs of inflammation and a number of plants have been screened for their ability to reduce mediators of inflammation, such as prostaglandins and nitric oxides (Sautebin, 2000).

Continuous oral administration of acetic acid, trinitrobenzene sulfonic acid (TNBS) or the sulphated polysacharide dextran sulphate sodium (DSS) in drinking water produces an acute distal colitis in rats and mice which shares clinical and histopathological characteristics in common with human UC (Okayasu et al., 1990; Cooper et al., 1993). They have been used as models for investigating the inflammatory mechanisms involved and for evaluating the effects of differing therapeutic strategies.

The DSS-induced colitis of rodents is characterized by initial acute colonic injury, followed by a slow colonic regeneration and concomitant chronic colitis, after stopping the administration of DSS in drinking water (Okayasu et al. 1990; Gibson et al., 1996). Colonic mucosa of rats treated with DSS for one week appeared edematous and with haemorragic erosions. The histological features of ulcerative colitis include destruction of epithelium and glands, crypt loss, inflammatory cells infiltration of the sub-epithelium and lamina propria (Gaudio et al., 1999).

1.2.9. Antioxidant and antiinflammatory mechanisms of the ginger family

Turmeric extract and curcumin were reported to possess substantial antioxidant properties as determined by inhibition of phospholipid peroxidation and of xanthine oxidase activity (Selvam et al., 1995) which are responsible for the generation of reactive oxygen species. Curcumin was also shown to induce gluthathione S-transferase (GST) and other gluthatione (GSH)-linked enzymes to detoxify electrophilic products of lipid peroxidation (Piper et al., 1998). Oyama et al. (1998) also found that 5' n-alkylated curcumins isolated from C. cassumunar had protective effects on living cells suffering from oxidative stress. Similar antioxidant activities were found in Z. officinale, [6]-gingerol attenuated TPA-stimulated production of superoxide generation in differentiated human premyelocytic leukemia (HL 60) cells (Surh et al., 1999).

Clinical and epidemiological data have indicated that nonsteroidal antiinflammatory drugs (NSAIDs) are inhibitors of cyclooygenase, induce a significant regression of colonic polyps in

patients with familial adenomatous polyposis (FAP) and are preventive in non familial adenomatous polyposis subjects (Thun et al, 1993). However NSAIDs inhibit COX-1 as well as COX-2 which in long term use cause side effects such as gastric irritation and renal malfunction. Members of Zingiberaceae also have been shown to have effects on prostaglandin endoperoxidase enzymes. Curcumin was a potent inhibitor of cyclooxygenase and lipoxygenase activities in TPA-treated mouse epidermis (Huang et al., 1991) and also in liver and colonic mucosa of AOM-induced F344 rats (Rao et al., 1995). Curcumin inhibited COX-2 but not COX-1 in HT-29 human colon cancer cells (Goel et al., 2001). Rat peritoneal macrophages preincubated with curcumin showed inhibition of PGE2, Leukotriene B4 and C4 (Joe and Lokesh, 1997). Curcumin also was reported to inhibit the cytokine tumor necrosis factor-α (TNF) which induces the production of the inflammatory mediator interleukin-1β (Chan, 1995). Alcoholic extract of *Z. officinale* inhibited COX and LOX activities (Katiyar et al.,1996).

According to Wattenberg (1985), curcumin is classified as a blocking agent, that is preventing the formation of carcinogenesis from precursor substances and/or preventing carcinogenic agents reaching or reacting with critical target sites in the tissues. Topical application of curcumin strongly inhibited TPA-induced inflammation and ornithine decarboxylase (ODC) activity (Lu et al., 1993; Mehta et al., 1997). It has been suggested that the inhibitory activity occurred by reducing synthesis and/or enhancing the breakdown of ODC mRNA. Rao et al (1993) also reported that dietary curcumin inhibited AOM-induced ODC and tyrosine phosphate kinase (TPK) in the rat colon. Hong et al. (1999) investigated the effects of curcumin on breast cancer cells and found that curcumin inhibited a 185 k-Da protein that had tyrosine kinase activity. Alcoholic extracts of *Z. officinale* also inhibited hyperplasia, edema and ODC activity (Katiyar et al., 1996). Extracts of ginger inhibited skin tumorigenesis by inhibition of cell proliferation.

The antiproliferative effect of curcumin occurred by preferentially arresting cells in the G2/S phase of the cell cycle (Mehta et al., 1997). In addition, Huang et al. (1997) reported that the

biosynthesis of DNA and RNA was also strongly inhibited 94% and 93% respectively by curcumin, at a concentration of 8 μM in HeLa cells.

Extracts and bioactive compounds of members of Zingiberaceae have been shown to induce apoptosis in *in vitro* and *in vivo* studies. Lee et al. (1998) reported that a methanolic extract of *Alpinia oxyphilla* induced apoptosis in HL-60 cells and Khar et al. (1999) reported that curcumin at a concentration of 10 μM, induced apoptosis in AK-5 tumor cells. Apoptotic bodies and cellular DNA fragmentation have been examined and that caspase-3 and reactive oxygen intermediates were involved in apoptosis induced by curcumin. These findings were also reported by Samaha et al. (1997) and supported by Bhaumik et al. (1999). However, pungent vanilloids: 6-gingerol and 6-paradol in ginger also induced apoptosis (Lee and Surh, 1998). Jee et al. (1998) reported that curcumin induced apoptosis in human basal cell carcinoma cells by upregulation of the tumor suppressor gene p 53.

Turmeric has low toxicity and is safe, used in certain regions of Asia as a dietary spice. Turmeric containing curcumin is consumed at the rate of up to 100 mg/day (Ammon and Wahl, 1991). In a phase I clinical trial in China, oral dose of 8000 mg/day showed no adverse effects (Chuang et al., 2000). Animals fed with diet supplemented with curcumin have shown no significant induction of chromosomal damage or change in mitotic index (Sukla et al., 2002). It also had antigenotoxic potential against cyclophosphamide (CP), a well-known antimutagen. Cancer cells are more susceptible to curcumin than are normal cells (Ramachandran and You, 2000; Lee and Surh, 1999).

1.3. Hypothesis

Members of Zingiberaceae (ginger family) have traditionally been used to treat inflammation in some societies, for example Indonesian, are considered to have benefit from their consumption. It is hypothesised that extracts of Zingiberaceae rhizomes, which have been used in traditional medicines especially for antiinflammation and as spices in food may have cancer protective

properties. The compounds found in the species most active in cancer cell screening test might have similar or different and unique chemical structures compared with the active compounds isolated from *C. longa*, which have been extensively studied. They may in fact have stronger anticancer activity than those found in *C. longa*.

1.4. Aims of study:

The objectives of this study were

- to screen the inhibitory activity of ethanol extracts of eleven ginger species using cancer cell lines *in vitro*: human HT-29 colon adenocarcinoma and MCF-7 breast cancer cells

- to evaluate the toxicity of extracts of eleven species using non-transformed skin fibroblast cells

- to fractionate, isolate and identify bioactive compounds found in the two most active species, *Z. aromaticum* and *B. pandurata*

- to evaluate anticancer activity of the bioactive compounds zerumbone (from *Z. aromaticum*) and panduratin A (from *B. pandurata*) using human HT-29 and CaCo2 colon and MCF-7 breast cancer cells

- to investigate the anti cancer activity of active species using azoxymethane (AOM) induced-aberrant crypt foci (ACF) in male Sprague Dawley rats

- to investigate antiinflammation activity and elucidate mechanisms of extract of *Z. aromaticum* using dextran sulfate sodium (DSS) induced ulcerative colitis using male Sprague Dawley rats

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. *Materials*

2.1.1. Ginger species

Roots and rhizomes of 10 species from the Zingiberaceae family, *Curcuma longa* LINN., *C. mangga* VAL., *C. xanthorrhiza* ROXB., *C. aeruginosa* ROXB., *Zingiber aromaticum* VAL., *Z. cassumunar* ROXB., *Z. officinale* ROSC., *Kaempferia pandurata* ROXB., *K. rotunda* LINN., *K. galanga* LINN. and seeds of *Amomum cardamomum* WILLD were purchased from the market place in Malang, East Java, Indonesia. They were sliced and air-dried and brought to Adelaide, South Australia with appropriate quarantine clearance.

2.1.2. Cell Cultures

HT-29 and CaCo2 human colon cancer cells and MCF-7 human breast cancer cells were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). Non-transformed SF 3169 skin fibroblasts were obtained from the Chemical Pathology Department of the Women's and Childrens's Hospital, South Australia.

2.1.3. Animals

Male outbred Sprague-Dawley rats were purchased from the Animal Research Centre at Murdoch University (Perth, Western Australia) and housed in wire cages to minimize coprophagy. They were maintained in an air-conditioned environment at 23 ± 2 °C with a 12:12-hour light-dark cycle. Rats were given free access to food and water. All experimental procedures involving animals had been approved by the Commonwealth Scientific and Industrial Research Organization (CSIRO)

Health Sciences and Nutrition Animal Experimentation Ethics Committee and The University of Adelaide Animal Experimentation Ethics Committee before commencing.

2.1.4. Experimental base diet

The experimental diets were modified forms of the AIN-93 (Reeves et al., 1993) semi-purified diet (Table 2.1). The vitamin and mineral mixtures were prepared according to the AIN 93 formula (Reeves et al., 1993) with modification as listed in table 2.3. and 2.4. respectively.

Table 2.1. AIN-93A diet (Reeves et al., 1993) and experimental base diet

Ingredient	(g/100 g)	AIN-93 (Reeves's)	Experimental diet
Protein	20.0	casein	casein
Sugar	20.0	sucrose	sucrose
Starch	45.0	dextrinized cornstarch	cornstarch
Fibre	5.0	Solko-floc	Alpha cell
Fat	5.0	soybean oil	Sunflowerr Seed Oil
Choline	0.2	choline bitartrate	choline
S-amino acid	0.3	L-cysteine	methionine
Mineral mix	3.5	See table 2.2	See table 2.2
Vitamin mix	1.0	See table 2.3	See table 2.3

Table 2.3. Mineral mix of AIN-93 (Reeves, et al 1993) and modified for experimental base diet

Ingredient	Reeves's	Experimental diet	mg/ kg diet
Calcium	Calcium carbonate	Calcium phosphate	4998.67
Phosporus	Potassium phosphate	As above	
Potassium	Potassium citrate	Citric acid	2787.40
Sulfur	Potassium sulfate	Potassium sulphate	820.17
Sodium	Sodium chloride	Sodium chloride	1003.60
Magnesium	Magnesium oxide	Magnesium oxide	496.00
Iron	Ferric citrate	Iron chloride	33.04
Zinc	Zinc carbonate	Zinc sulphate	25.16
Manganese	Manganous carbonate	Manganous sulphate	9.97
Copper	Cupric carbonate	Copper sulphate	5.38
Iodine	Potassium Iodate	Potassium iodate	0.22
Selenium	Sodium selenate	Sodium selenate	0.15

Table 2.4. Vitamin mix of AIN-93 (Reeves, et al 1993) and modified for experimental base diet

Ingredient	Reeves's	Experimental Base diet	(mg/ kg diet)
Nicotinic acid	Nicotinic acid	Nicotinic acid	30
Pantothenate	Ca Pantothenate	Pantothenic acid	16
Thiamine	Thiamine	Thiamine	6
Riboflavin	Riboflavin	Riboflavin	6
Pyridoxine	Pyridoxine	Pyridoxine	7
Folic acid	Folic acid	Folic acid	2
D-Biotin	D-Biotin	D-Biotin	0.2
Vit A	Palmitate	Palmitate	4
Vit E	α Tocopheryl acetate	di α tocopherol	50
Vit B 12	cyanocobalamin	Cyanocobalamin[a]	0.01
Vit K	Phylloquinone	K1 phylloquinone[b]	0.05
Vit D	D3 Cholecalciferol	D3 Cholecalciferol[c]	0.1

Note: [a] Vit B12 Cyanocobalamin 5 mg/ml in Milli Q water
  [b] Vitamin D Cholecalciferol 5 mg/ml in ethanol
  [c] Vit K1 5 mg/ml in SSO (Sigma)

2.1.5. Haematoxylin (Lillie-Mayers) and Eosin staining.

Haematoxylin solution:

Five milligrams of hematoxylin (Sigma) was dissolved in 10 ml of absolute ethanol. Aluminium ammonium sulfate (Alum) (50 g) was dissolved in water (250 ml) with heating. The haematoxylin solution was then added, together with sodium iodide (1 g), glacial acetic acid (20 ml), glycerol (300 ml) and made to 1 L. The solution was then filtered (Whatman no 1) and stored in a dark bottle.

Eosin solution:

Ten milligrams of Eosin (Sigma) and 5 mg of potassium dichromate were dissolved in saturated aqueous solution of picric acid (100 ml) which was then mixed with 100 ml in absolute ethanol (100 ml) and made up to 1 L with distilled water. One part of this eosin solution was mixed with one part of distilled water and then filtered (Whatman no 1) and stored.

2.2. *General Methods*

2.2.1. Preparation of extracts, fractions and bioactive compounds

2.2.1.1. Extraction

One hundred grams of dried, sliced roots and rhizomes or seeds were homogenised and extracted with absolute ethanol (250 ml) overnight at room temperature. The alcoholic extract was then vacuum filtered. The extraction was repeated three times and the combined ethanolic extract was evaporated to dryness at 35°C under vacuum and nitrogen (gas) to avoid oxidation. These extracts were used for cell culture investigations.

2.2.1.2. Analytical High Performance Liquid Chromatography (HPLC).

Analytical HPLC (Waters) was routinely used to analyse compounds in the ginger extracts, to measure purity of bioactive compounds and also to quantify bioactive compounds in the diets. The

method was a modification of He et al (1998). The HPLC system consisted of a Licrosphere (Supelco Inc., USA) RP C18 column (particle size 5μm, 250 x 3.2 mm) equipped with a guard column containing the same material. The HPLC was run under gradient concentrations of 0.25% acetic acid and 100% acetonitrile (solvent B) with flow rate of 0.77 mL/min. at 48°C (table 2.5).

Table 2.5. Gradient Concentration

Time (min)	Solvent A (%)	Solvent B (%)
0	60	40
17	40	60
32	0	100
38	0	100
38	60	40
45	60	40

2.2.1.3. Preparative Liquid Chromatography

Preparative LC was used to fractionate compounds from the extracts and to isolate bioactive compounds. The crude ethanol extract was fractionated in to fractions A and B by preparative reversed phase HPLC using two different eluents; fraction A was eluted with 1:1 methanol/water containing 0.025% v/v trifluoroacetic acid and fraction B was then eluted with 100 % methanol. The HPLC system consisted of a Waters (Waters, Milford, MA) Prep Nova-Pak HR PrepLC Column (particle size 6μm, 200 x 25 mm) equipped with a guard column containing the same material. The HPLC was run with flow rate of 20 mL/min.

2.2.2. Cell culture studies

Tumor cell lines and skin fibroblasts were maintained as monolayer cultures in Dulbecco Minimum Essential Medium (DMEM) supplemented with 10 % heat-inactivated foetal bovine serum (FBS), 20mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, 0.1% Amphostat B and 5μg/ml gentamycin in a humidified atmosphere of 5% CO_2 / 95% air at 37° C.

Dulbecco Minimum Essential medium (DMEM), heat-inactivated FBS, HEPES, gentamycin, Amphostat B, PBS, trypsin were all obtained from Trace, Australia. MTT-tetrazolium salt (3-(4,5-dimethylthiasol-2yl)-2,5-diphenyl tetrazolium bromide), N,N-dimethyl formamide, and sodium dodecyl sulfate (SDS) were purchased from Sigma (St Louis,MO).

2.2.2.1. Assessment of cell viability

Exponentially growing cells were dispersed with a phosphate-buffered saline solution (PBS) of 0.25% trypsin and suspended at a density of 2-4 X 10^4 cells/ml in DMEM. After overnight attachment they were treated with serial dilutions of the ginger extract at a range of concentrations in 96 well flat bottom plates for 72 hours. Controls were treated with ethanol alone. The viability of the cells was assessed by the MTT tetrazolium salt assay. The tetrazolium salt assay is based on the reduction of MTT by mitochondrial dehydrogenase of intact cells to a purple formazan product (Hansen et al., 1989). The amount of formazan was determined by measuring the absorbance at 570 nm using Spectra Max 250 (Microplate Spectrophotometer, Molecular Devices, USA).

2.2.2.2. Determination of IC_{50} values

For each ethanolic extract, three sets of experiments measuring the cell growth inhibition of cancer cell lines and the fibroblasts were completed using the MTT assay. The percentage of viable cells was determined by taking the optical density of each treated cell row and dividing it by the optical density of the vehicle-treated cells in the same 96 well plate. Each concentration of extract of each species was then plotted against the percentage cell survival. In this manner, a dose-response curve was generated and the IC_{50}, ie the concentration of the extract required to inhibit cell growth by 50%, was determined.

2.2.2.3. Morphological examination of cancer cells

The morphological examination of cells was carried out using several stains including Diff Quick stain, fluorescent Hoechst 3355, and 4,6-diamino-2-phenylindole (DAPI).

Cancer cells were grown on cover slips at a density of 100,000 cells/ml in six well plates and allowed to attach for 24 hours. The cells were washed and then treated with the extracts or active compounds and incubated for 24, 48 and 72 hours. Five hundred μl of cell suspension were obtained from the wells and slides were prepared using a cytocentrifuge (Shandon Southern Products, Cheshire, UK) to examine the floating cells. Cover slips and slides were air-dried, fixed in methanol and stained using DiffQuick stain (Sigma, St Louis MO). Slides were examined under a light microscope.

For fluorescent stains, cytospin preparations of control cells and of cells treated with extracts were examined using DAPI and Hoechst-335. Cytosmears were stored at 4°C prior to fixing and analysis. For DAPI staining, cells were fixed in Carnoy's fixative (6:1 ethanol:acetic acid) for 10 min, washed 2X5 min in PBS, rinsed in sterile H_2O and air dried. Cells were then stained in DAPI (8μM) (Sigma) for 1 min, rinsed 3 X in sterile H_2O, air dried, mounted in anti-fade medium (1% propylgalate, 86% glycerol) and directly visualised using a Fluorescence microscope. For Hoechst-335 stain, cytosmears were fixed in ethanol for about 10 min at room temperature, cells were then washed with PBS and then stained with 8 μg/ml of Hoechst-33258 (2'-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1Hbenzimidazole) (Sigma) for 15 min at room temperature and then directly visualized under a fluorescence microscope.

2.2.2.4. Cell Cycle Analysis

Propidium iodide (PI) staining analysis was used to determine cell cycle distribution of treated and untreated samples. HT-29 cells in 24 well plates (5X10^5 cells/well) were cultured overnight and then exposed to bioactive compounds. After 24 hours, cells were harvested by trypsinization and

resuspended in 300 μl PBS and fixed with 70% cold ethanol slowly while vortexing for 30 mins. The suspension was then centrifuged at 150 g for one minute and washed with 1 ml of PBS. DNA fluorescence of PI stained cells was evaluated by excitation at 488 nm and monitoring through a 630/22 nm band pass filter using a Becton-Dickinson FACS-Calibur flow cytometer. Ten thousands cells were analysed per sample and the DNA histograms were analysed further using CellQuest software (Becton Dickinson) to estimate the percentage of cells in various phases of the cell cycle. About 200,000 cells/well were plated in 6 well plates overnight to allow cells to attach and treated with zerumbone dissolved in DMSO at concentrations as above. After 24 h of treatment cells were harvested by using 0.01% trypsin/EDTA and then resuspended in 1% FBS in PBS. The cells were fixed by dropwise addition of cold 70% ethanol while vortexing to avoid aggregation and kept at –20 °C for at least 30 min. The cells were then washed with PBS twice, suspended in 40μg/ml PI and 200 μg/ml RNAse (Sigma) in PBS and incubated at room temperature for 30 min. DNA content was then analysed by Flow Cytometry.

2.2.2.6. Apoptosis Assays

200,000 cells/well were plated in 6 well plates overnight to allow cells to attach and treated with the active compounds dissolved in DMSO (the final concentration of DMSO was 0.5%) at concentrations of 10 - 65 μM. After 48 h of treatment, both adherent and floating cells were harvested and then double-labelled with Annexin-V-Fluos (Roche) and propidium iodide (PI) (Sigma) as described by the manufacturer. Cells (10,000) were analysed using a FACScan Flow Cytometry equipped with FACStation running Cell Quest software (Becton Dickinson, San Jose, CA).

2.2.3. Animal studies

2.2.3.1. Aberrant Crypt Foci

After two weeks on experimental diets, male SD rats were injected subcutaneously with azoxymethane (AOM) (Sigma Chemical, St Louis, MO) dissolved in normal saline at a dosage of 15 mg/kg body weight once weekly for 2 weeks. Animal were killed after 6 (for short term experiment) and 12 (for long term experiment) weeks of consuming experimental diets for ACF identification. Rats were killed with halothane/oxygen anaesthesia followed by abdominal aortic exsanguination.

2.2.3.1. 1. Aberrant Crypt Foci (ACF) assay

ACF formation was assayed according to the method of McLellan and Bird (1998). Colons were removed from the animal, flushed with PBS (pH 7.4) and expanded with 10% formalin-phosphate buffered saline for a few minutes. They were then slit open longitudinally and fixed flat between two pieces of filter paper in 10% buffered formaldehyde. After minimum of 24 h in formalin, they were kept in 70% ethanol until ACF were counted. The aberrant crypts were analysed between the distal Peyers patch and the beginning of the Herring bone musculature. Each colon was placed in a Petri dish containing 0.2% methylene blue dissolved in PBS for 7-10 min. It was then placed mucosa-side up on a microscope slide and ACF were scored using a low power light microscope (X 10 magnification). Aberrant crypts were identified by alterations in size, shape of luminal opening and stain intensity according to the method of Bird (1987).

2.2.3.2. Inflammatory Bowel Disease: Ulcerative colitis

After two days on experimental diets, male SD rats were given 2% (w/v) of dextran sulfate sodium (DSS) (MW 36-40 kDa, sulphur content 15-20 %, ICN Biochemicals, Cleveland, Ohio) in drinking water ad libitum for 5 days in metabolic cages. Body weight, food intake, fluid intake, stool and blood in the faeces were recorded. Animals were put in a wire cage and killed after 3 days

recovery. Organs were taken and weighed. Colons were measured and removed for biochemical and histological examinations.

2.2.3.2.1. Preparation of sample of colon tissue

Colons were removed from the caecal end and slit open longitudinally, cleaned of faecal material and cut into pieces for histological and biochemical analysis as shown in figure 2.1.
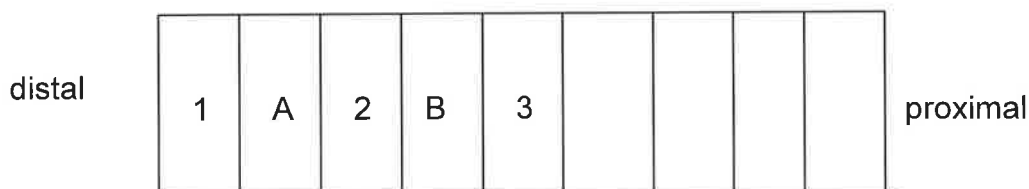

Figure 2.1.
1,2,3 for histological investigation, A for MPO assay, and B for PGE-2 and TXB-2 assay.

Colon tissues, which were used for biochemical analysis, were frozen in liquid nitrogen and stored at -70° C until used. Colon tissues for histological examination were fixed flat between two pieces of filter paper in 10% buffered formaldehyde overnight and stored in 70% ethanol.

2.2.3.2.2. Histopathological examination: Hematoxylin and Eosin

Three approximately one centimetre pieces of colon were cut for histological examination. The flat tissues were processed for paraffin embedding using solutions as shown in table 2.6. Tissue was blocked by transferring it from the final wax bath to a mould filled with molten wax, orientating the surface to be cut on the base of the mould. The block was then quickly cooled down, trimmed and cut at 5 μm and mounted on slides. The slides were processed for dehydration procedures as listed on table 2.6. The tissues were removed from the wax with xylene, and brought back to water before staining with H & E, differentiated and rehydrated as procedures listed on table 2.7. The tissue were

then dehydrated and cleared with xylene as listed on table 2.8. and finally mounted with DePex (Sigma).

Table 2.6. Dehydration

solution	Time (mins)
70% ethanol	30
80% ethanol	30
90 % ethanol	30
95% ethanol	30
100 % ethanol (1)	30
100 % ethanol (2)	30
100 % ethanol (3)	45
Chloroform (1)	30
Chloroform (2)	45
Wax (1)	30
Wax (2)	45

Table 2.7. Rehydration, staining and differentiation procedures.

solution	Time (mins)
Xylene-1	3
Xylene-2	3
100 % ethanol	3
90 % ethanol	3
70 % ethanol	3
RO water	3
Haematoxylin solution	10
Rinsing in running RO water	
1% HCl in 70% ethanol	1-2-3 dip
RO water	2
Eosin solution	3
Rinsing in running RO water	

Table 2.8. Dehydration and clearing processes.

solution	Time (mins)
Absolute ethanol-1	2
Absolute ethanol-2	2
Absolute ethanol-3	3
Xylene-1	5
Xylene-2	10

CHAPTER 3

EXTRACTION OF RHIZOMES OF GINGER SPECIES, FRACTIONATION OF ETHANOL EXTRACTS AND ISOLATION AND IDENTIFICATION OF THE ACTIVE COMPOUNDS

3.1. *Introduction*

Traditional medicines have been used since ancient time in almost every culture including Europe, Asia, Africa and the Americas (Wargovich et al., 2001). A recent survey suggested that up to 50% of cancer patients in America used herbal medicine as alternative medicine (Richardson et al., 2000). In Indonesia, herbal medicines are still used in everyday life. However, some practitioners would not suggest the use of traditional medicine since they lack scientific data. The investigation of herbal medicine should follow a standard structured design, the isolated bioactive agents being assayed for efficacy, toxicity, biological mechanisms assessed and pharmacokinetics determined (Gescher et al, 2001).

The yellow pigment curcumin (diferuloylmethane) is a bioactive compound isolated from *C. longa* and has been extensively studied (Rao et al., 1993; Ruby et al., 1995). Each species of ginger contains a unique set of compounds with structures similar to curcumin such as "yakuchinones" in *A. oxyphylla* (Chun et al., 1999), and cassumunin A and B from *Z. cassumunar* (Oyama et al., 1998) or compounds with completely different structures from curcumin such as paradol and gingerol from *Z. officinale* (Lee and Surh, 1998).

As part of screening program of natural products for antitumour and anti-HIV activities by the National Cancer Institute (NCI), zerumbone (figure 3.1), a sesquiterpenoid compound, has been isolated from *Z. aromaticum* and *Z. zerumbet.*
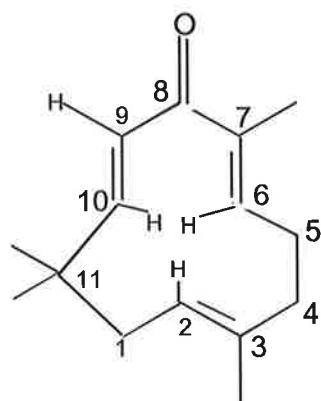
Figure 3.1. Chemical structure of zerumbone (2,6,9 humulatriene-8-one)

B. pandurata, which is called "fingeroot", is one of the main spices used in Thailand. Potential bioactive compounds from Thai *B. pandurata* include essential oils, flavanones, and chalcones. These have been intensively isolated and identified (Trakoontivakorn et al., 2001). The structures of some of the active compounds isolated from *B. pandurata* are shown in figure 3.2. Jantan et al. (2001) investigated the constituents of *B. pandurata* from Malaysia, Indonesia and Thailand and found that the composition and concentration of essential oils of this species varied depending upon their origin. The fact that concentrations of compounds are variable depending upon the condition where they grow may have an influence on the bioactivity of the plants.

The aims of this study were to extract eleven species of Zingiberaceae most frequently used in local traditional medicine and cooking and to isolate and identify the active compounds in these species. This work was carried out in conjunction with investigation of the extracts or fractions in *in vitro* cell culture studies.
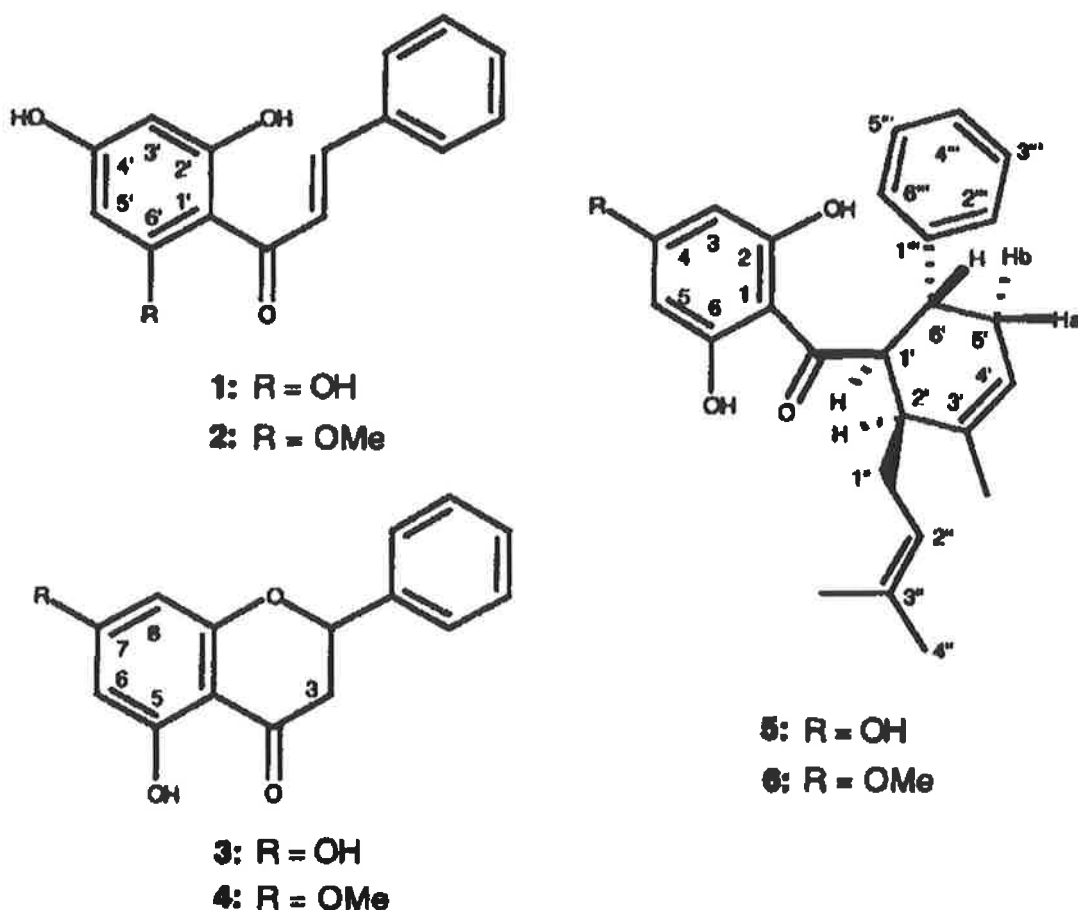
Figure 3.2. Chemical structures of active compounds isolated from B. pandurata: 1= pinocembrin chalcone; 2= cardamonin; 3= pinocembrin; 4=pinostrobin; 5= 4-hydroxypanduratin A; 6= panduratin A.

3.2. *Experimental Designs*:

3.2.1. Experiment : Preparation and analysis of extracts

Preparation of ethanol extracts is described in General Method (2.2.1.1.). The dried ethanolic extracts of gingers were dissolved in methanol and then applied on to silica gel 60 F_{254} precoated TLC plates (Merck, Darmstadt). The solvent was developed with 93:7 toluene/ethylacetate v/v. After drying to removed the developing solvent the plate was sprayed with anisaldehyde-sulphuric acid (AS) reagent (0.5 ml anisaldehyde was mixed with 10 ml of glacial acetic acid followed by 85 ml methanol and 5 ml of concentrated sulphuric acid). The plate was sprayed with about 10 ml of the solution and heated at 100° C for 5- 10 min. Spots were examined visually or under a UV lamp (365 nm).

The methanolic solutions were also analysed by HPLC to determine the composition of the extracts.

3.2.2. Experiment: Fractionation of ethanol extracts using Preparative Liquid Chromatography

Ethanol extracts of *C. longa*, *K. pandurata* and *Z. aromaticum* were separated into 2 fractions using different solvents and methods described in General Methods (2.2.1.3). Fraction A was eluted with a mobile phase containing 50% v/v aqueous methanol containing 0.025% v/v TFA and fraction B was eluted with 100% methanol. Fractions were then evaporated to dryness using a rotary evaporator, and analysed by HPLC (see 2.2.1.2).

3.2.3. Experiment: Isolation of active compounds using preparative Liquid Chromatography

The active compounds of *Z. aromaticum* and *B. pandurata* were purified from the active fractions by preparative reverse phase LC as described in General Method (2.2.1.3.) with a mobile phase consisting of a stepwise gradient of 60; 70; 75 and 80% v/v aqueous methanol containing 0.025% v/v TFA. Isolated compounds were lyophilized to a dry powder and used for characterisation and identification.

3.2.4. Experiment: Characterisation and Identification of active compounds using Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS)

Isolated compounds were characterized by ^{1}H and ^{13}C NMR (600 MHz, Varian Inova) using acetone-d_6 or DMSO-d_6 as solvents and LC ESI-MS (API-300, PE Sciex) operated in the positive ion mode (ion spray voltage 5300 V; orifice potential, 50 V). The molecular formula was determined by HR-FAB MS (Kratos Concept ISQ). Data were compared with previously published values.

3.3. *Results:*

3.3.1. Extracts of 11 species of Zingiberaceae

The amount of ethanol extracts of eleven species of Zingiberaceae varied from 4.2 to 21.2 % by weight of the dried material (Table 3.1).

Table 3.1: The amount of ethanol extract of 11 species of Zingiberaceae

No	Species (*)	Weight of dried extract (%)
1.	*Amomum cardamomum* (C,M)	5.8
2	*Curcuma aeruginosa* (M)	4.6
3.	*C. longa* (C,M)	21.5
4.	*C. mangga* (M)	12.9
5.	*C. xanthorrhiza* (M)	8.5
6.	*Kaempferia galanga* (C,M)	21.2
7.	*Boesenbergia pandurata* (C,M)	13.6
8.	*K. rotunda* (M)	4.2
9.	*Zingiber aromaticum* (M)	8.6
10.	*Z. cassumunar* (M)	5.3
11.	*Z. officinale* (C,M)	12.0

Note * Usage of rhizomes in Indonesia: C=cooking; M=medicine

Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), the ethanol extracts of different species were shown to contain different compounds (Figure 3.3). The chromatographic data of HPLC of *Z. aromaticum* and *B. pandurata* is given in figure 3.4 and 3.5. The chromatographic data of 10 mg/ml of extracts of ginger species is given in Appendix A.

From the screening study (Chapter 4) of the growth of cancer cells *in vitro*, ethanol extracts of two species, *B. pandurata* and *Z. aromaticum*, were found to be the most active species and had similar activity to extract of *C. longa*. The area of the peaks in analytical HPLC chromatograms were used to approximate concentrations of curcuminoids in the extracts of *C. xanthorriza*, *B. pandurata* and *Z. aromaticum*. The concentrations of curcuminoids from the chromatograms of extract of *C.*

longa were used as a comparison. Extracts of *B. pandurata* and *Z. aromaticum* contain no curcuminoids (Table 3.2).
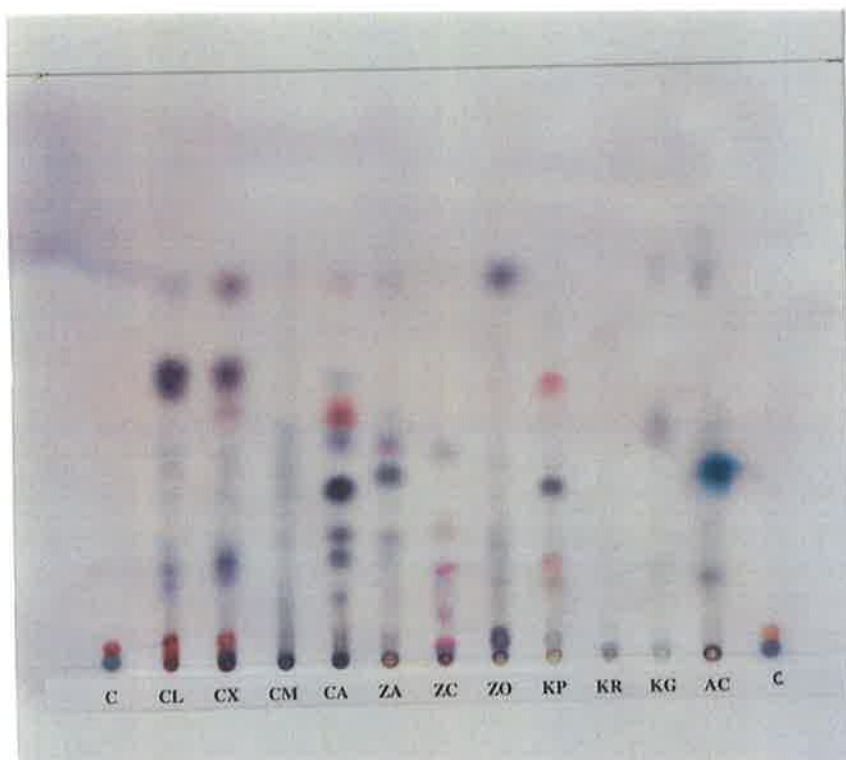

Figure 3.3. Ethanol extract of CL (*C. longa*); CX (*C. xanthorrhiza*); CM (*C. mangga*); CA (*C. aeruginosa*); ZA (*Z. aromaticum*); ZC (*Z. cassumunar*); ZO (*Z. officinale*); KP (*Boesenbergia pandurata*); KR (*Kaempferia rotunda*); KG (*K. galanga*); AC (*A. cardamomum*); C (Curcumin) on a silica gel 60 F$_{254}$ precoated TLC plate run in 93:7 toluene/acetylacetate and sprayed with anisaldehyde-sulphuric acid reagent.

Table3.2: The amount of curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) in the extracts (10 mg/ml) of *C. longa, C. xanthorrhiza, B. pandurata* and *Z. aromaticum* based on an approximate of area of peaks of chromatograms (see General Method 2.2.1.2 and Appendix 1)

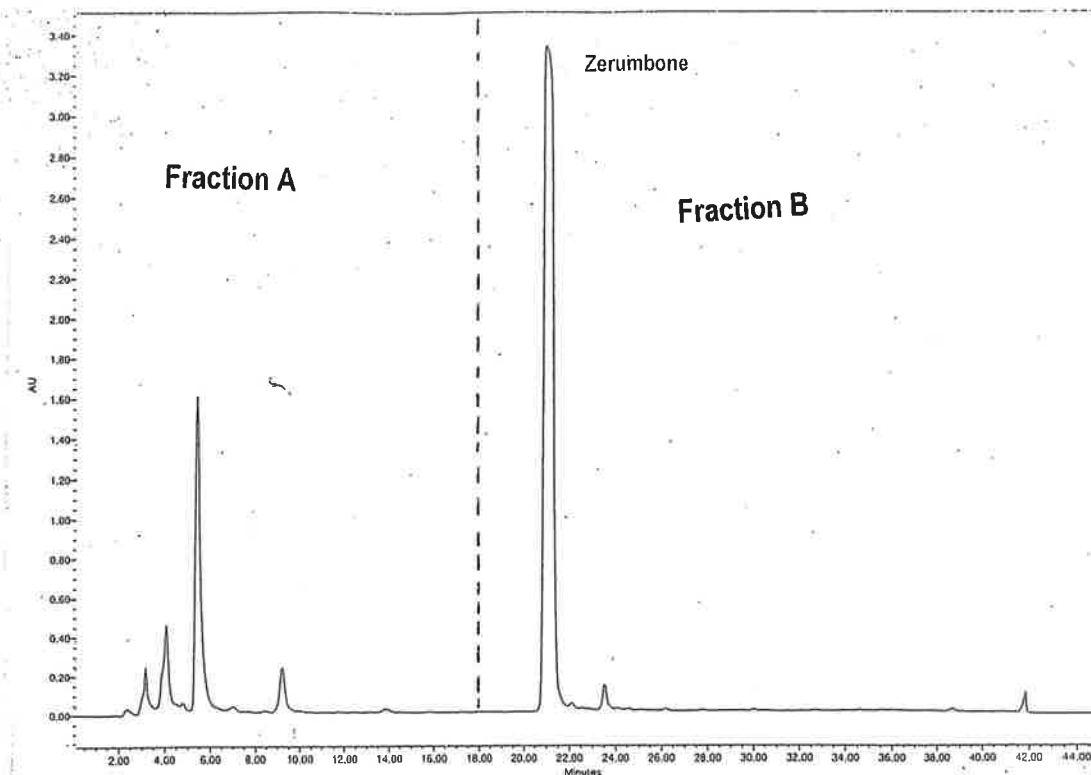|	Curcumin	Demethoxycurcumin	Bisdemethoxycurcumin
C. longa	+++	++	++
C. xanthorrhiza	+++	+	trace
B. pandurata	-	-	-
Z. aromaticum	-	-	-

Figure 3.4.
A chromatographic data of analytical HPLC of ethanolic extract of Z. aromaticum at 254 nm.
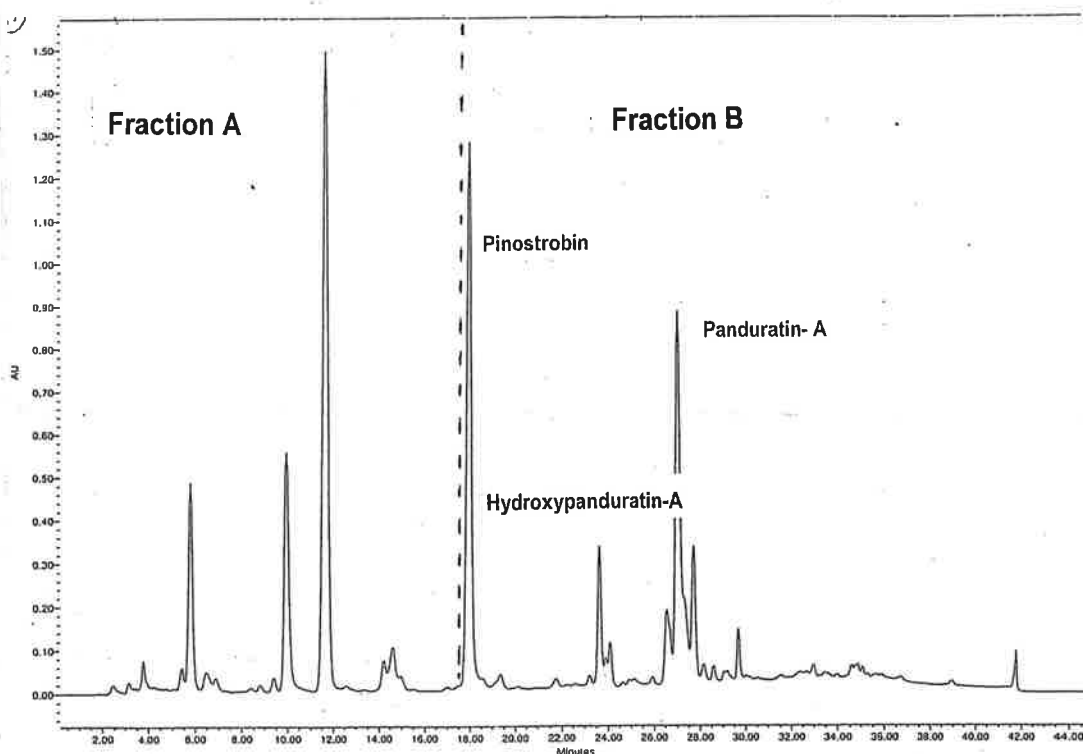

Figure 3.5.
A chromatographic data of analytical HPLC of ethanolic extract of B. pandurata at 254 nm. These compounds were on the bases of retention times and identified (Trakoontivakorn et al., 2001). Fraction A was eluted with a mobile phase containing 50% v/v aqueous methanol containing 0.025% v/v TFA and Fraction B was eluted with 100% methanol.

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3.3.2. Identification of active compounds

Fraction B of *Z. aromaticum* or *B. pandurata* were more active than fraction A of the respective species (Chapter 4). A compound from fraction B of *Z. aromaticum* or *B. pandurata* was isolated and purified using preparative LC and identified using ^{1}H and ^{13}C NMR and data was compared to values of published data.

Zerumbone was the most prominent peak in fraction B of *Z. aromaticum*. Zerumbone contains three double bonds; an isolated one at C2 and two at C6 and C9 which are part of a cross-conjugated dienone system and has the molecular formula of $C_{15}H_{23}O$. The ^{13}C NMR data of zerumbone is shown in table 3.3. The mass spectrum of zerumbone showed [MM + Na]$^+$ at m/z 459.6, [MM + H]]$^+$ at m/z 437.6, [M + Na]$^+$ at m/z 241.4 and [M + H]$^+$ at m/z 219.4 (figure 3.6).

Table 3.3. ^{13}C NMR Data (ppm) of zerumbone recorded at 150.85 MHz on a Varian Inova 600 spectrometer and compared to that of Dai et al (1997) and of Kitayama and Okamo (1999) recorded at 125 MHz.

Assignment	current data	Dai et al (1997)	Kitayama and Okamoto (1999)
Solvent	Acetone-d$_6$	CDCl3	CDCl3:CCl4 (7:3)
C13	10.5	11.8	11.8
C12	17.6	15.2	15.2
C15	22.7	24.2	24.2
C5	25.6	24.4	24.4
C14	28.3	29.4	29.4
C11	36.5	37.9	37.9
C4	39.5	39.5	39.4
C1	42.1	39.5	42.2
C2	125.3	125.0	125.0
C9	126.9	127.2	127.1
C3	136.5	136.2	136.3
C7	137.0	138.0	137.9
C6	147.0	148.8	149.0
C10	165.2	160.9	160.9
C8	206.3	204.4	204.4

Panduratin A has the molecular formula $C_{26}H_{30}O_4$ including 12 alipathic carbons (3CH3, 2CH2, 3CH, 2C=CH-), 12 aromatic carbon and one methoxyl carbon (Tuntiwachwuttikul et al., 1984).

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The NMR data of panduratin A and 4-hydroxypanduratin were very similar. The only difference was there was no signal at 55 ppm for the 4-MeO of hydroxypanduratin A (Trakoonvitakorn et al., 2001). The^{13}C NMR data of panduratin A is shown in table 3.4.

Table 3.4. ^{13}C NMR Data (ppm) were recorded at 150.85 MHz on a Varian Inova 600 spectrometer and data of Panduratin A (Tuntiwachwuttikul et al., 1984) and of 4-hydroxypanduratin A (Trakoontivakorn et al., 2001).

Assignment	current data	Tuntiwachwuttikul et al (1984)	Trakoontivakorn et al (2001)
Solvent	DMSO-d$_6$	CDCl3	DMSO
3"-Me	17.62	17.9	17.6
3'-Me	22.53	22.8	22.6
C4"	25.45	25.7	25.5
C1"	28.41	28.9	28.4
C5'	35.58	35.9	35.7
C6'	36.47	37.2	36.4
C2'	41.11	42.8	42.1
C1'	52.96	54.1	52.8
4-MeO	55.25	55.5	-
C3, C5	93.30	94.6	94.8
C1	105.44	106.4	104.7
C4'	120.76	121.3	120.9
C2"	124.11	124.4	124.3
C4'''	125.54	125.7	125.3
C2''', C6'''	126.86	127.3	126.9
C3''', C5'''	128.13	128.0	128.1
C3"	130.73	132.0	130.6
C3'	136.61	137.3	?
C1'''	146.96	147.2	?
C2, C6	163.86$^+$	163.2	164.1
C4	165.17	165.3	164.3
C=O	206.25	206.6	205.7

$^+$ peak was broad
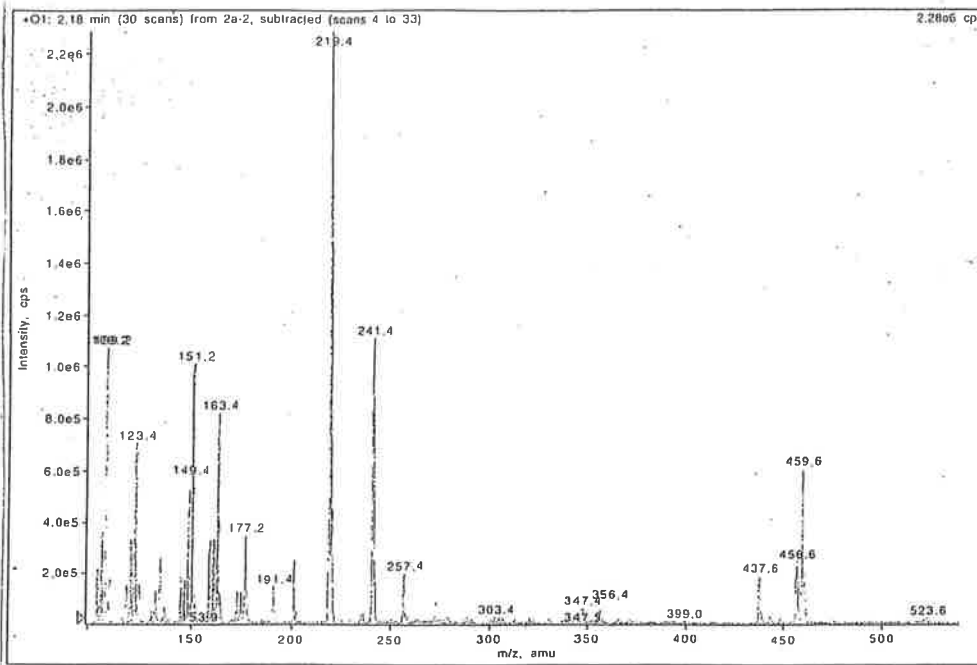
Figure 3.6. Mass Spectrum of zerumbone

gb01june25-2#65-74 RT: 1.09-1.24 AV: 10 SB: 18 0.72-1.01 NL: 4.46E7
T: + c EI [49.96-800.01]

gb01june25-2#65-74 RT: 1.09-1.24 AV: 10 SB: 18 0.72-1.01 NL: 4.46E7
T: + c EI [49.96-800.01]
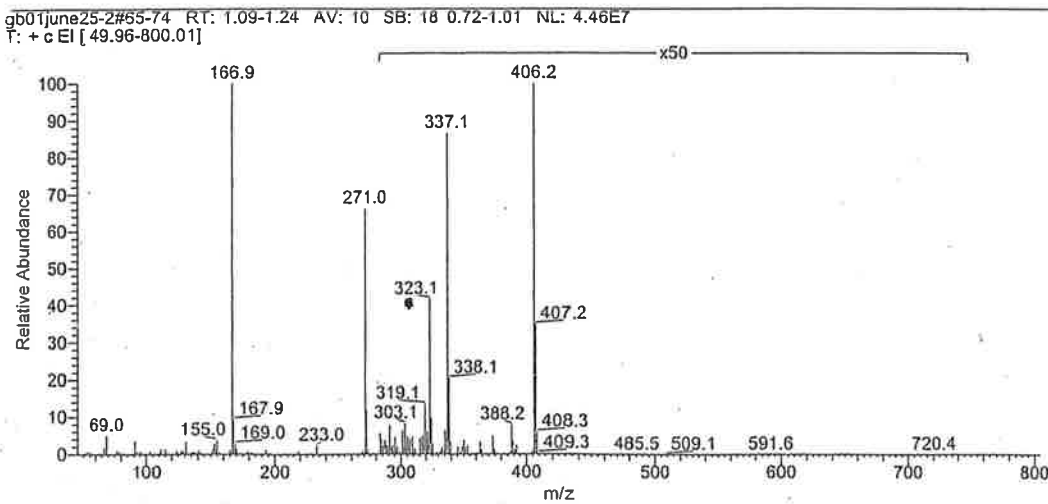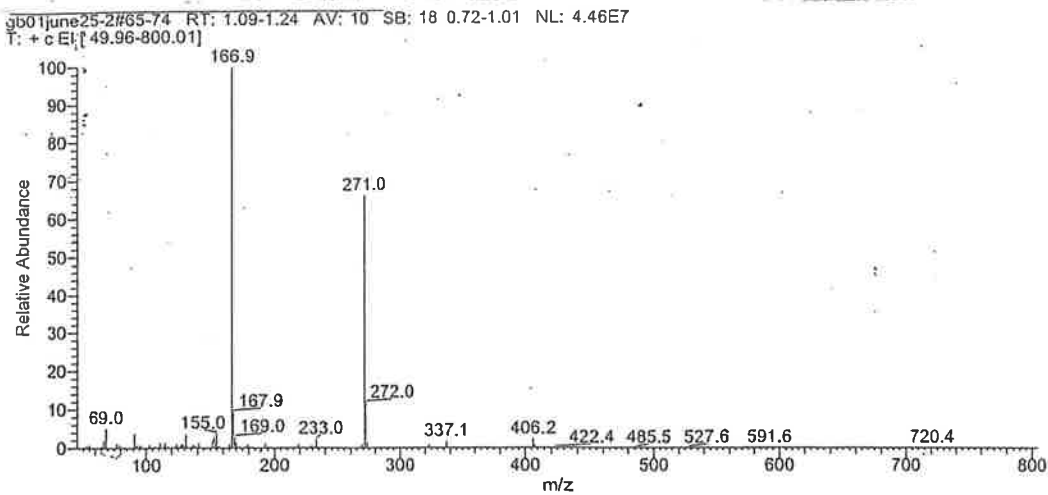
Figure 3.7. Mass Spectra of panduratin A

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Fraction B of *B. pandurata* contains both 4-hydroxypanduratin and panduratin A. At 163.9 ppm, the peak was broad, possibly due to H bonding to the solvent. The mass spectrum of panduratin A showed [M + H]$^+$ at m/z 407.2, M$^+$ at m/z 406.2, the base peak at m/e 166.9 and other prominent peaks at m/z 337.1, 271.0 and 323.1 (figure 3.7).

3.4. Discussion

Compared to the number of family members of Zingiberaceae only a few have previously been investigated and our studies has identified that some have potential anticancer activity (Chapter 4). Extracts of the 11 species of Zingiberaceae family were shown to contain a range of different compounds. *C. longa* and *C. xanthorrhiza* showed similarity on morphological features of their rhizomes and both contain yellow pigment curcumin, which is suggested to be the bioactive compound. *C. longa* is called long turmeric and *C. xanthorrhiza* is called round turmeric. *C. xanthorrhiza* is only used for medicine. The concentration of curcumin in the extract of *C. xanthorrhiza* was similar to that of *C. longa*, however the concentration of demethoxycurcumin and bisdemethoxycurcumin in *C. xanthorrhiza* was much lower than those found in *C. longa*. Demethoxycurcumin and bisdemethoxycurcumin have been reported to be potential anticancer agents in *C. longa* (Simon et al., 1998).

Due to shortages in supply, *B. pandurata* has often been used interchangeable with *K. rotunda* as the main component of popular traditional tonics and medicines used especially for women. *B. pandurata* contains different compounds than *K. rotunda*. Extract of *K. rotunda* had no effect in inhibiting the growth of cancer cells (see Chapter 4). Regardless the purposes of the folk medicine, these two species contain different compounds therefore use of *K. rotunda* could result a different and or lesser effect.

Extracts of *K. pandurata* and *Z. aromaticum* had similar or greater inhibitory effect than the extract of *C. longa* in the growth of cancer cell lines (Chapter 4). Curcuminoids was not detected in the

extracts of *K. pandurata* and *Z. aromaticum*. Fractionation of extracts showed that fraction B of *K. pandurata* and *Z. aromaticum* were more active than fraction A of the respective species (Chapter 4). Zerumbone was found to be the most prominent peak in fraction B of the extract of *Z. aromaticum*. Dai et al. (1997) have previously isolated and identified from zerumbone from *Z. aromaticum* and *Z. zerumbet* and reported that zerumbone was a major antiviral and cytotoxic compound in these species.

Various compounds have been identified from *B. pandurata* which is used in Thailand in cooking and folk medicine. They are flavononones, pinostroin and pinocembrin and chalcones boesenbergin A and cardamonin (Jaipetch et al., 1982). Murakami et al (1993) suggested that cardamonin isolated from *B. pandurata* has potential anticancer activity. In this study, panduratin A, a chalcone derivative, was isolated from fraction B and identified. Tuntiwachwuttikul et al have identified panduratin A from *B. pandurata* in 1984.

3.5. Summary

Extracts of ginger species showed a range of different compounds. Fraction A of *C. longa* was found to contain curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin). *C. xanthorrhiza* contained curcumin with a concentration similar to that in *C. longa* but less amount of demethoxycurcumin and no bisdemethoxycurcumin. *Z. aromaticum* and *B. pandurata* contain no curcuminoids. Zerumbone was isolated from fraction B of *Z. aromaticum* and panduratin A was isolated from fraction B of *B. pandurata*.

CHAPTER 4

ANTICANCER STUDIES OF RHIZOMES EXTRACTS OF GINGER SPECIES AND ACTIVE COMPOUNDS ZERUMBONE AND PANDURATIN A IN *IN VITRO* CELL CULTURE

4.1. *Introduction*

As many as 63 species of the family Zingiberaceae (ginger) have been identified in Indonesia (Hyene, 1987), of which about 20 are regularly used for culinary or medicinal purposes. However, only a few members have been studied for their potential anticancer activities.

Ethanol extracts of ginger have been reported to inhibit skin tumorigenesis (Katiyar et al., 1996). *Alpinia oxyphilla* Miquel, a member of Zingiberaceae, is used as a traditional oriental medicine, and has been shown to inhibit skin tumorigenesis (Lee et al., 1998). Murakami et al. (1998) and Vimala et al. (1999) reported that rhizomes of species of Zingiberaceae possess antitumor potentials as determined by inhibition of 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced Epstein-Barr virus activation in Raji cells. However, there has been no report on the activity of these members of the family of ginger against established cancer cell lines.

The aims of this study were to screen the inhibitory activity of the 11 species of Zingiberaceae using influence on the growth of HT-29 colon and MCF-7 breast cancer cells. The second objective was to establish toxicity of extracts using non-transformed human skin fibroblasts. Extracts of *C. longa,* which contains curcumin, an established anticancer agent, was used for comparison. Morphological changes of the cells treated with extracts of ginger species were also examined. The third objective was to investigate the inhibitory activity of fractions from the active species on the growth of HT-29 colon cancer cells to further determine active compounds from the extracts. The fourth objective was to investigate anticancer activity including growth inhibitory activity, cell cycle analysis and apoptosis of active compounds using colon and breast cancer cell lines.

4.2. Experimental Designs:

4.2.1. Ethanol extracts of ginger species

Inhibitory activity of ethanol extracts of ginger species was assessed using MTT tetrazolium salt assay which is described in General Method (2.2.2.1). HT-29 colon and MCF-7 breast cancer cells were treated with serial dilution of the ginger extracts at concentration range of 15 to 250 μg/ml. The extraction procedures were standardised by weighing extracts and assaying extracts using analytical HPLC to identify presence and constancy of all components in the extracts. Morphology of cancer cells treated with extracts of the active ginger species was examined using different stains as described in General Methods (2.2.2.3).

Inhibitory activity of ethanol extracts of gingers was measured as the viability cells after 72 h incubation and assessed by the MTT tetrazolium salt assay on the growth of SF 3169 skin fibroblasts as described in General Method (2.2.2.1) at concentration range of 15 to 250 μg/ml. In this manner, a dose-response curve was generated and the IC_{50} ie. the concentration of the extract required to inhibit cell growth by 50% was determined. The higher the IC_{50}s represent a less toxic effect of the extract to the non-transformed cells.

4.2.2. Fractions of extracts of ginger species

Inhibitory activity of extracts was assessed using the MTT tetrazolium salt assay and described in General Method (2.2.2.1). Exponentially growing HT-29 cells were treated with dilutions of fraction A and B of *Z. aromaticum*, *B. pandurata* and *C. longa* at concentration ranges from 15 to 37.5 μg/ml.

4.2.3. Bioactive compounds

Isolated and purified panduratin A from *B. pandurata* and zerumbone from *Z. aromaticum* were dissolved in DMSO (final concentration of DMSO was 0.5%) and used for cell culture investigations.

Inhibitory activity of the active compounds on HT-29 and Caco-2 colon and MCF-7 breast cancer cells was assessed using MTT tetrazolium salt assay which is described in General Methods (2.2.2.1).

Exponentially growing cancer cells in DMEM were treated with zerumbone at concentrations of 1, 5, 10 and 25 µM or panduratin A at concentrations of 9, 23, 28 and 46µM for 24, 48 and 72 h.

Cell cycle analysis was carried out on HT-29 colon cancer cells. About 200,000 cells were treated with zerumbone at concentrations of 10, 12.5, 25 µM or panduratin A at concentrations of 9, 23, 28, 46 µM for 24 h. The cell cycle assay is described in General Methods (2.2.2.4)

Apoptosis was assayed using double-labelled stains using a FACScan Flow Cytometry as described in General Methods (2.2.2.6) and the morphology of the cells undergoing apoptosis was observed following staining with Diff Quick as described in 2.2.2.3. About 200,000 HT-29 cells were treated with zerumbone at concentrations of 10, 12.5, 25 and 50 µM or panduratin A at concentrations of 28, 46, 65 µM for 48 h to investigate apoptosis.

4.2.4. Statistical analysis

Values of Z score were means of three independent experiments ± SE. Extract of *C. longa* has been extensively studied and has showed anticancer activity *in vivo* and *in vitro*, therefore values of IC_{50} of C. longa was used as comparison for other species. The differences between *C longa* and other species were performed using Z score calculation as follow:

$$Z \text{ score} = \frac{X-Y}{\sqrt{SE(X)^2+SE(Y)^2}}$$

X = mean of IC_{50} of extract of C. longa

Y = mean of IC_{50} of extract of other species

SE(X) = standard error of IC_{50} of extract of C. longa

SE(Y) = standard error of IC_{50} of extract of other species

Two values are significantly different if $-2 < Z < 2$

4.3. Results

4.3.1. Cytotoxicity of extracts of 11 species of Zingiberaceae on cancer cells and non transformed skin fibroblast cells.

Figure 4.1 shows the growth of cancer cells and fibroblast cells in the media after 72 h. The growth of HT-29 colon adenocarcinoma cells was about four times faster than MCF-7 breast cancer cells while non-transformed SF3169 skin fibroblast cells showed little division for up to 72 h.

The anticancer activity of 11 species of Zingiberaceae was assessed using two different cell lines: HT-29 and MCF-7 cancer cells by using the MTT tetrazolium salt assay. The ethanolic extracts of eight species of Zingiberaceae showed strong inhibitory effects on the growth of both cell lines at concentrations of 10-100 µg/ml. They were *A. cardamomum, C. longa, C. xanthorriza, C. mangga, Z. officinale, Z. cassumunar, Z. aromaticum,* and *K. pandurata.* An extract of *C. aeruginosa* was less active and extracts of two species (*K. galanga* and *K. rotunda*) had no effect on the growth of either cell lines at concentration up to 250 µg/ml. The IC_{50} of the extracts are presented in Table 4.1.
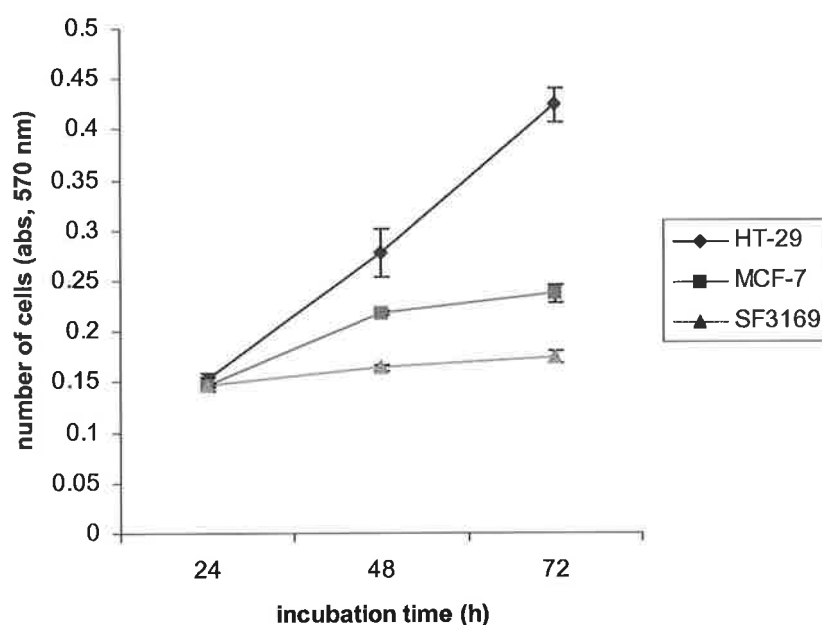

Figure 4.1.:
The growth of HT-29, MCF-7 cancer cells and SF3169 cells in the media for up to 72 h. Values are means ± SE of three sets experiments

The IC_{50} of eight active species were at concentrations between 10-100 µg/ml while the IC_{50} of the less active species fell between concentrations of 100-120 µg/ml. The IC_{50} of extracts of *C. longa*, *K. pandurata* and *Z. aromaticum* showed that they were the most active species. The IC_{50} of extract of *K. pandurata* was not significantly different from that of C. longa while the IC_{50} of *Z. aromaticum* was significantly less than that of C. longa on HT-29 cells. Using MCF-7 cells the IC_{50} of *K. pandurata* and *Z. aromaticum* were significantly less than that of *C. longa*. These species had a very similar inhibitory effect on both cell lines. Individual dose response curves are presented in Figures 4.2 and 4.3.

Table 4.1. The IC_{50} (µg/ml) of extracts of the gingers on MCF-7 and HT-29 cancer cells and non-transformed SF3169 skin fibroblasts*, activity assessed relative to *C. longa*

Species	MCF-7 cells	HT-29 cells	SF 3169 cells
A. cardamomum	76.2 ± 11.7[a]	79.2 ± 23.2[a]	84.6 ± 7.8[a]
C. aeruginosa	119 ± 5.8[a]	103.8 ± 16.5[a]	> 150[a]
C. longa	31.0 ± 3.3	28.1 ± 2.7	30.9 ± 3.8
C. mangga	44.7 ± 2.7[a]	91.0 ± 5.9[a]	77.6 ± 5.3[a]
C. xanthorrhiza	47.2 ± 7.9	39.9 ± 3.1[a]	60.6 ± 4.2[a]
K. galanga	> 250[a]	> 250[a]	ND
B. pandurata	21.3 ± 0.3[b]	32.5 ± 1.5	49.5 ± 2.6[a]
K. rotunda	> 250[a]	> 250[a]	ND
Z. aromaticum	20.2 ± 1.8[b]	11.8 ± 1.0[b]	70.9 ± 2.5[a]
Z. cassumunar	54.5 ± 11.5	84.9 ± 8.3[a]	> 100[a]
Z. officinale	40.6 ± 1.4[a]	53.5 ± 5.0[a]	72.7 ± 8.2[a]

*Data represent the means of ± SE obtained from three separate determinations for each species, ND not done, Z score was calculated to compare the activity of extract of each species with extract of *C. longa*. The analysis is applied within each columns, [a] significantly greater than *C. longa*; [b] significantly less than *C. longa*

Toxicity of extracts of ginger species was assessed on non-transformed skin fibroblast cells SF 3169. It appeared that cancer cell lines were more susceptible to ethanol extracts of species of Zingiberaceae than non-transformed skin fibroblasts. The IC_{50} shows the 50% growth of cells at a given concentration. In this case the higher the IC_{50} value means that the extract is less toxic. The IC_{50} of the extracts on SF 3169 skin fibroblasts is shown in Table 4.1. The IC_{50} of extracts of other species was significantly greater than that of *C. longa* indicated that those extracts were less toxic than extract of *C. longa*. With the exception of the extract of turmeric (*C. longa*) (IC_{50} = 31 μg/ml), extracts of other species had an IC_{50} on non-transformed fibroblasts higher than those on cancer cell lines.
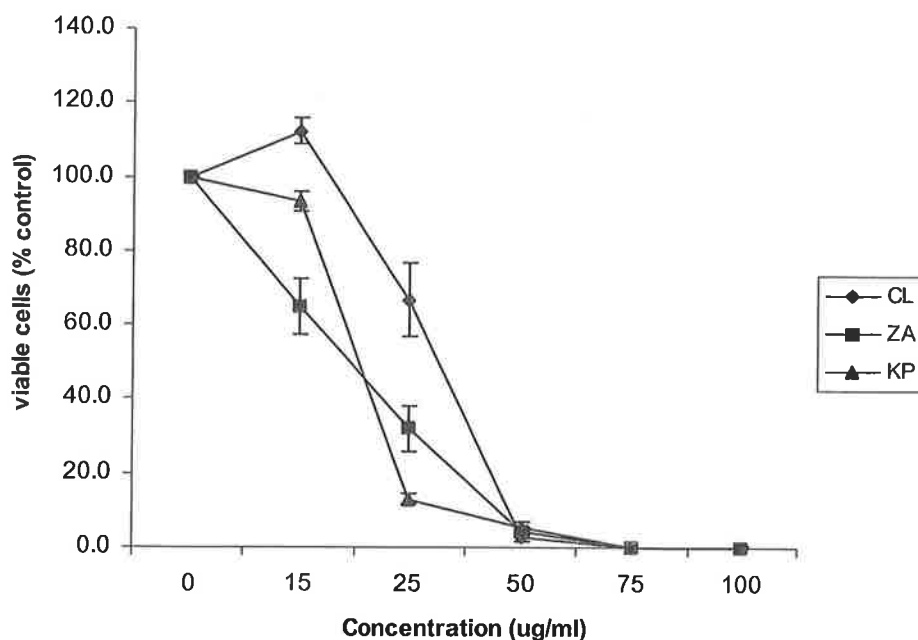

Figure 4.2. The growth of MCF-7 breast cancer cells exposed to ethanol extracts of *C. longa* (CL), *Z. aromaticum* (ZA) and *K. pandurata* (KP) after 72 hours. Values are means ± SE of three independent experiments
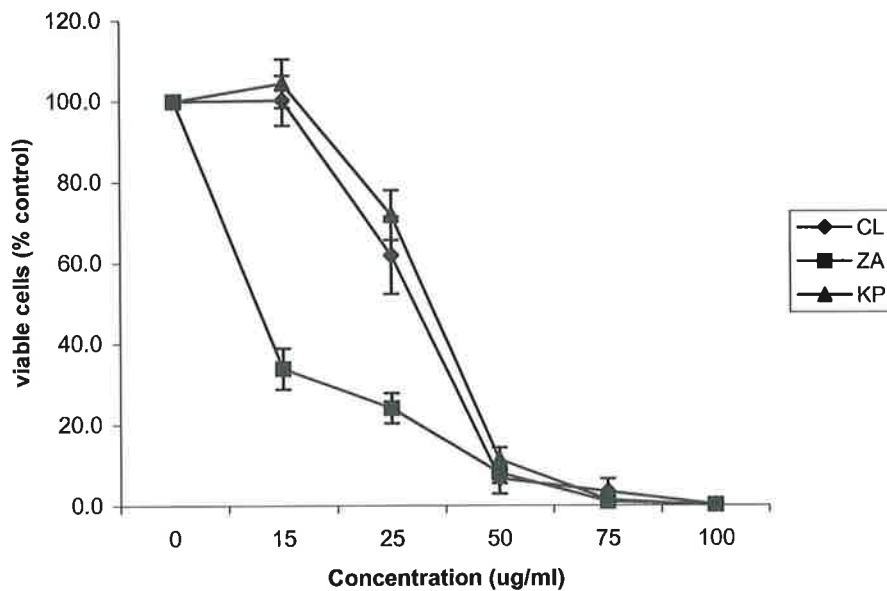
Figure 4.3. The growth of HT-29 colon cancer cells exposed to ethanol extracts of *C. longa* (CL), *Z. aromaticum* (ZA) and *K. pandurata* (KP) after 72 hours. Values are means ± SE of three independent experiments.

Cancer cells treated with extracts of active species showed features of apoptosis such as membrane blebbing, chromatin condensation and/or nucleus fragmentation (figure 4.4 and 4.5).

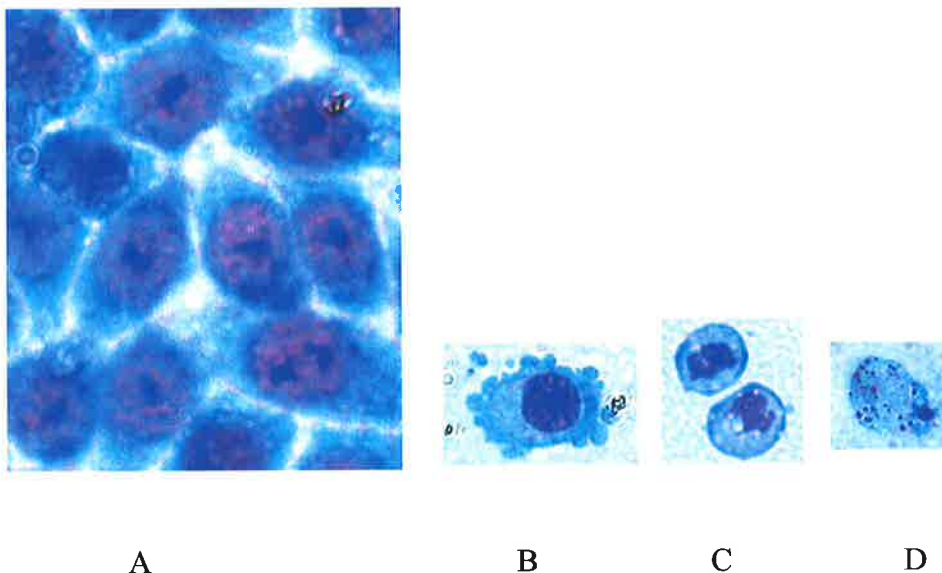A   B   C   D

Figure 4.4. (A) Normal HT-29 colon cancer cells, (B) features of apoptosis of HT-29 cancer cells after treatment with an extract of *K. pandurata* stained with DiffQuick, membrane blebbing, nucleus shrinkage and chromatin condensation (B); chromatin condensation and pyknotic nuclei (C) and nucleus fragmentation into smaller nuclear bodies (apoptotic bodies) (D). Original magnification X 100.
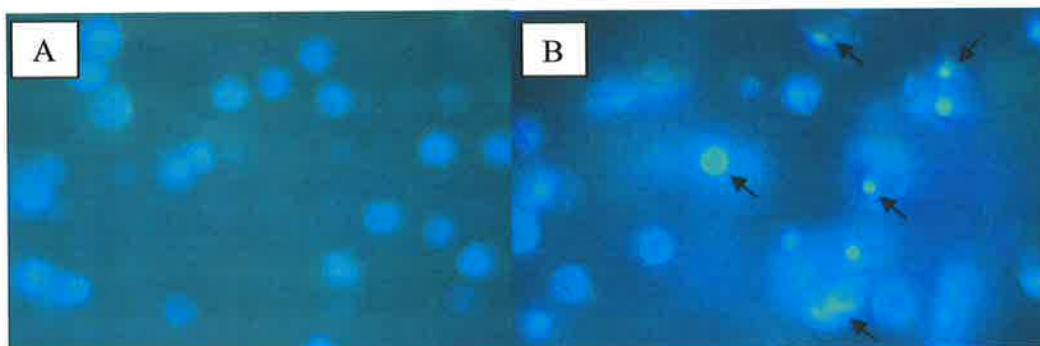
Figure 4.5. (A) Normal HT-29 colon cancer cells, (B) features of apoptosis of HT-29 cancer cells after treatment with an extract of *Z. aromaticum* for 72 h and stained with Hoechst-33258, chromatin condensation and nucleus fragmentation into smaller nuclear bodies (apoptotic bodies). Original magnification X 400.

4.3.2. Inhibitory activity of fractions A and B of *C. longa, Z. aromaticum* and *B. pandurata*

By comparison with a standard curcuminoids, fraction A of *C. longa* contained curcumin, demethoxycurcumin and bisdemethoxycurcumin. Fraction A of *Z. aromaticum* and *K. pandurata* contained no curcuminoids (Chapter 3).

Fraction A of *C. longa* had the greatest anti proliferative activity fraction against HT-29 cells compared to those of *Z. aromaticum* and *B. pandurata* (Figure 4.6). The IC_{50} of the *C. longa* extract was 15.0 μg/ml.

Fraction B of *Z. aromaticum* and *K. pandurata* were more active than that of *C. longa* (figure 4.7). The IC_{50} of fraction B of *Z. aromaticum* and *K. pandurata* were about 10 μg/ml and that of *C. longa* was 22.2 μg/ml.

Fraction A of *C. longa* was more active than fraction B, while fraction B of *Z. aromaticum* and *B. pandurata* was more active than its respective fraction A.
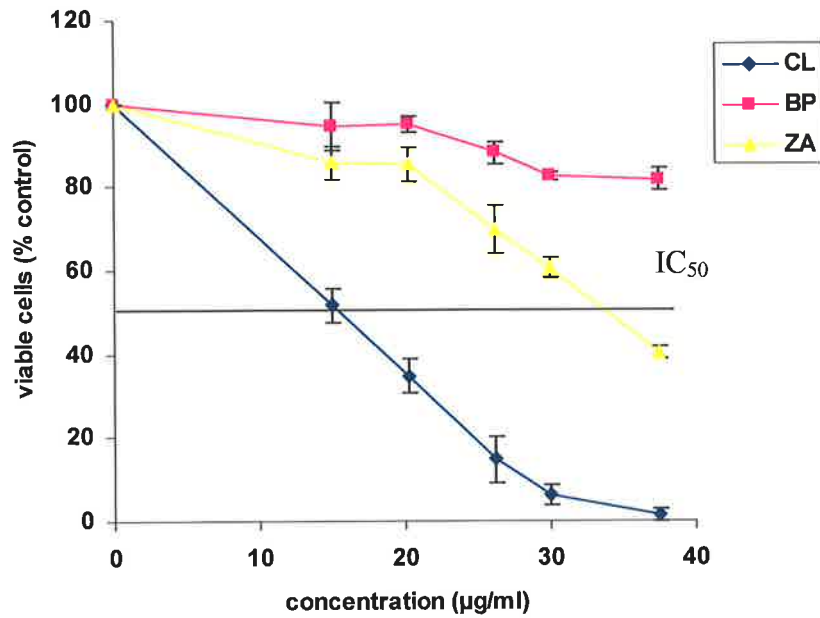
Figure 4.6.: The growth of HT-29 cells exposed to fraction A (eluted with a mobile phase containing 5% v/v aqueous methanol containing 0.025% v/v TFA) of different species (CL=*C. longa*; BP= *B. pandurata*; ZA= *Z. aromaticum*) for 72 h. Values are means ± SE of three independent assays.
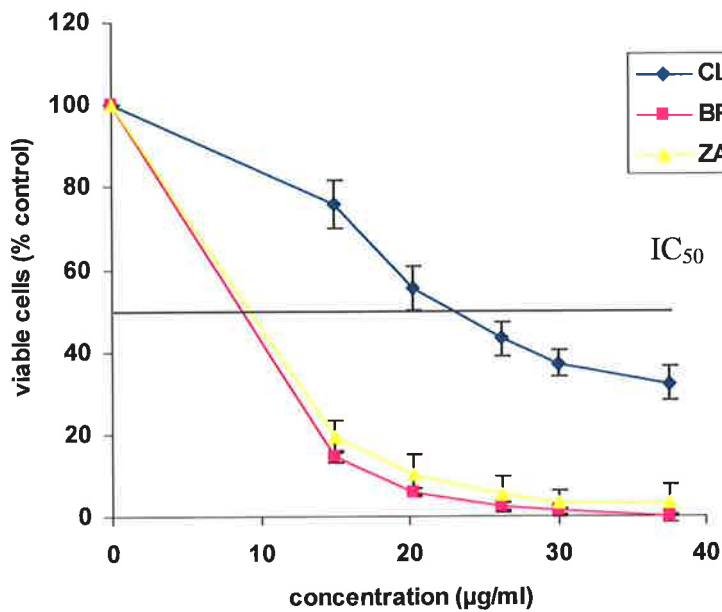

Figure 4.7: The growth of HT-29 cells exposed to fraction B which was eluted with a mobile phase containing 100 % methanol of different species (CL=*C. longa*; BP= *B. pandurata*; ZA= *Z. aromaticum*) for 72 h. Values are means ± SE of three independent assays

4.3.3. The anticancer activity of zerumbone

The inhibitory activity of zerumbone was assessed using three different cell lines: HT-29 colon cancer, CaCo-2 cells and MCF-7 breast cancer cells. Figure 4.8. shows the viability of three cancer cell lines treated with zerumbone at concentrations of 1, 5,10, and 25 μM for 72 h. Zerumbone inhibited the proliferation of all three cell lines to a similar extent. The IC $_{50}$ of zerumbone in each of the cell lines was about 10 μM. The IC $_{50}$ of curcumin on HT-29 cells was 25 μM.
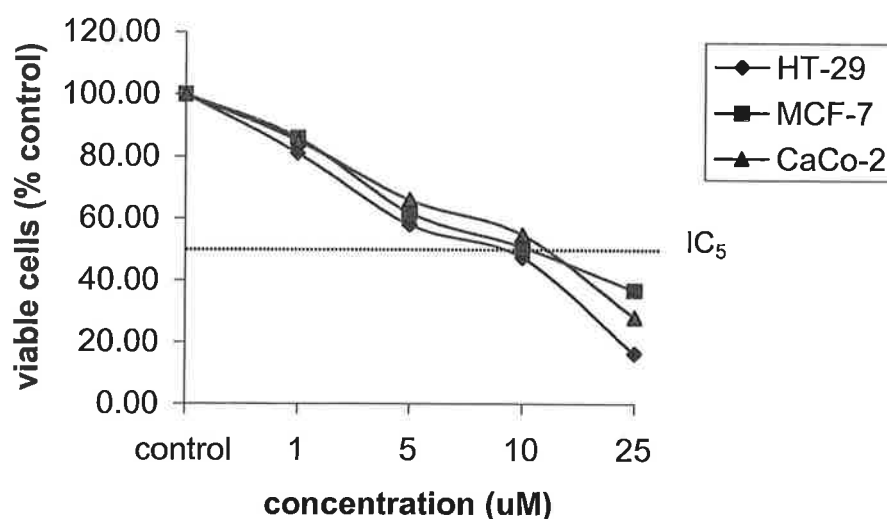

Figure 4.8. The viability and IC50 of MCF-7, HT-29 and CaCo-2 cells at 72 h after treating with zerumbone. Values are means of three independent experiments

Figure 4.9 shows the effect of zerumbone to inhibit the growth of HT-29 cells when incubated at 24, 48 and 72 h. A cytostatic effect of zerumbone on HT-29 cells was at 50 μM.

Cell cycle analysis of HT-29 cancer cells treated with zerumbone at 10, 12.5 and 25 μM for 24 h showed alterations of the distribution of DNA content (Figure 4.10). The proportion of cells in the S phase was reduced from 18.7 % in untreated cells to 10.2 % at 10 μM and 3.1 % at 25 μM respectively. By comparison, there was an increase in the G2/M phase from 18.5 ± 0.3 % in cells treated with 10 μM to 40 ± 0.1 % at a concentration of 25 μM. Chromosomal morphology of HT-29

cells treated with zerumbone suggested that cells were arrested in G2 phase of the cell cycle (Figure
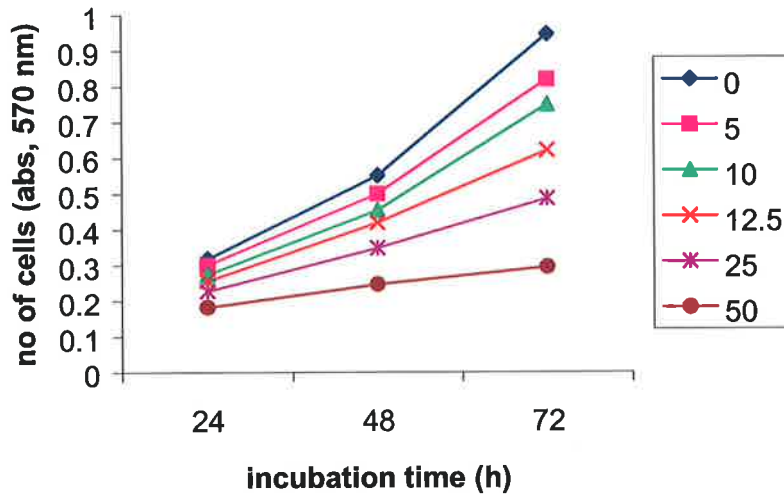
4.11B).

Figure 4.9 The growth of HT-29 colon cancer cells treated with zerumbone up to 72 h. Values are means of three independent experiments
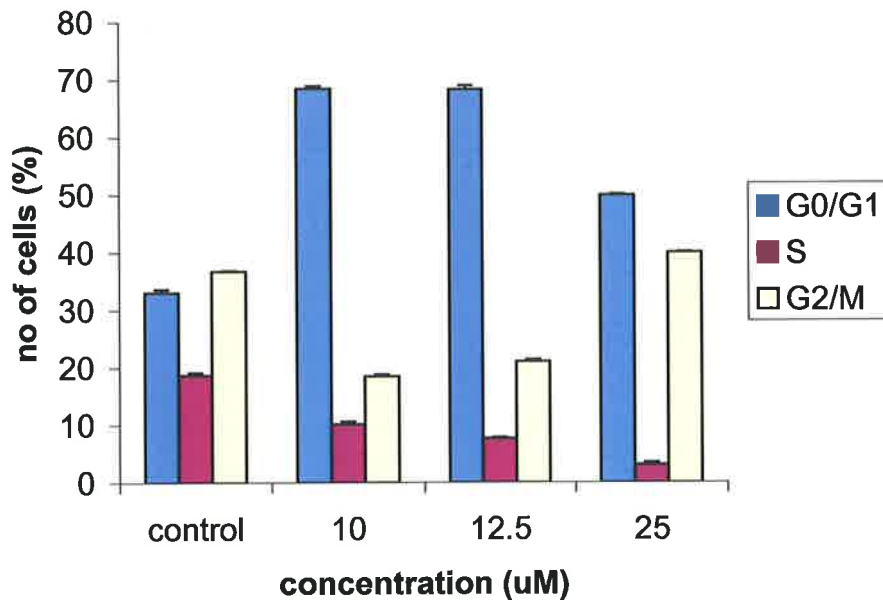

Figure 4.10. Cell cycle analysis of HT-29 colon cancer cells treated with zerumbone for 24 h. Values are means ± SE of three independent experiments

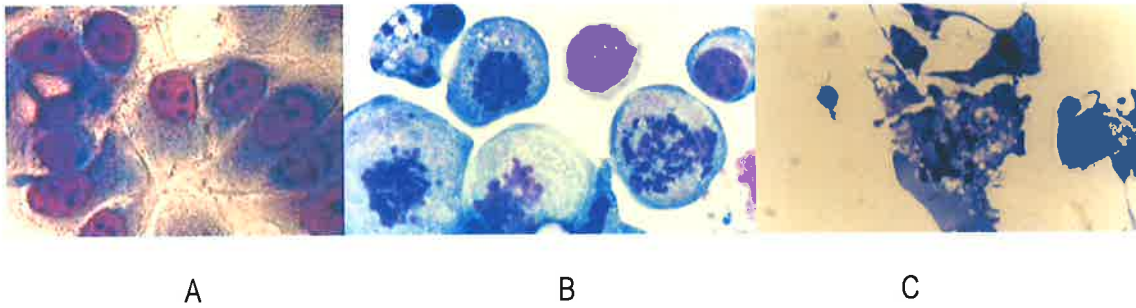A   B   C

Figure 4.11.
Morphology of HT-29 cells stained with DiffQuick : untreated cells (A); treated with zerumbone for 24 h showing chromosomes due to cell cycle arrest in G2 phase (B); and treated with zerumbone at 48 h showing features of apoptosis such as chromatin condensation and apoptotic bodies (C) (1000 X).
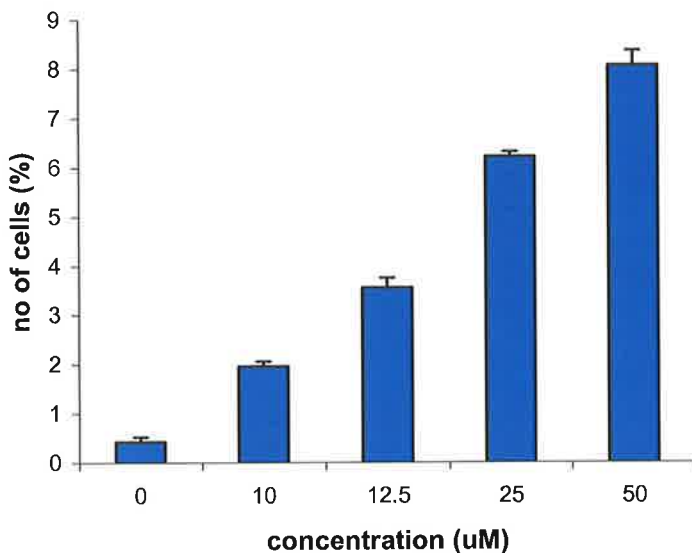

Figure 4.12. Zerumbone induced apoptosis in HT-29 colon cancer cells after 48 h. Values are means ± SE of three independent experiments

Morphological changes of cells treated with zerumbone showed features of apoptosis such as membrane blebbing, chromatin condensation and apoptotic bodies (Figure 4.11C) compared to untreated cells (Figure 4.11A). Flow cytometry analysis using Annexin-V stain further revealed that zerumbone induced apoptosis in HT-29 cells in a dose dependent manner (Figure 4.12). At 48 h, 2% of cells treated with 10 μM of zerumbone underwent apoptosis increased to 6% when treated with 25 μM.

4.3.4. The anticancer activity of panduratin A

The inhibitory activity of panduratin A was assessed using HT-29 colon and MCF-7 breast cancer cells. Figure 4.13 shows the viability at 72 h of the cell lines treated with panduratin A at concentrations of 9, 23, 28, and 46 μM. The IC_{50} of panduratin A in HT-29 cells was 16 μM, 17.2μM in CaCo 2 cells and in MCF-7 cells was 9 μM. A cytostatic effect of panduratin A on both HT-29 and MCF-7 cells was at 9 μM (figure 4.14 and 4.15).
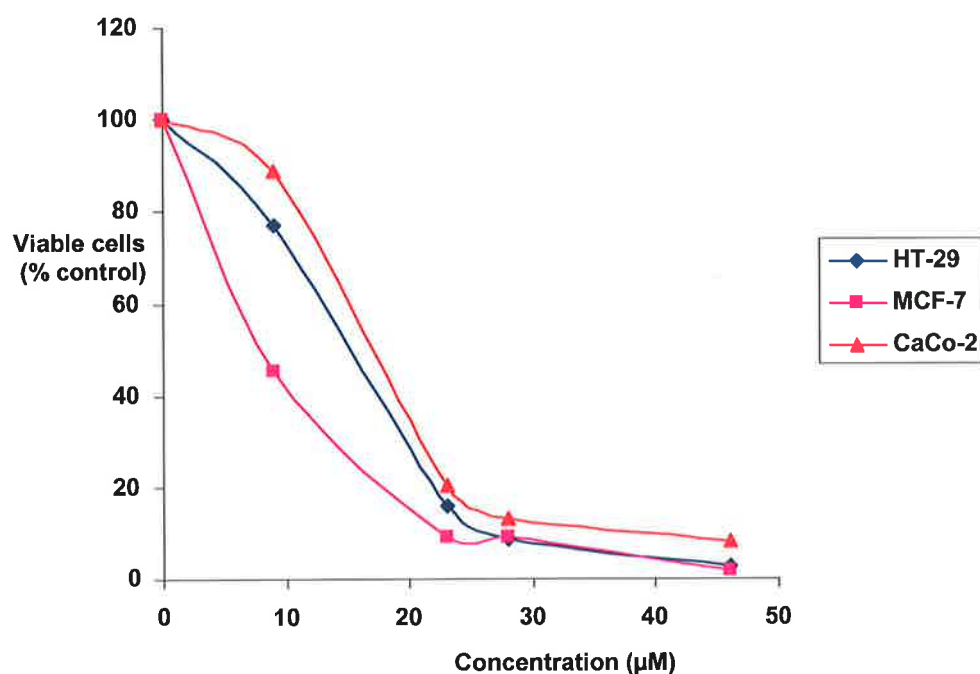
Figure 4.13.: The viability and IC50 of MCF-7 and HT-29 cells after 72 h treatment with different concentration of panduratin A. Values are means ± SE of three independent experiments.

Cell cycle analysis of HT-29 cancer cells treated with panduratin A at 9, 23, 28, 46 and 65 μM for 24 h showed alterations of the distribution of DNA content (Figure 4.16). There was an increase in the G0/G1 phase from 33 % in untreated cells to 71 % at the highest concentration used. The proportion of cells in the S phase was slightly reduced from 18.7 % in untreated cells to 10.9 % at 65

μM. By comparison, there was a decrease in the G2/M phase from 36.8 % in untreated cells to 15.4 % at a concentration of 65 μM.

Figure 4.14. The growth of MCF-7 cells treated with panduratin A at different concentrations (μM) for up to 72 h.
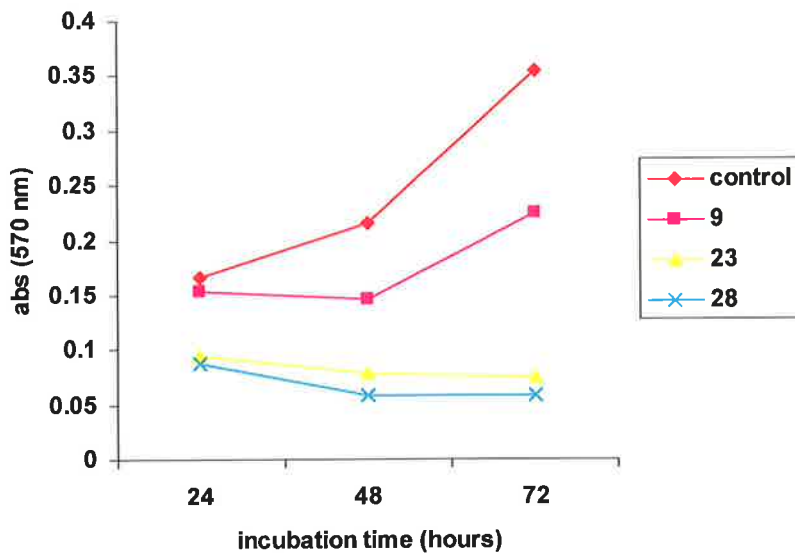
Figure 4.15. The growth of HT-29 cells treated with panduratin A at different concentrations (μM) for up to 72 h.
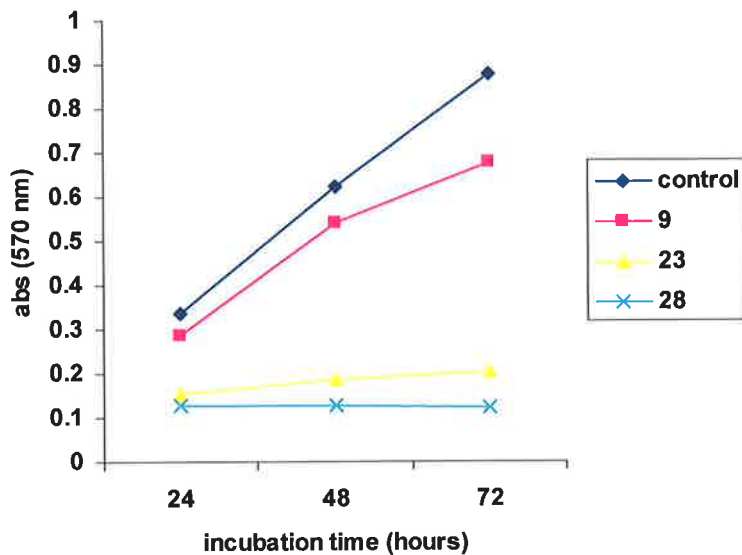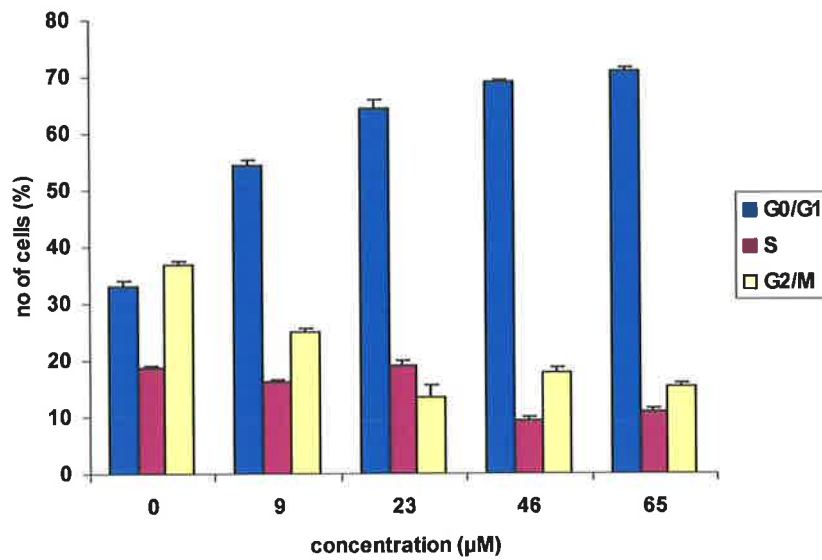
Figure 4.16: HT-29 cancer cells in different phases of cell cycle after 24 h treatment with different concentrations of panduratin A. Values are means ± SE of three independent experiments.
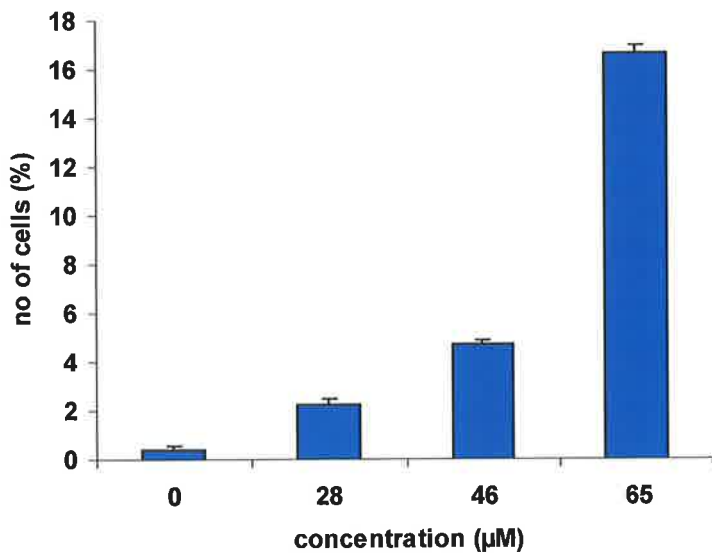
Figure 4.17: Panduratin A induced apoptosis in HT-29 cancer cells after 48 h. Values are means ± SE of three independent experiments

Chromatin condensation and/or nuclear fragmentation were observed in the cells treated with panduratin A when stained with Hoechst 33258. Morphological changes of cells treated with panduratin A showed features of apoptosis such as membrane blebbing, chromatin condensation and

apoptotic bodies compared to untreated cells. Flow cytometry analysis using Annexin-V stain further revealed that panduratin A induced apoptosis in HT-29 cells. At 48 h, 2.2% of cells treated with 28 μM of panduratin A underwent apoptosis which increased to 16.7% when treated with 65 μM (figure 4.17).

4.4. Discussion

We have used established colon and breast cancer cells to screen ethanol extracts of eleven species of Indonesian Zingiberaceae. The extracts of eight species of Zingiberaceae were found to strongly inhibit the growth of MCF-7 and HT-29 cells. They were *A. cardamomum, C. longa, C. xanthorrhiza, C. mangga, Z. aromaticum, Z. cassumunar, Z. officinale*, and *K. pandurata*. Five of these: *C. mangga, C. xanthorrhiza, C. aeruginosa, Z. aromaticum, Z. cassumunar* have been used for medicinal purposes, while *Z. officinale* and *K. pandurata* and *A. cardamomum* have been used as traditional medicine as well as in cooking. The IC_{50} of extracts of these species were between 10 and 100μg/ml. A comparison of the IC_{50} values represents a means of determining the potency of a given extract in inhibiting cell growth by 50%. The lower the IC_{50} value, the more potent is the extract as an inhibitor of tumor cell growth. Extract of *C. aeruginosa* was found to be less active with the IC_{50}s at 100-120μg/ml, while extracts of *K. rotunda and K. galanga* were not active at concentrations up to 250 μg/ml. We found that the values of IC_{50} of some extracts were quite different on different cell lines. For example extracts of C. mangga (44.7 μg/ml for MCF-7 and 91.0 μg/ml for HT-29 cells) and *Z. cassumunar* (54.5 μg/ml for MCF-7 and 84.9 μg/ml for HT-29 cells). This effect could be due to the sensitivity of cell lines to the nature of active compounds present in the extracts and could represent a tissue-specific response. Screening for antitumor activity of some members of Zingiberaceae has been conducted using activated Epstein Bar Virus (EBV) on Raji cells (Murakami et al., 1998; Vimala et. al., 1999). It was reported that an extract of *K. galanga* was less active compared to the other

species (Murakami et al., 1998) and the ethanol extract showed inhibitory activity at a concentration of 320 µg/ml (Vimala et al., 1999).

Demethoxycurcumin was reported to have the same potent antiinflammatory activity as (Huang et al. 1995) or greater activity than curcumin (Ruby et al. 1995). The amount of curcumin in the extract of C. xanthorrhiza was similar to that of C. longa, but the concentration of demethoxycurcumin was much lower (Chapter 3). The composition of curcuminoids in these two species may explain the difference in their anticancer activity.

The inhibitory activity of curcumin has been reported to result from an inhibition of protein kinase C (PKC) (Liu et al., 1993) and phosphorylase kinase (Reedy and Agarwal, 1994) which are involved in the regulation of cell proliferation and growth and induction of apoptosis in various cancer cell lines (Jiang et al., 1996; Kuo et al., 1996). Lee et al. (1998) found that a methanol extract of A. oxyphylla inhibited the growth of HL 60 cells and was also found to induce apoptosis. Gingerol, vanilloids and paradol isolated from Z. officinale have also been shown to induce apoptosis in vitro (Lee and Surh, 1998). In the present study, apoptosis were also observed following incubation of either tumor cell lines with extracts of active species.

Extracts of the active ginger species were found to be more active inhibiting cancer cell lines than non-transformed fibroblast cells with an exception of extract of C. longa which showed no difference on both cell lines. The IC_{50} on HT-29 and MCF-7 cells was 28 and 31 µg/ml respectively and on skin fibroblast was 31µg/ml. Gautam et al. (1998) reported that at concentrations higher than 25 µM, curcumin had a similar effect at inhibiting the growth of both cancer cells and normal fibroblast cells. This can be explained by non-transformed skin fibroblast being very slow growing cells compared to cancer cells. Therefore at the same concentrations, the extract was regarded not toxic on the normal cells. The Jiang et al. (1996) and Ramachandran and You (1999) reported that curcumin inhibited the growth of cancer cells more effectively than normal cells by elevating apoptosis in cancer cells.

Fraction A of *C. longa* was more active than fraction B, however fraction B also appeared to contain active constituents which inhibited the growth of HT-29 colon cancer cells. Curcumin, which has been found to be the major active compound in *C. longa* and intensively investigated for anticancer activity in *in vitro* and *in vivo* studies, was found in fraction A. The dried rhizomes of *C. longa* have been reported to contain 3-5% essential oils and 0.02-2% aromatic yellow curcuminoids (Gopalan et al., 2000). He et al. (1998) reported that the major constituents of turmeric were sesquiterpenoids including ar-turmerone, curlone, α-turmerone, bisacumol, zingiberene, curcumenone, curcumenol, procurcumenol and dehydrocurdione. Using HPLC and comparing to the chromatogram from He et al. (1998), these sesquiterpenoid compounds were present in fraction B. Terpenes are the largest group of compounds isolated from plants, which have been identified as having pharmacological activities especially antioxidant activity (Dillard and German, 2000). Other terpenes such as limonene were also reported to have anticancer activity by inhibiting phase I and II enzymes (Fahey and Stephenson, 2002). The presence of these terpenes in fraction B of *C. longa* suggests that the antiproliferative activity of this fraction may be due to these compounds.

By contrast, fraction B of *K. pandurata* and *Z. aromaticum* were more active in inhibiting the growth of HT-29 cells than those of their respective fraction A. Zerumbone, a sesquiterpenoid compound was isolated from fraction B of *Z. aromaticum*. Dai et al. (1997) reported that zerumbone was a major antiviral and cytotoxic compound from *Z. aromaticum* and *Z. zerumbet*. Zerumbone isolated from *Z. zerumbet* was also reported to inhibit the growth of cancer cells (Murakami et al., 2002).

Zerumbone inhibited the growth of three human cancer cell lines, HT-29 colon, CaCo-2 colon and MCF-7 breast cancer cells. In this study HT-29 cells treated with zerumbone for 48 h induced apoptosis in a dose dependent manner. Apoptosis induction by zerumbone is partly caused by dysfunction of the mitochondrial transmembrane as observed by Murakami et al (2002). The antiproliferative activity of zerumbone appeared to alter the distribution of DNA in HT-29 cells by

inhibition of DNA synthesis and blocking cells at the G0/G1 at lower concentrations and G2/M phase at higher concentrations. Curcumin also had a blocking effect in the G2/M phase of cell cycle in many types of cancer cells (Holy, 2002). In cell culture experiments zerumbone was found to inhibit superoxide anion (O_2^-) formation (Murakami et al., 2002), which is easily converted to the more reactive intermediates causing DNA mutation and leading to tumor promotion. Murakami et al. (2002) reported that α-humulane, a structural analog lacking only the carbonyl group in zerumbone was inactive and they have suggested that the α,β-unsaturated carbonyl group of zerumbone plays an important role in its anticancer activity.

In a screening program of extracts from natural products for antiviral and antitumor activity by the National Cancer Institute (NCI), it was found that zerumbone which was isolated from Z. aromaticum and Z. zerumbet had antiviral and cytotoxic properties against HL-60 cell line ($GI_{50} = 0.33$ μg/mL) (Dai et al., 1997). Sawada and Hosokawa (2000) reported that zerumbone from Z. zerumbet showed very strong inhibitory effect on the growth of EL4 cells, a mouse T-lymphoma cell. Murakami et al (2002) also reported that zerumbone from Z. zerumbet had an antiproliferative effect on various human colonic adenocarcinoma cells (LS174T, LS180, COLO205 and COLO320DM) but less effect on the growth of normal cells. They attributed these effects of zerumbone to the induction of apoptosis.

Panduratin A has been reported to possess antiinflammatory activity in the TPA-induced ear edema model in rats (Tuchinda et al., 2002). However, there has been no study on the anticancer activity of the compound. In this study panduratin A inhibited the growth of HT-29 colon and MCF-7 breast cancer cells. This chalcone was more active in inhibiting the growth of MCF-7 cells ($IC_{50} = 9$ μM) than HT-29 cells ($IC_{50} = 16$ μMl) or CaCo-2 cells ($IC_{50} = 17.2$ μMl). The antiproliferative activity of panduratin A appeared to be due to alteration in the distribution of DNA in HT-29 cells by blocking cells at G0/G1 phase and inhibiting S phase. Panduratin A also induced apoptosis. Dysregulation of the cell cycle may directly affect the apoptotic stimuli. The tumor suppressor gene p53 has been

suggested to play a crucial role to detect DNA damage and subsequently arrest the cells in G1 phase and further induce apoptosis (Mendoza-rodriguez and Cerbon, 2001). Panduratin A has also been reported to induce phase II enzymes such as quinone reductase (QR) (Trakoontivakorn et al., 2001). Phase II enzymes are very important in cellular defence and metabolism including detoxification of electrophilic species and thereby preventing induction of oxidative stress (Schultz et al., 1997). The potential anticancer activity of panduratin A needs further evaluation.

4.4 Summary of experiments

The ethanolic extracts of eight species of Zingiberaceae showed strong activity in inhibiting the growth of HT-29 and MCF-7 cells. They were A. cardamomum, C. longa, C. xanthorriza, C. mangga, Z. officinale, Z. cassumunar, Z. aromaticum, and K. pandurata. An extract of C. aeruginosa was less active and extracts of K. galanga and K. rotunda had no effect on the growth of either cell lines at concentrations up to 250 µg/ml. The extracts with the greatest activity were Z. aromaticum, and K. pandurata which showed similar inhibitory activity to extract of C. longa. Ethanol extracts of eleven species of Zingiberaceae were less toxic to non-transformed cells. Ethanol extracts of the eight active species were shown to induce apoptosis of cancer cells.

Fraction A and fraction B of C. longa showed some growth inhibitory activity against HT-29 cells in vitro. Fraction A of C. longa was found to contain curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) and was more active than fraction B. Fraction B of Z. aromaticum and B. pandurata were more active that fraction A respectively. Zerumbone was isolated from fraction B of Z. aromaticum and panduratin A was isolated from fraction B of B. pandurata. Fraction A of Z. aromaticum however showed only slight inhibitory activity.

Zerumbone showed antiproliferative activity by alteration of the cell cycle in G2 phase and induction of apoptosis. The IC_{50} of zerumbone on HT-29, CaCo-2 colon cancer and MCF-7 breast cancer cells was 10 µM.

Panduratin A showed antiproliferative activity by alteration of the cell cycle in G0/G1 phase and induction of apoptosis. The IC_{50} of panduratin was 9, 16 and 17 µM on MCF-7, HT-29 and CaCo-2 cells respectively.

CHAPTER 5

THE INFLUENCE OF EXTRACTS OF

ZINGIBER AROMATICUM AND BOESENBERGIA PANDURATA ON AZOXYMEHTANE (AOM)-

INDUCED ABERRANT CRYPT FOCI (ACF) IN RAT COLON CANCER MODEL

5.1. Introduction

Colorectal carcinogenesis is a complex multisep process, and aberrant crypt foci (ACF) are thought to be an early stage of colon cancer which then to proliferate by crypt fission to form microadenoma (Archer et al., 1992). ACF induced by the carcinogen AOM (McClellan and Bird, 1988) and DMH (Goldin, 1988) have been used and provide a useful means for assessing the protective potential of dietary components against chemically induced carcinogenesis.

The epidemiological data which showed that the incidence and mortality of colon cancer differ from country to country and the fact that cancer rates of migrants moving from low risk countries to high risk countries soon come to resemble that of the host country have led to the suggestion that colon cancer is associated with environmental factors including diet (Bruce et al., 1993).

From a previous study, ethanol extracts of Z. aromaticum and B. pandurata and their active compounds zerumbone and panduratin A respectively have been shown to have potential anticancer activity in in vitro cell culture studies.

The aim of this study was to investigate the anticancer activity of extracts of Z. aromaticum and B. pandurata using the AOM-induced abberant crypt foci (ACF) colon cancer model in rats in a short (5 weeks) and long term (13 weeks) study. The anticancer effect of extracts of Z. aromaticum and B. pandurata was compared to that of an extract of C. longa and standard curcumin as a positive control.

5.2. *Experimental Designs:*

Four weeks old male outbred Sprague-Dawley rats were purchased from the Animal Research Centre at Murdoch University (Perth, Western Australia), housed in wire cages (5 rats per cage) to minimize coprophagy and maintained in an air-conditioned environment of 23 ± 2 ^{0}C with a 12:12-hour light dark cycle. Rats were given free access to diet and water.

5.2.1. Short term (five week) study

Beginning at 6 weeks of age rats were given different experimental diets which based on AIN-93 specification (Reeves et al., 1993). There were 4 groups of 10 animals (see table 5.1).

Table 5.1. Ethanolic extract of dried rhizomes of Z. *aromaticum* (ZA), B. pandurata (BP) and C. *longa* (CL) added to semipurified rodent diet.

Group	Equivalent dried rhizome weight (% diet w/w)
AIN 93	-
AIN 93 + ZA extract	2
AIN 93 + BP extract	2
AIN 93 + turmeric extract	2

Extracts were prepared from the equivalent of 2% by weight of dried rhizomes of *Z. aromaticum, B. pandurata* and ground *C. longa* (turmeric). The dried material was extracted with ethanol three times (material : solvent = 1:6) at room temperature. The ethanol extracts were combined and evaporated to reduce the amount of ethanol. Extracts were then mixed with the AIN 93 diet and dried at 35 ^{0}C in an oven overnight. The active compounds of extracts in the diet were not quantified as the active compounds in the extract of *Z. aromaticum* and *K. pandurata* had not been identified at the commencement of the experiment. At eight weeks of age, rats received two subcutaneous injections of AOM one week apart at a dose rate of 15 mg/kg body wt. Rats were

weighed weekly and sacrificed three weeks after the second AOM injection (figure 5.1). Colons were

removed for ACF counting.

Figure 5.1. Diagram of short term (five week) experiment

Week of age
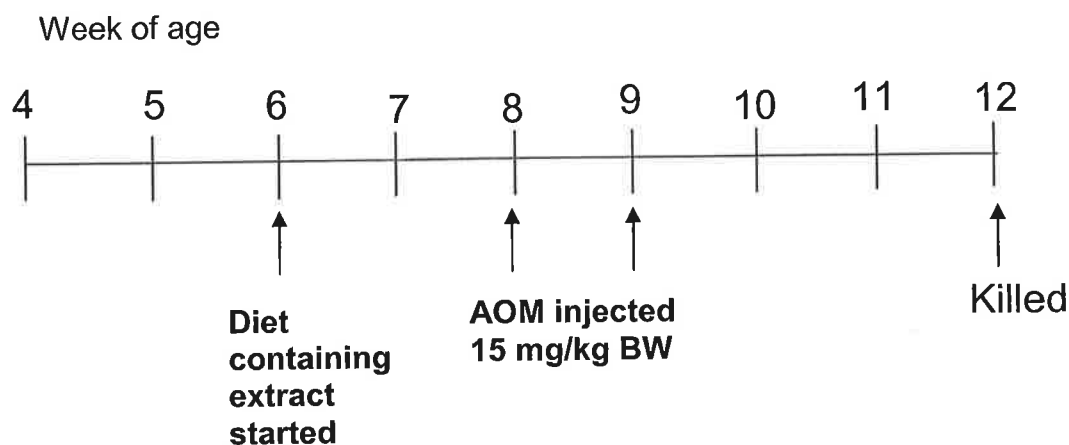
5.2.2. Long term (thirteen week) experiment

Beginning at 5 weeks of age rats were given different experimental diets which based on AIN-93 specification (Reeves et al., 1993). There were 5 groups of 14 animals (see table 5.2).

Extracts were prepared from the equivalent of 4% by weight of dried rhizomes of Z. aromaticum, B. pandurata and turmeric. The dried material was extracted with ethanol as described earlier. Extract from dried rhizomes of Z. aromaticum, B. pandurata or turmeric (34; 54; 86% w/w respectively) were added to AIN diet. The concentration of zerumbone in the diet containing extract of Z. aromaticum was measured using HPLC and found to be 300 ppm. The concentration of curcumin in the diet containing turmeric extract was 300 ppm. The concentration of panduratin A in the diet containing extract of B. pandurata was not quantified as the compound had not been identified at the commencement of the experiment. Diets were made fortnightly and kept in the freezer until required. At 7 weeks of age, rats received two subcutaneous injections of AOM one week apart at a dose rate of 15 mg/kg body wt. Rats were weighed weekly. Rats were sacrificed 10 weeks after the second AOM injection (figure 5.2) and their colons removed for ACF counting.

Table 5.2. Ethanolic extract[*] of dried rhizomes of *Z. aromaticum* (ZA) and *C. longa* (CL) added to semipurified rodent diet.

Group	Equivalent dried rhizome weight (% diet w/w)	Ethanol extract[*] (% diet w/w)	Active compound[¥] (ppm in diet)
AIN 93	-	-	-
AIN 93 + ZA extract	4	0.34	300 ppm as zerumbone
AIN 93 + BP extract	4	0.54	NA
AIN 93 + CL extract	4	0.86	300 ppm as curcumin
AIN 93 + Cur 2000 ppm	-	-	2000 ppm curcumin

Note: [*] weight of extract added to diet (%); [¥] concentration in diet of bioactive component assay by HPLC in extract; ZA = *Zingiber aromaticum*; BP= Boesenbergia pandurata; CL = *Curcuma longa*; Cur = Curcumin; AIN 93 is American Institute of Nutrition semipurified rodent diet (19). NA = not measured.

Figure 5.2. Diagram of long term (13 week) experiment

Week of age

Diet containing extract started

AOM injected 15 mg/kg BW

Killed
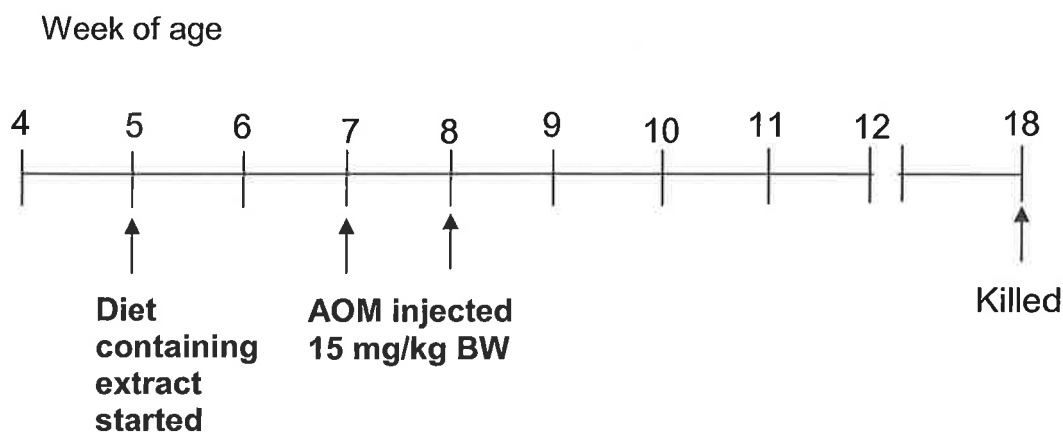
5.2.3. Statistical analysis

Data are reported as mean ± SEM and comparisons were analysed by a one-way analysis of variance (ANOVA), with Bonferroni post hoc test to identify between-group differences ($p < 0.05$) using Graphpad Prism (version 2.0).

5.3. Results:

5.3.1. Short term (five week) experiment

In a short-term experiment (5 weeks), body weights of rats did not differ between the dietary treatment groups over the experiment period (figure 5.1). Weights of spleen and liver and length of colon between groups were also not significantly different (Table 5.3.).

Diets containing extract from 2% dried rhizomes of *Z. aromaticum*, *B. pandurata* and *C. longa* did not affect the formation of ACF in rats compared to those fed the control diet (figure 5.2).
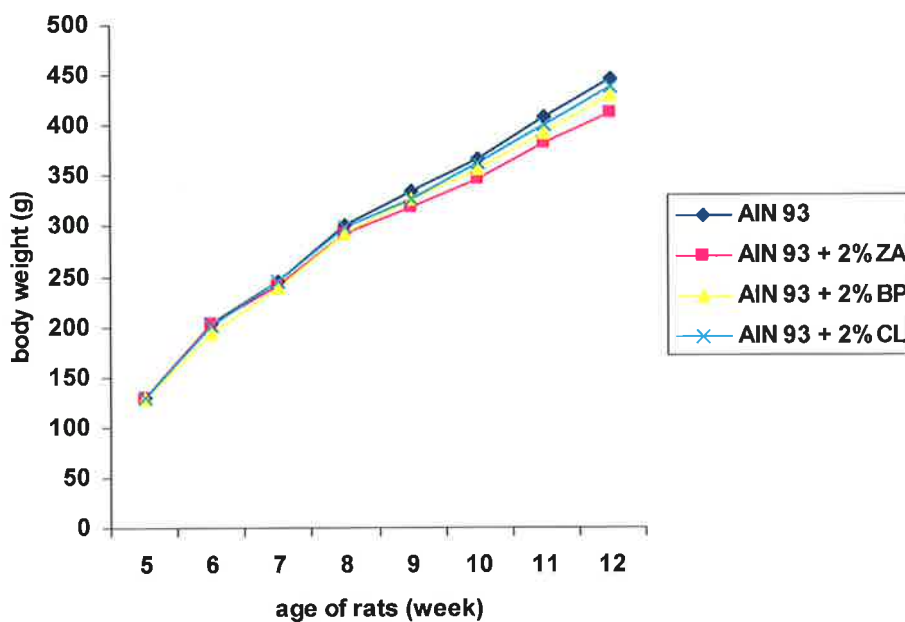

Figure 5.1. Body weight of rats before and during the five week experiment

The ACF formation in this experiment was dominated by small numbers of aberrant crypts (1 or 2) per focus. The number of ACF with 2 and 3 and 4 aberrant crypts per focus in rats fed diet with *Z. aromaticum and B. pandurata* extract were slightly decreased compared to the control diet, but this also was not statistically significant (Figure 5.3). The longer (13 weeks) experiment was conducted to provide larger ACFs which are better predictive of cancer. The amount of extracts in the diet was increased from 2% (short term five week experiment) to the equivalent of 4% by weight of dried rhizomes in the diet to increase the bio-potency range.

Table 5.3. Weight of organs (g) and length of colon (cm) of rats between groups

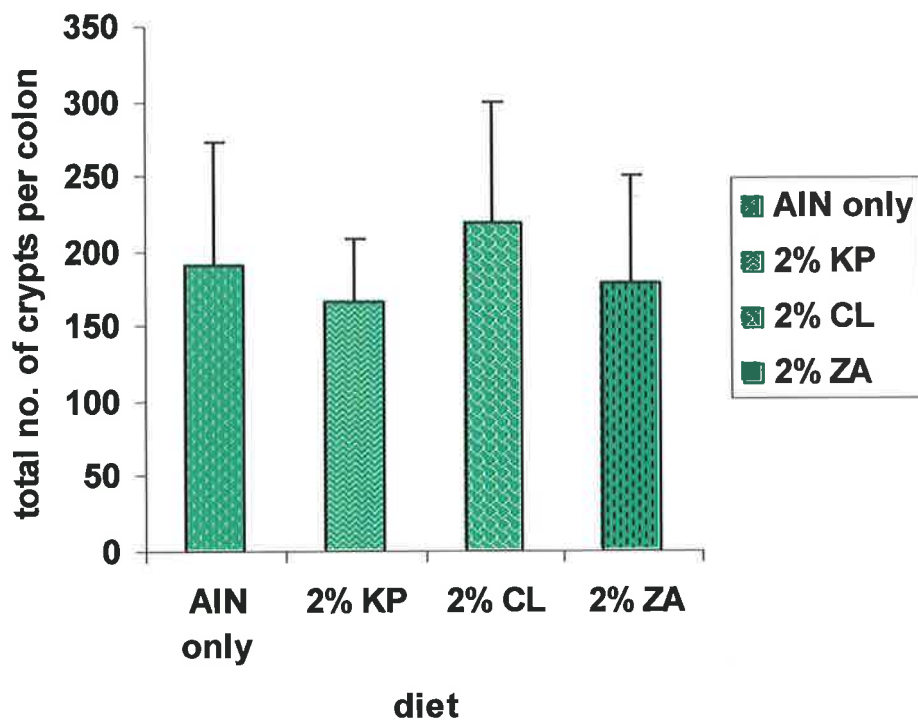| Groups	Length of Colon (cm)	Weight of	
		Spleen (g)	Liver (g)
AIN 93	23 ± 0.27	0.96 ± 0.03	18.5 ± 0.9
AIN 93 + 2% ZA	23 ± 0.37	0.91 ± 0.03	18.2 ± 0.8
AIN 93 + 2% BP	23 ± 0.37	0.96 ± 0.03	16.1 ± 0.7
AIN 93 + 2% CL	24 ± 0.32	0.92 ± 0.03	18.2 ± 0.8

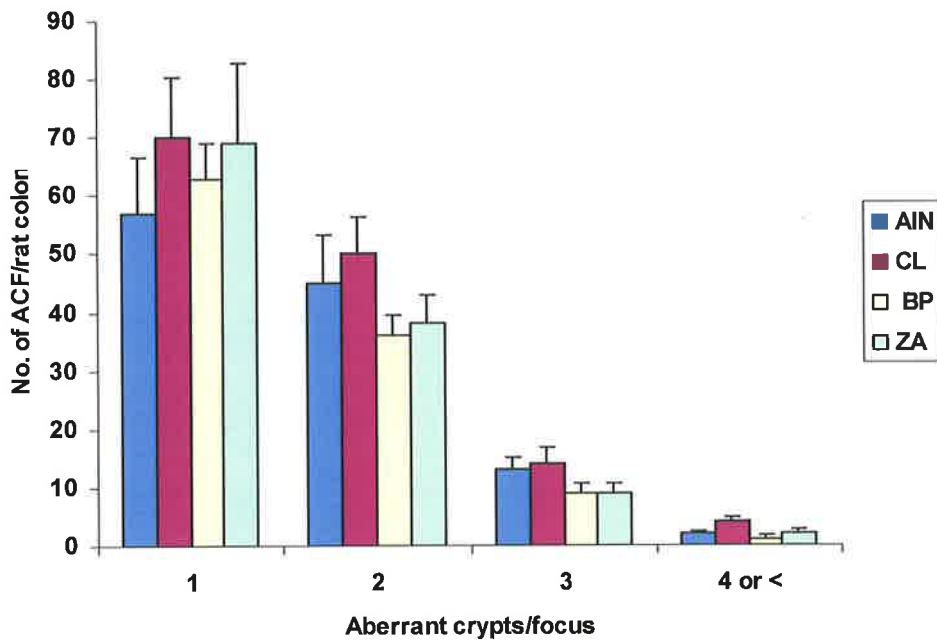Figure 5.2. Total ACF per colon of rats in the short term study

Figure 5.3: Aberrant crypt foci (ACF) formation in the colon of rats treated with different diets after 5 weeks of the second injection of AOM at 15 mg/kg BW (short term study). Treatments were not significantly different from the control AIN diet.

Diet based on AIN 93, extract of ZA, BP and CL were prepared from 2% of dried material of Z. *aromaticum, B. pandurata* and *C. longa.*

Values are means ± SE, n = 10

5.3.2. Long term (thirteen week) experiment

In long term experiment (13 weeks), body weights of rats did not differ between the dietary treatment groups over the experiment period (figure 5.4). The final body weights were (means ± SE): 509 ± 8 g in Group 1 (control), 492 ± 11 g in Group 2 (4% ZA), 519 ± 8 g in Group 3 (4% CL) and 520 ± 9 g in Group 4 (Curcumin).

Weights of spleen and the length of colon of rats between groups were not significantly different. Weight of livers of rats fed with diet containing 4% extract of CL was significantly different from those of rats fed with AIN only (Table 5.4.).
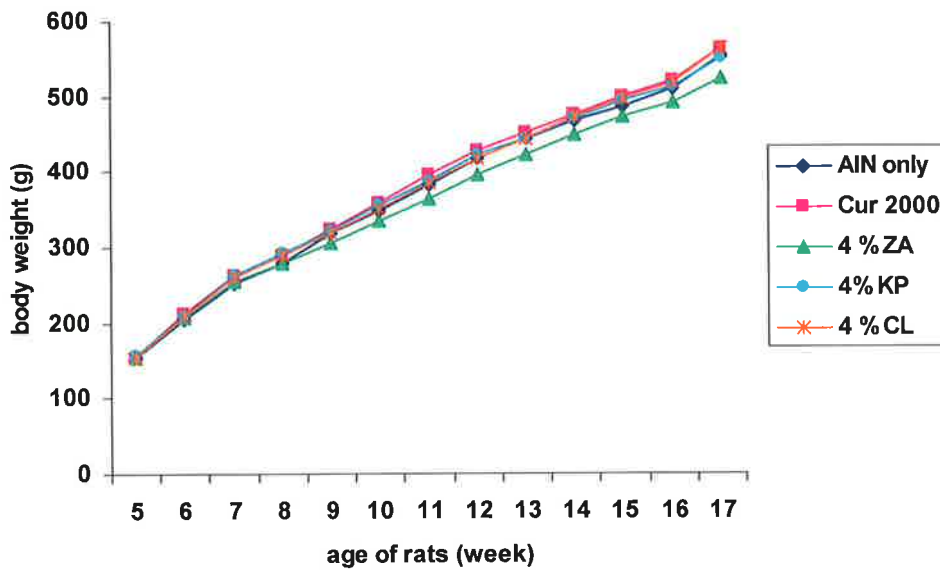
Figure 5.4. Body weight of rats before and during the long term study

Table 5.4. Weights of spleen and liver (g) and length of colon (cm) of rats between groups

Groups	Length of Colon (cm)	Weight of	
		Spleen (g)	Liver (g)
AIN 93	23 ± 0.38	0.94 ± 0.04	17.20 ± 0.58
AIN 93 + 4% ZA	23 ± 0.35	0.87 ± 0.03	18.69 ± 0.86
AIN 93 + 4% BP	23 ± 0.50	0.92 ± 0.06	18.25 ± 0.93
AIN 93 + 4% CL	24 ± 0.44	0.96 ± 0.04	20.37 ± 0.49***
AIN 93 + Cur 2000	23 ± 0.40	0.97 ± 0.05	18.16 ± 0.52

Significant different from AIN group *** ($p < 0.05$)

The effect of dietary treatments on colonic ACF formation is summarized in figure 5.17. Total ACF in AOM-induced SD rats fed with the extract of Z. aromaticum was significantly reduced (21%, $P < 0.05$) compared to rats fed the control diet. There was no significant difference in small ACFs (1-2 aberrant crypts/focus) between dietary treatments. The number of foci containing 3-4 aberrant crypts

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(AC) per focus was significantly reduced (35%, P<0.001) in animals fed the Z. aromaticum extract. The total number of ACF containing 5 or more aberrant crypts per focus was 41% lower than control (P<0.05). The reduction of total ACF in rats fed the extract of Z. aromaticum was similar to that seen in rats fed curcumin at 2000 ppm as a positive control. The concentration of zerumbone in the Z. aromaticum diet was 300 ppm.

Incorporation of extract of 4% B. pandurata extract did not affect the formation of ACF compared to the control AIN diet.
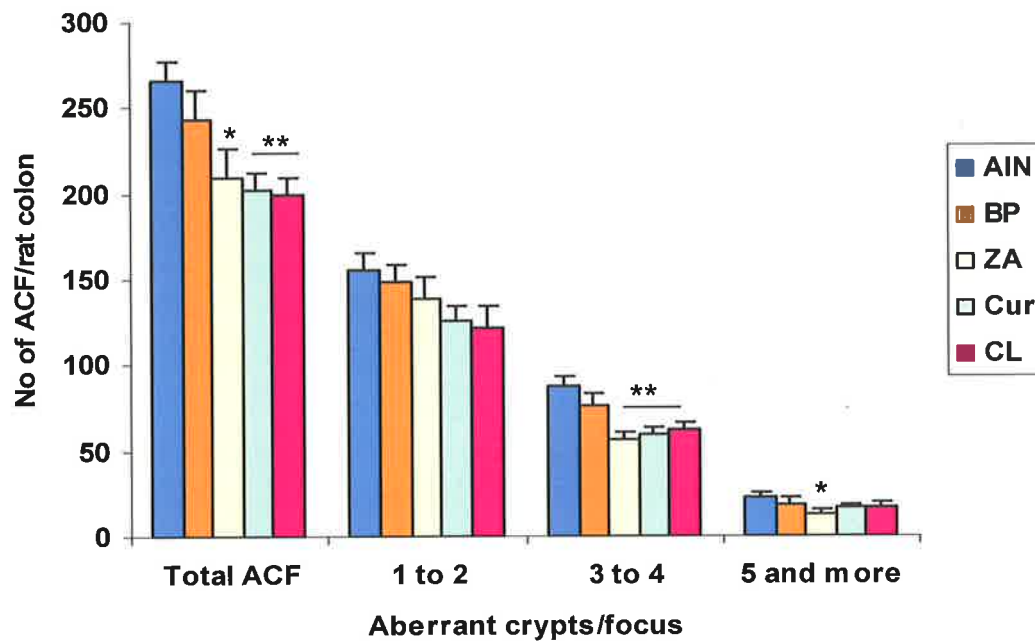

Figure 5.5. Aberrant crypt foci (ACF) formation in the colon of rats treated with different diets (AIN 93; AIN added with extract of B.pandurata (BP); Z. aromaticum (ZA), curcumin (Cur), and C. longa (CL) in the long term study.
Diet based on AIN 93, extract of ZA, BP and CL were prepared from 4% of dried material of Z. aromaticum, B. pandurata and C. longa, curcumin diet was 2000 ppm.

Values are means ± SE, n = 14, Significantly different from control * (p<0.05), ** (p<0.01), *** (p<0.001)

The number of ACF in the colon of rats given the diet containing an extract of C. longa (containing 300 ppm curcumin) was similar to those treated with diet containing pure curcumin at 2000

ppm. Total ACF in AOM-induced SD rats fed with the *C. longa* extract was significantly reduced (24%, P< 0.01) compared to rats fed the control AIN diet. The number of foci containing 3-4 aberrant crypts per focus was significantly reduced (34%, P<0.001) in animals fed the extract of *C. longa*. There was also a trend to a reduction in the number of ACF containing 5 or more aberrant crypts per focus (22%, not significant) relative to control.

5.4. Discussion

Diets containing 2% turmeric have been shown to suppress 7,12-dimethyl- benz[a]anthracene (DMBA)-induced skin tumors and to inhibit benzo[a]pyrene-(BP) induced forestomach tumors in mice (Azuine and Bhide, 1992). However, in the present study, the diets containing extracts of 2 % *Z. aromaticum* and *C. longa* failed to affect the formation of ACF in the short-term experiment (5 weeks). The majority of the ACF at this stage were small, containing mainly 1 and 2 aberrant crypts (ACs)/focus. It is generally recognised that large ACFs are better predictors of neoplasia risk. Uchida et al. (1997) and Jenab et al. (2001) found that large ACFs (4 or more ACs/focus) correlated with a greater rate of cell proliferation and a higher degree of dysplasia and, therefore provided a better predictor of colon tumorigenesis. Initially ACFs appear as single crypts, which then expand by crypt branching (or multiplication) to larger ACFs. ACF with a large number of ACs (four or more ACs) per focus are referred as microadenomata (Bird and Good, 2000).

Diet containing extracts from the equivalent of 4% by weight of *Z. aromaticum* and from 4% *C. longa* however significantly inhibited the formation of ACF in our AOM-induced colon cancer model in the 13 week experiment. Tanaka et al (2001) reported that a diet containing 500 ppm zerumbone significantly inhibited the formation of ACF in AOM-induced rats in a short term experiment (5 weeks). In this study the concentration of zerumbone in diet containing extract of *Z. aromaticum* was 300 ppm. The effect of zerumbone was clearly more pronounced in the larger ACFs (≥3 AC/focus). This

therefore represents a moderately effective chemopreventive agent as assessed using this model (Corpet and Tache, 2002).

The activity of zerumbone inhibiting ACF formation was similar to that of the positive control curcumin at a concentration of 2000 ppm, which has been found to effectively reduce ACF and tumors in previous carcinogen-induced colon cancer animal studies (Rao et al., 1993; Kawamori et al., 1999, Corpet and Tache, 2002).

We also investigated the effect of an extract of *C. longa* for chemoprevention and compared its activity with curcumin. Turmeric is used in cooking, as a natural coloring agent and preservative, in medicine and as a tonic in several Asian countries. In westernised societies, there is considerable interest in the preparation of purified compounds derived from natural products for use as medicinal agents. However in some societies, particularly those in Asia and South East Asia, the whole plant material containing close related natural products is regarded as more beneficial than the isolated and purified individual components. Thus their belief is in the benefit of the total balance of constituents in nature (Kobayashi, 1999). For example Miquel et al (2002) reported that a daily intake of 200 mg of extract of *C. longa* in healthy subjects had a beneficial effect in decreasing cardiovascular risk.

Interestingly, the effect on total ACF as well as numbers of AC/focus in rats given the turmeric extract was very similar to that seen in the curcumin diet group, and yet the concentration of curcumin in the turmeric extract diet was only one seventh (300 ppm) that of the curcumin 2000 ppm group. In the cell culture experiment, we found that fraction A, which contained curcuminoids, as well as fraction B inhibited the growth of cancer cells. He et al (1998) have reported that the major constituents in turmeric are curcuminoids and essential oils. Fraction B of *C. longa* contained sesquiterpenes such as α- and β-turmerone, ar-turmerone, zingiberene, bisacumol, curcumenol. The presence, therefore, of other compounds in turmeric with potential anticancer effects could have a synergistic effect, and may give a better performance with regard to chemoprevention than pure curcumin.

A possible mechanism whereby zerumbone may act involves cyclooxygenases (COX) and phase II enzymes (Murakami et al., 2002). COX-1 and COX-2 are key enzymes in arachidonate metabolism, and may be involved in many inflammatory processes. Unlike COX-1, COX-2 is induced in inflammation and in tumorigenesis (Prescott and Fitzpatrick, 2000). Nonsteroidal anti-inflammatory drugs (NSAIDs) which inhibit both COX-1 and COX-2 may result in unwanted side effects, such as gastrointestinal ulceration and bleeding. Zerumbone has been reported to inhibit prostaglandin synthesis and suppress COX-2 but not COX-1 activity (Tanaka et al., 2001), suggesting that it may be more effective than some NSAIDs currently in common use. Curcumin has also been found to effectively inhibit COX-2 but not COX-1 in both *in vitro* (Goel et al., 2001) and *in vivo* studies (Zhang et al., 1999). Tanaka et al (2001) also showed that zerumbone increased levels of glutathione S-transferases (GST) and quinone oxidoreductase (QR) in liver and colonic mucosa. GST and QR are phase II enzymes which are important in cellular defence and metabolism, including detoxification of electrophilic species and thereby preventing induction of oxidative stress (Schultz et al., 1997). Curcumin has been shown to enhance the activities of detoxifying enzymes such as GST (Piper et al., 1998). In this respect then these agents may offer similar protective effects.

In cell culture investigations, panduratin A was very effective in inhibiting the growth of cancer cells but appears to have different mechanisms from zerumbone and curcumin. In the animal study, diet containing extract from the equivalent of 4% by weight of *B. pandurata* did not affect the formation of ACF induced by AOM. The concentration of panduratin A was not assayed in the diet due to late identification of the active compound.

5.5. *Summary of experiments and suggestions*

Zerumbone in the extract of *Z. aromaticum* has a beneficial effect by inhibiting the formation of chemically induced colonic preneoplastic ACFs in rats, suggesting that *Z. aromaticum* may have benefits as a chemopreventative agent. However further studies are needed to elucidate the

mechanisms of zerumbone and other compounds within the extracts of *Z. aromaticum* and *C. longa*, to understand their pharmacological actions and their anticancer effect using differing cancer models. Bioavailability of active compounds needs to be assayed to understand the efficacy of extracts/active compounds in cancer prevention.

An extract of *B. pandurata* showed no effect on the formation of chemically induced colonic preneoplastic ACFs in rats. Further studies on panduratin A and *B. pandurata* are needed to elucidate the mechanisms of their pharmacological actions and the effect on different cancer models.

CHAPTER 6

THE ANTIINFLAMMATORY ACTIVITY OF EXTRACT OF <u>ZINGIBER</u> <u>AROMATICUM</u> USING DEXTRAN SULFATE SODIUM (DSS)-INDUCED ULCERATIVE COLITIS (UC) IN RATS

6.1. *Introduction*

Traditionally ginger species have been used to treat various ailments that have inflammatory symptoms (Hyene, 1987, Rosita et al., 1993). There have been a great number of papers in the literature describing the extracts and bioactive compounds isolated from members of Zingiberaceae which are potent antiinflammatory agents. For example gingerol, a pungent principal from ginger inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema (Park et al., 1998) and in a clinical study curcumin completely regressed a non-neoplastic orbital inflammatory lesion with no adverse side effects (Lal et al., 2000). Generally two models of antiinflammatory measurement are used to investigate the extracts or bioactive compounds from these ginger species. They are (1) a chronic model using cotton pellet and granuloma pouch and (2) an acute model using chemically-induced rat paw edema (Araujo and Leon, 2001). Zerumbone isolated from Z. *zerumbet* has also been shown to inhibit the increased expression of iNOS and COX2 which are associated with inflammation (Murakami et al., 2002).

Ulcerative colitis is a chronic relapsing inflammatory disease process of the large bowel of unknown etiology. However, it has been suggested that the disease is caused by adverse environmental factors which insult enteric microflora in genetically susceptible individuals and then activate an immune response (Farrel and Peppercorn, 2002).

From epidemiological studies, it has been reported that patients with inflammatory bowel disease (IBD), especially those with long-standing and extensive ulcerative colitis, have an increased risk of developing colorectal cancer (Wong and Harrison, 2001). There has been

no previous research of the potential of antiinflammatory activity by members of the Zingiberaceae family using a model of inflammatory bowel disease.

An animal model of human IBD having the pathology, pathophysiology and histopathology as well as clinical spectrum has been described by Strober (1985). Inflammation is induced in the bowel by administering chemicals such as acetic acid, trinitrobenzene sulfonic acid (TNBS) or dextran sulfate sodium (DSS) (Elson et al., 1995). The DSS model has been shown to be very useful, with many phenotypic characteristics that resemble the human disease and therefore has value for testing the efficacy of a variety of pharmacological agents. The DSS model is easily applied, reliable and highly reproducible (Murthy and Flanigan, 1999). Intermittent and prolonged administration of DSS produces dysplasia and carcinomas and has been used to study the development of colorectal neoplasia from chronic inflammation (Okayasu et al., 2002). The mechanism whereby DSS produces colonic inflammation is not fully understood. However, it has been suggested that the dissociation of sulfate from DSS by colonic bacteria which then produces hydrogen sulphide (H_2S) may significantly interfere with cellular metabolism and induce an inflammatory response in the epithelium (Murthy and Flannigan, 1999).

The aim of this study was to investigate the antiinflammatory activity of an ethanolic extract of Z. aromaticum using the DSS-induced ulcerative colitis model in male rats. Sulfasalazine (2-hydroxy-5[[4-[(2-pyridinylamino) sulfonyl]phenyl]azo]benzoic acid; SASP), a widely used compound to treat IBD in humans was used as a positive control. The clinical spectrum of IBD including weight change, loose stools, diarrhea and faecal blood were observed daily and histopathological features of the colon were examined. Biomarkers for inflammation such as myeloperoxidase, prostaglandin E2, thromboxane and COX-2 were also measured.

6.2. *Experimental Design*

6.2.1. Experiment: Ulcerative colitis using DSS

Six week old male SD rats with body weights of about 164 g were used for this study. There were four groups of 9 and 8 animals (table 6.1). Controls were fed a diet based on the AIN 93 diet and as positive controls rats were fed the AIN 93 diet containing 0.05% sulfasalazine (Sigma, St Louis, MO).

Extracts were prepared from the equivalent of 4% and 8% by weight of dried rhizomes of *Z. aromaticum* in the diet. The dried material was extracted with ethanol three times (material:solvent = 1:6) at room temperature. The ethanol extracts were combined and evaporated to reduce the amount of ethanol. Extracts were then mixed with the AIN 93 diet and dried at 35°C in an oven overnight. The experimental design was described in General Methods (2.2.3.2). Fluid and food intake, body weight and inflammatory signs such as loose stools, diarrhea and faecal blood were recorded daily. Colons were removed and the length of colons was measured. Thymus, kidneys, liver and spleen of rats were removed and weighed.

6.2.2. Scoring of disease activity index

In all animals body weight, presence of blood and stool consistency were assessed daily. Blood in the faeces was tested using the fecal occult blood (ColoScreen Lab Pack, Helena Laboratories, Beaumont, Texas). The disease activity index (DAI) was scored according to Murthy et al. (1993). The DAI combines the scores of weight loss, stool consistency, and blood in stool and divides by 3 as listed in table 6.2.

Table 6.1. Composition of the diets

Group	Number of rats	Diet
Control	9	AIN 93
0.05% SLZ	8	AIN 93 + 0.05% Sulfasalazine
4% ZA	9	AIN 93 + extract prepared from equivalent of 4% dried rhizome of Z aromaticum
8% ZA	9	AIN 93 + extract prepared from equivalent of 8% dried rhizome of Z aromaticum

Table 6.2. Criteria for scoring disease activity index (DAI) (Murthy et al., 1993)

Score	Weight loss	Stool Consistency**	Occult/gross bleeding
0	None	Normal	Normal
1	1 – 5%	Loose stool (+)	Negative
2	5 – 10%	Loose stool (++)	Hemoccult positive (+)
3	10 – 20%	Diarrhea (+)	Hemoccult postive (++)
4	> 20%	Diarrhea (++)	Gross bleeding

**Normal stool=well formed pellets; loose stools=pasty and semi formed stool which do not stick to the anus; diarrhea=liquid stool that sticks to the anus

6.2.3. Myeloperoxidase (MPO) assay

Myeloperoxidase activity was assayed on the supernatants from the homogenates of the fresh colon tissue. The tissues were placed in 1.5 ml of 50 mM potassium phosphate buffer, pH 6 containing 0.5% hexadecyl-trimethylammonium bromide (HTAB) (Sigma, St Louis, MO), homogenized for 45 s on ice (Ultra Turrax, Janke and Kunkel, IKA Labortechnik, Germany) and then centrifuged at 900 g for 60 seconds.

The myeloperoxidase assay was adapted from that described by Fernandez et al (2001) using a 96-well microplate reader (Spectra Max 250, Microplate Spectrophotometer, Molecular Devices, USA). The reagents were added in the following order: 50 µl of supernatant, 50 µl phosphate buffer containing 0.5% HTAB, 50 µl O-dianisidine (0.68 mg/ml in distilled water), and to start the reaction 50 µl of freshly prepared 0.003% hydrogen peroxide. The optical density at 450 nm was read immediately and thereafter at 2 min intervals. The

activity of MPO in the supernatant samples was compared to that obtained from animals fed the basal diet with DSS.

The activity of the enzyme in the samples was obtained by comparison with the rate of reaction in wells containing supernatant from colonic tissue of the control AIN group.

6.2.4. Histological Examination

Colon tissue fixed in formalin at autopsy was processed for histological examination and stained using H & E as described in General Methods (2.2.3.2.2.). The sections were graded as a crypt score and as other histopathological scores such as damage of epithelium, depletion of goblet cells and infiltration of inflammation cells. The crypt scoring was based on a method used by Cooper et al. (1993) in table 6.3 and the other score was based on a method used by Iba et al. (2003) as listed on table 6.4.

Table 6.3. Crypt scoring (Cooper et al. ,1993).

Grade	Changes of crypt
0	Intact crypt
1	Loss of basal one-third of the crypt
2	Loss of the basal two-third of the crypt
3	Loss of entire crypt with the surface epithelium remaining intact
4	Loss of both the entire crypt and surface epithelium (erosion)

The crypt changes are quantified as to the percentage involvement by the disease process as:

Score	Area of involvement
1	1-25 %
2	26 – 50%
3	51 – 75%
4	76 – 100%

Each piece of tissue was scored with a grade and percentage area involvement with the product of the two being a crypt score.

Table 6.4. Histopathological scoring of colitis (Iba et al., 2003)

Feature	Score	Description
Loss of epithelium	0	None
	1	0 – 5% loss of epithelium
	2	5 – 10% loss of epithelium
	3	Over 10% loss of epithelium
Depletion of goblet cells	0	None
	1	Mild
	2	Moderate
	3	Severe
Infiltration of inflammatory	0	None
	1	Mild (mucosa)
	2	Moderate (submucosa)
	3	Severe (muscularis externa)

6.2.5. Prostaglandin E_2 (PGE$_2$) and thromboxane (TXB$_2$) assay

The method for determination of PGE$_2$ was adapted from Ackerman et al (2002) and performed using a radioimmunoassay (RIA). Thawed colon tissues were placed in 1 ml potassium phosphate buffer (50 mM/l, pH 7.4). They were minced with scissors on ice, homogenized for 10 s (Ultra Thurax) and centrifuged in an eppendorf centrifuge for 10 s. The pellet was resuspended in 1 ml of the buffer, vortexed for 1 min, and 100 µl of indomethacin (100 mM) added (Sigma) and the tubes were then centrifuged for 60 s. PGE$_2$ standard (Cayman chemical, Ann Arbor, MI). PGE$_2$ and TXB$_2$ were measured by radioimmunoassay (RIA) carried out at the Department of Rheumatology, Royal Adelaide Hospital, South Australia.

6.2.6. Statistical analysis

Data were analysed by one-way analysis of variance and Tukey's post-hoc test to identify group differences (p<0.05) and grouping factor of treatments were analysed using repeated measures analysis of variance by BMDP Statistical software package (BMDP

Statistical software, Inc., CA, U.S.A). Differences between groups of repeated measures were performed using a Z score calculation as follows:

$$Z \text{ score} = \frac{X-Y}{\sqrt{SE(X)^2 + SE(Y)^2}}$$

X = mean of estimate of repeated measures of a group
Y = mean of estimate of repeated measures of other group
SE(X) = asymptotic standard error of X
SE(Y) = asymptotic standard error of Y
Any two values were considered to be significantly different if $-2 < Z < 2$

6.3. *Results*

6.3.1. Body weight

Body weights of the rats between groups were not statistically different over 5 days of DSS treatment. The body weights of rats (g) on the first day of DSS treatment were not different: 199 ± 2; 199 ± 2; 197 ± 2; 197 ± 2 for the AIN group; 0.05% Sulfasalazine; 4% ZA and 8 % ZA respectively. Body weights of rats at the end of experiment (three days after DSS was removed) was also not significantly different although there was a slightly lower average body weight of rats given the diet containing 8% extract of ZA. The average body weights (g) at termination of the study were 268 ± 5; 264 ± 5; 262 ± 6; 266 ± 3 for group of rats given AIN; 4% ZA, 8% ZA and 0.05% Sulfasalazine respectively.

6.3.2. Weight of organs

The weight of thymus, spleen, kidneys and liver of rats did not differ between the dietary treatment groups (table 6.4). The length of colon of DSS-treated rats did not differ between the dietary treatment groups at the end of experiment.
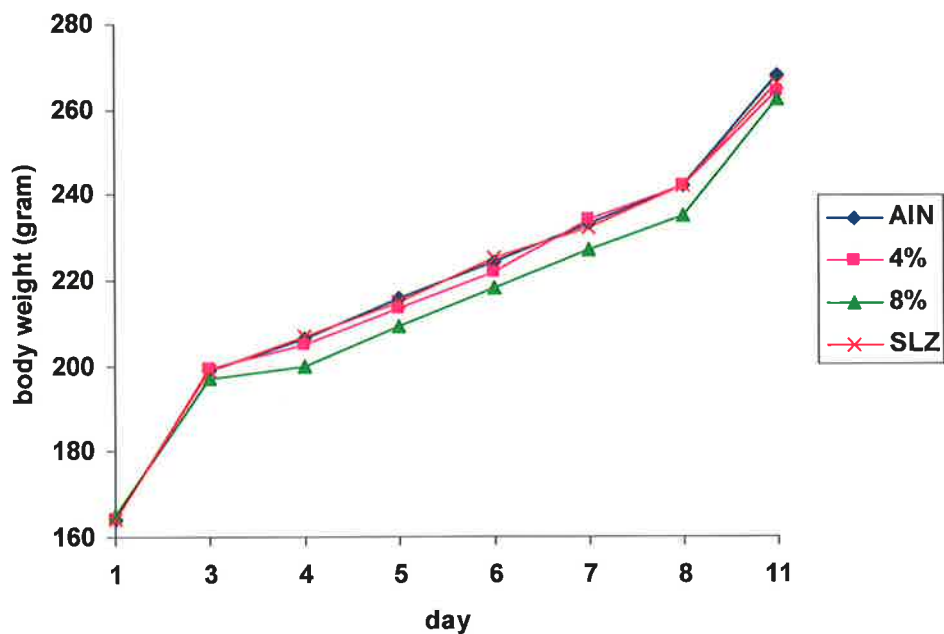
Figure 6.1. Body weight of rats during the experiment

Table 6.4. The weight of organs and length of colon

Treatment	Length (cm) of colon	Weight (g)			
		thymus	spleen	kidneys	liver
AIN only	17.3 ± 0.7	0.75 ± 0.03	0.77 ± 0.03	2.24 ± 0.06	13.4 ± 0.4
AIN + 4% ZA	17.4 ± 0.7	0.72 ± 0.04	0.76 ± 0.03	2.24 ± 0.05	13.8 ± 0.6
AIN + 8% ZA	17.3 ± 0.5	0.67 ± 0.06	0.78 ± 0.04	2.23 ± 0.04	14.8 ± 0.7
AIN + 0.05% S(*)	16.8 ± 0.5	0.66 ± 0.03	0.75 ± 0.04	2.13 ± 0.03	13.1 ± 0.4

Note S=sulfazalasine (Sigma); Values are means ± SEM; n = 9 or 8 [*]

6.3.3. Liquid intake

Fluid intake by rats during DSS administration was not statistically different between dietary treatment groups (figure 6.2).

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6.3.4. Food intake

The amount of food intake by rats fed with diet containing extract of 8% ZA was significantly less compared to those in the group fed with AIN diet and diet containing 0.05% sulfasalazine (Fig. 6.3). On the first day of DSS administration, rats in the group given 8% ZA ate 16.4 g food while those fed the AIN group and 0.05% Sulfasalazine ate 20.0 and 19.3 g respectively. The rats fed with AIN diet and diet containing extract of Z. aromaticum ate more over time, while rats in the group given 0.05% sulfasalazine ate the same amount of food over 5 days (Fig. 6.3).
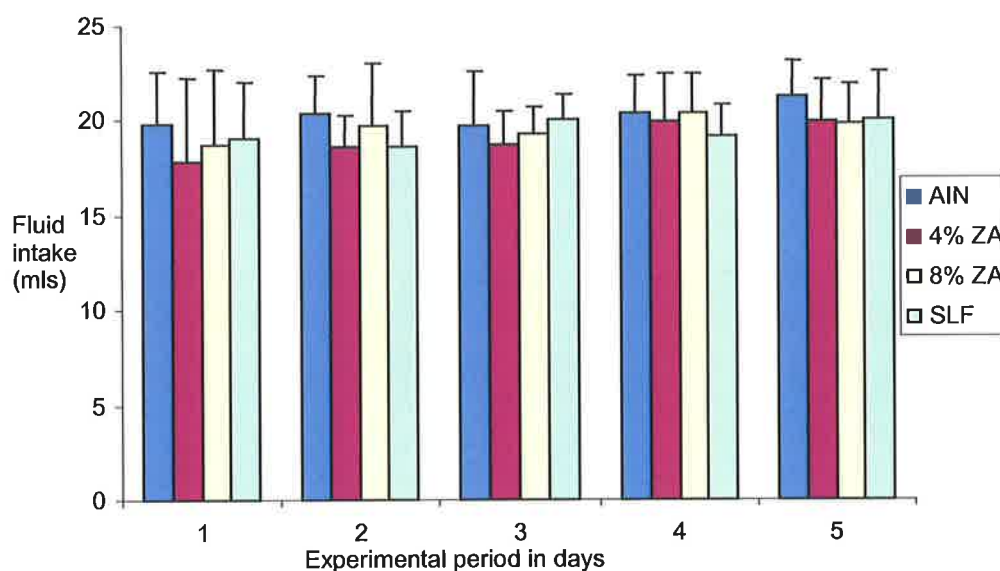
Figure 6.2. Average daily fluid intake by rats during DSS treatment period. Values are means ±
SE
(n = 9 or 8 animals)
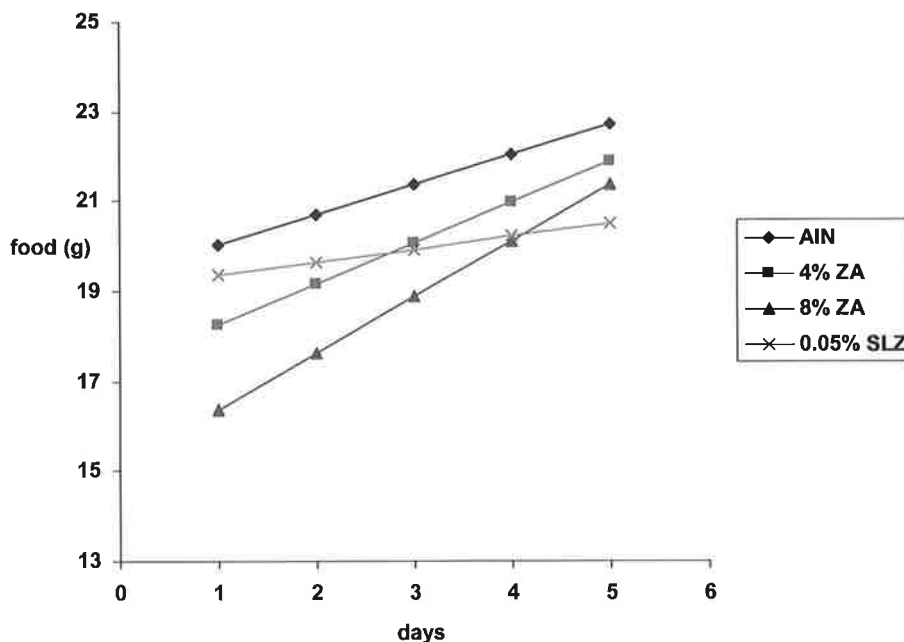
Figure 6.3. Daily food intake of rats during DSS treatment period

6.3.5. Disease activity index (DAI)

Weight loss, stool consistency and blood in faeces are clinical parameters which are somewhat analogous to clinical symptoms observed in human IBD.

The average daily body weight gain of DSS-treated rats over five days were 8.6 ± 1.1 g, 8.6 ± 2.3 g, 7.7 ± 2.6 g and 8.6 ± 0.8 g for AIN, 4% ZA, 8% ZA and 0.05% SLZ group respectively.

Disease activity in the control rats was apparent from day 2 after DSS administration with 11% hemoccult test positive (1 of 9 animals), 67% positive by day three and 100% positive and/or with gross bleeding by day 5 (figure 6.4). In the groups of rats given diet containing 4% ZA, 8% ZA and 0.05% sulfasalazine, 22%, 11% and 11% of the animals were hemoccult positive respectively on day 3 after DSS treatment with. On day 5, only 22% of animals given 0.05% sulfasalazine were hemoccult positive, 33 % of rats in the group fed with 4% ZA and 67% of rats in the 8% ZA group.

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Rats fed with AIN diet reached a DAI score of 1.4 on day 5 while those fed diet containing extract from 8% dried *Z. aromaticum* had a DAI of 1.3. The DAI of rats fed with the diet containing 4% dried *Z. aromaticum* was 0.4 i.e. very similar to that of rats treated with 0.05% sulfasalazine (figure 6.5).
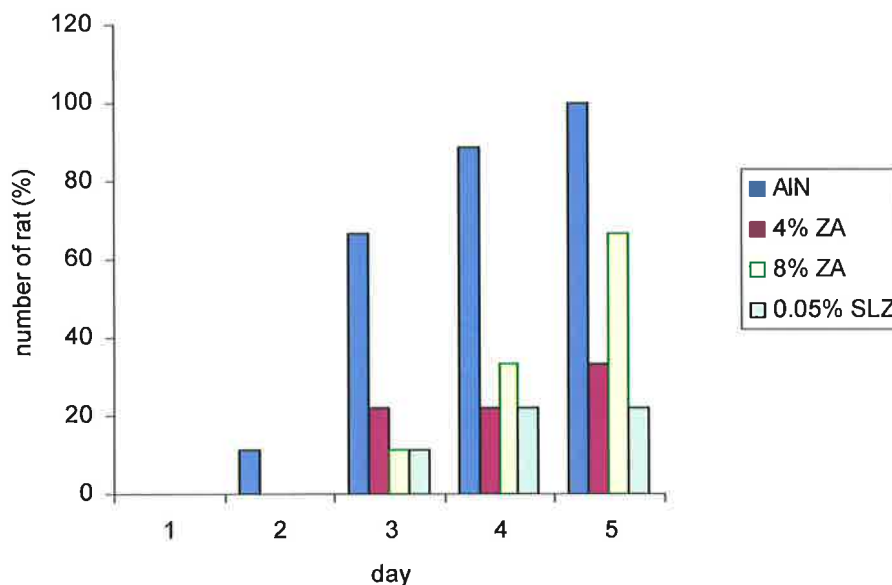

Figure 6.4. Incidence of blood in the faeces and/or diarrhoea in rats (%) during DSS treatment period

6.3.6. Histological observation

No damage was observed in the colonic mucosa of normal rats and those fed with diet containing extract prepared from the equivalent of 4% dried rhizome of *Z. aromaticum* (figure 6.6). The histology of normal mucosa contains straight, tubular glands (crypts) on the muscularis mucosa. The lumen of the glands is narrow except in the deepest part of the gland. The cells that line the surface of the colon and the glands are absorptive cells with thin striated borders and goblet cells. Between the glands is the lamina propria which contains considerable numbers of lymphocytes and other cells of the immune system.
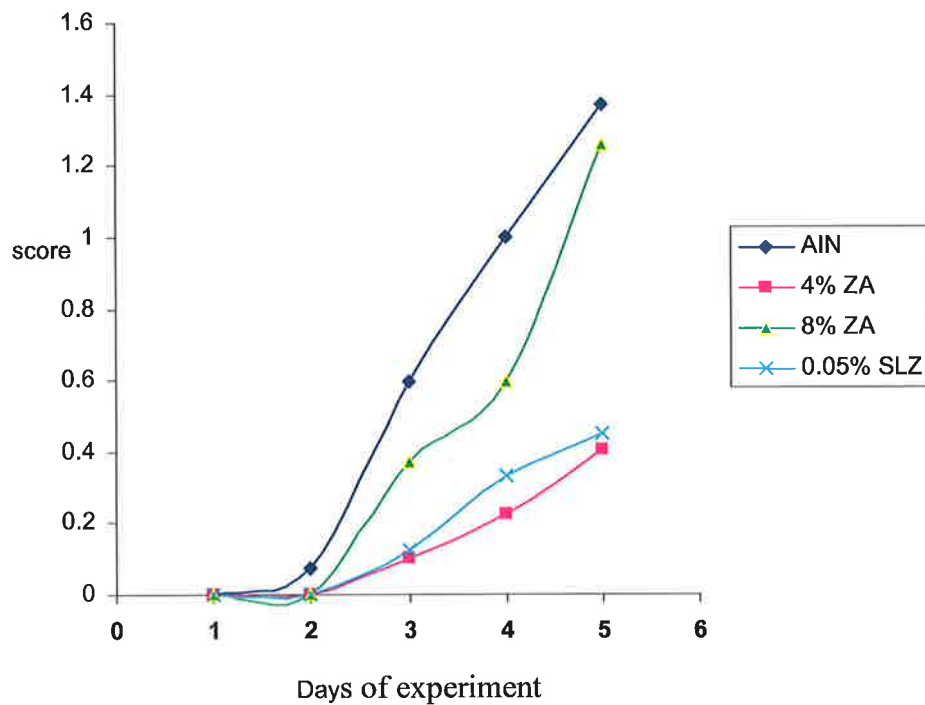
Figure 6.5. Disease acitivity index (combines scores of weight loss + stool consistency + bleeding divided by 3)
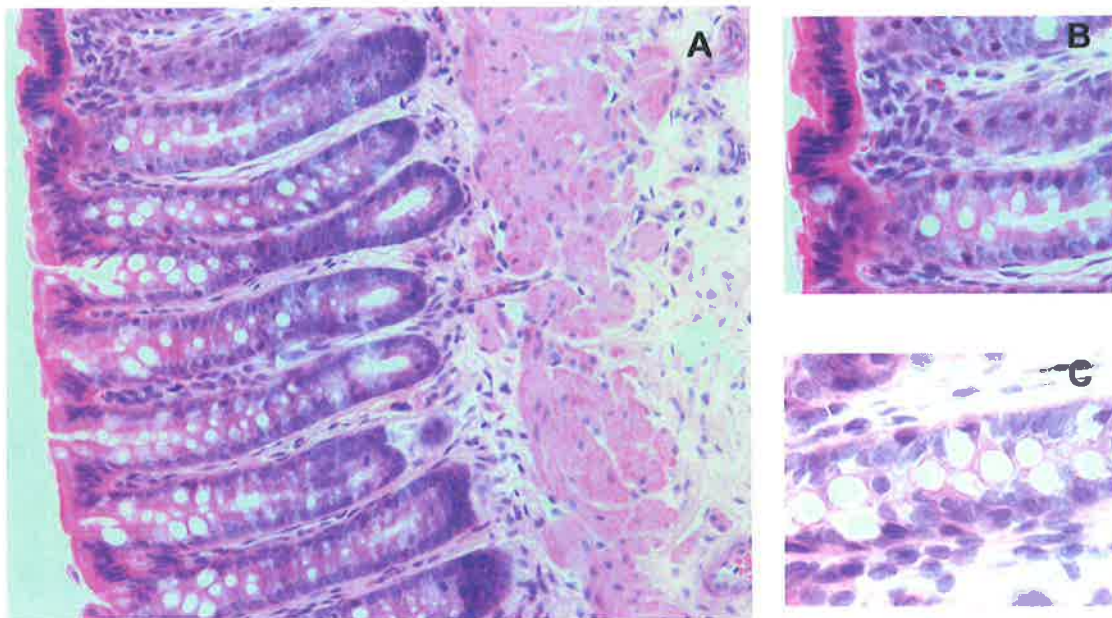

Figure 6.6. Histological appearance of colonic mucosa of normal rat shows straight, tubular crypt with a thin striated border absorptive cells and goblet cells with narrow lumen. Tissue was stained with H&E. A (X 20); B (X60); C (X 100). B and C shows sections of the crypts in higher power

After 5 days administration of DSS, histological examination of colonic mucosa demonstrated shortening of crypts with an area of separation between the base of the crypt and the muscularis mucosa (fig. 6.7 B), erosion of the epithelium with slight crypt dilatation (fig. 6.7 A, arrow), loss of goblet cells, crypt erosion (fig. 6.7 C), abscess formation (fig 6.7, arrow head) and crypt loss (fig 6.7 D). Acute inflammatory infiltration of small round cells and polymononuclear leucocytes and red blood cells was observed in the lamina propria, muscularis mucosa and submucosa and muscularis mucosa.

All treatment groups had a slightly (but not significant) lower crypt score than the controls (figure 6.8).
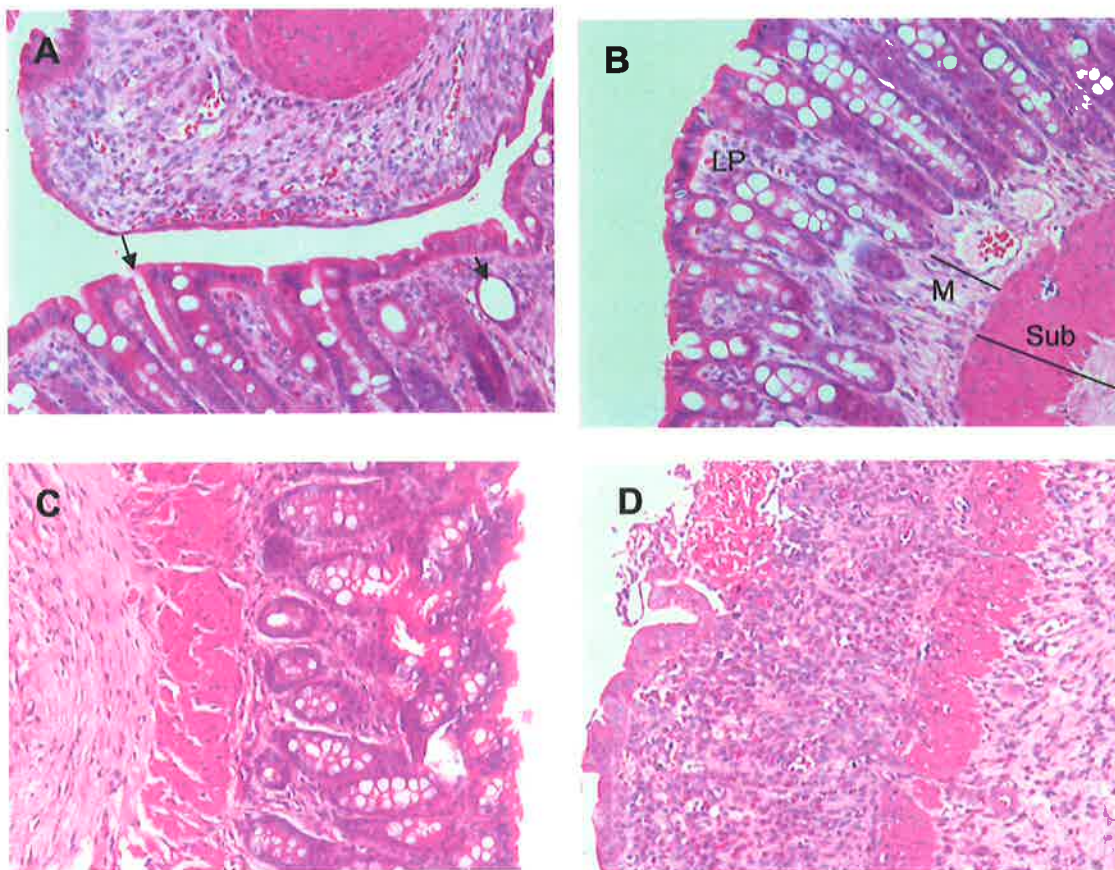

Figure 6.7. Histological appearance of colonic mucosa of rat treated for 5 days with 2% DSS in drinking water shows (A-D) shortening of crypts, loss of goblet cells, crypt dilatation (arrow), crypt abscesses (arrow head), crypt erosion and loss of crypt structure. Inflammatory cells infiltrated into lamina propria (LP), muscularis mucosa (M) and submucosa (Sub). Tissue was stained with H&E. (X 20).
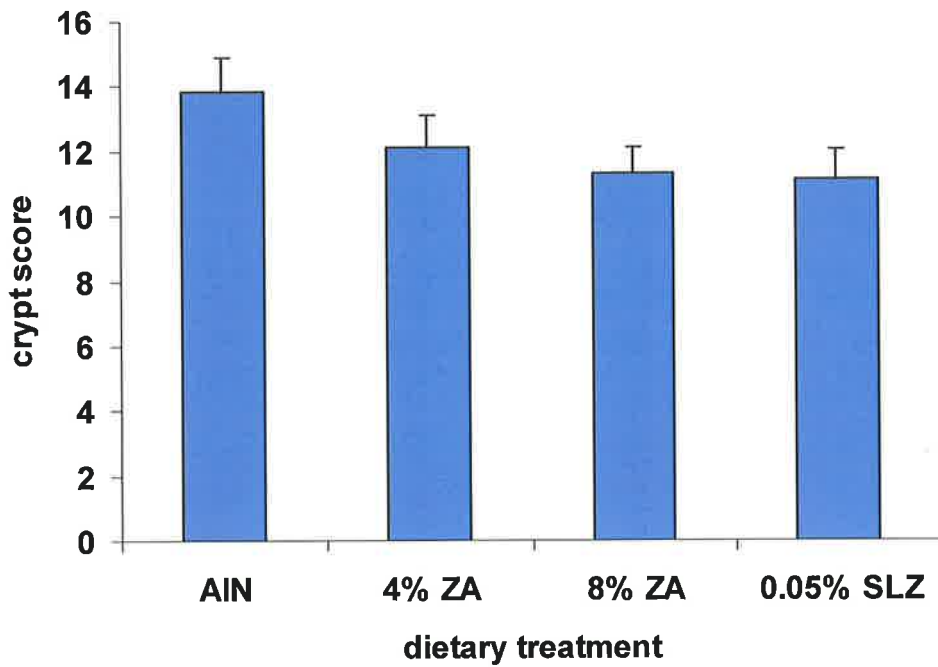
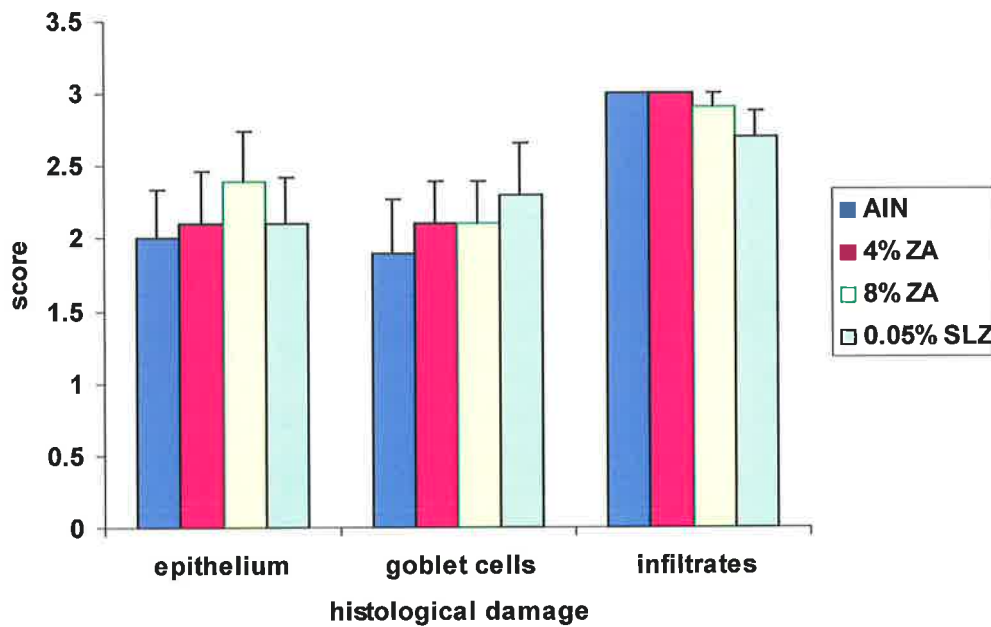Figure 6.8. The effect of dietary treatment on crypt lesions induced by DSS. Values are means ± SE

Figure 6.9. The score of loss of epithelial, loss of goblet cells and infiltration of inflammation cells induced by DSS on different dietary treatment. Values are means ± SE

The other histological score such as loss of epithelium, goblet cells and infiltration of inflammation cells of tissue from distal colon of rats between groups were also not significantly different (figure 6.9).

6.3.7. Myeloperoxidase in the colon tissue

Total MPO activity per 100 mg colon tissue showed that treatment with 0.05% sulfasalazine suppressed the increase of MPO activity in the colonic mucosa. The MPO activity of colonic tissue of rats treated with Z. aromaticum at either concentration was not different to that of rats fed with AIN only.
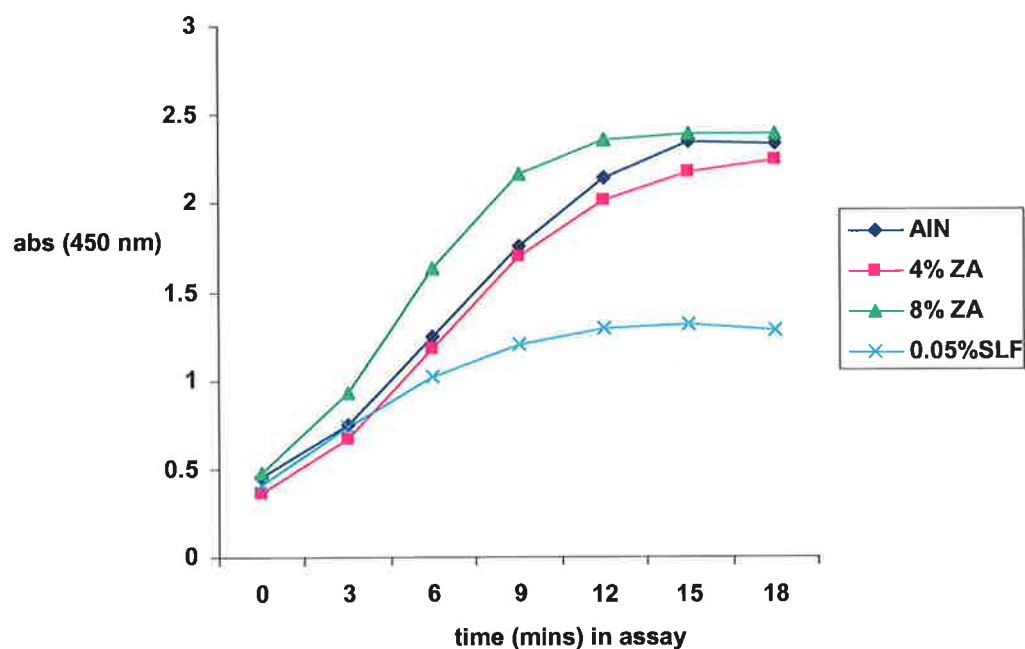

Figure 6.10. Myeloperoxidase activity (abs, 450 nm) per 100 mg of colon tissue

6.3.8. PGE-2 and TXB-2 content in the colon tissue

Inclusion of Z. aromaticum in the diet tended to decrease the TXB-2 concentration in the colon tissue of rats but these were not significantly different (figure 6.11). The concentrations of TXB-2 were 3.3 ± 0.3; 2.8 ± 0.4; 2.4 ± 0.4 and 3.3 ± 0.3 ng/ml of rats fed

with AIN, extract prepared from 4% and 8% dried *Z aromaticum* and 0.05% sulfasalazine respectively.

Figure 6.11. The concentration of TXB-2 (ng/ml) in inflammation colon tissue of rats induced by DSS and fed with different dietary treatments. Values are means ± SE
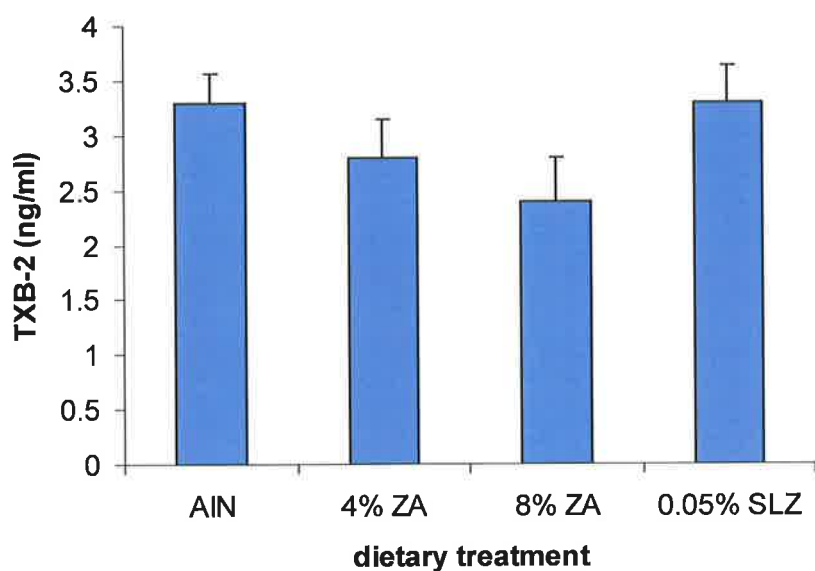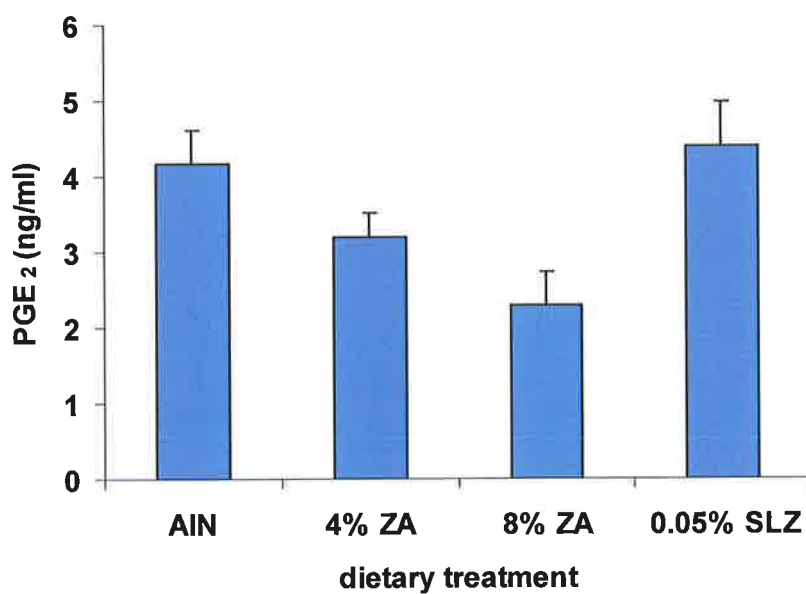

Figure 6.12. The concentration of PGE-2 (ng/ml) in inflammation colon tissue of rats induced by DSS and fed with different dietary treatments. Values are means ± SE

The concentration of PGE-2 in the colon of rats fed with extract of Z. aromaticum was significantly decreased compared to that in the colon of rats fed the AIN diet alone. The concentrations of PGE-2 were 4.2 ± 0.5; 3.2 ± 0.3; 2.3 ± 0.4 and 4.4 ± 0.6 ng/ml in the colon of rats fed with AIN, extract prepared from 4% and 8% dried Z. aromaticum and 0.05% sulfasalazine respectively. Sulfasalazine had no effect on the concentrations of PGE-2 and TXB-2.

6.4. Discussion

Medical treatment of UC relies mainly on the use of sulfasalazine, salicylates and immunomodulators such as azathioprine and cyclosporin. The therapeutic treatments frequently fail and the disease relapses. There is also a concern about the toxicity of immunomodulator agents at a high dose (Guslandi, 1998) and sulfasalazine has been claimed to cause male infertility (Marmor, 1995) and non steroid antiinflammatory drugs (NSAIDs) which have been suggested to prevent colorectal cancer cause exacerbations of UC and induce de novo colitis (Farrel and Peppercorn, 2002). The limited efficacy of standard medical therapies for IBD has resulted in a continuing search for alternative treatments as well as to prevent colorectal cancer.

In this study sulfasalazine was employed as a positive control at a dose of 0.05% as suggested by Ahn et al. (2001) and Oketani et al. (2001). Sulfasalazine has been used for more than 55 years to treat IBD (Hoyt et al., 1995) and 5-aminosalsalicylic acid (5-ASA), an active metabolite of sulfasalazine has been shown to possess several actions including inhibition of eicosanoid production (Vilaseca et al., 1990) and significantly suppressed lipid peroxidation (Ahn et al., 2001). Rats given the diet containing sulfasalazine from two days before DSS administration, throughout the DSS treatment and three days of recovery period had suppressed clinical symptoms of IBD such as rectal bleeding, consistency of stool and

diarrhea. The amount of myeloperoxidase, a marker enzyme of neutrophils, in the tissue of rats treated with 0.05% sulfasalazine was also significantly lower than that of untreated animals. Krawisz et al. (1984) found that the amount of myeloperoxidase was directly correlated to the number of neutrophils in the inflamed tissue.

The inflammatory mechanisms and clinical symptoms have indicated that eicosanoids are modulators of inflammation and involved in the pathogenesis of IBD. Vilaseca et al. (1990) reported that eicosanoid productions such as PGE-2, 6-keto PGF $_{1\alpha}$, TXB-2 and leukotriene B$_4$ increased significantly in colonic tissue after induction by TNBS. In this study, sulfasalazine at 0.05% did not have any effect on PGE-2 and TXB-2. Oketani et al. (2001) also found that sulfasalazine did not affect the concentration of PGE-2 and TXB-2 in inflamed colon.

The histological scores of rats treated with sulfasalazine, however did not differ compared to those of untreated rats. In this experiment, it was found that rats with no sign of blood in the faeces and had normal stools had severe histological evidence of severe colonic tissue damage.

The extract prepared from the equivalent of 4% dried rhizome of Z. aromaticum in the diet reduced clinical symptoms of IBD such as rectal bleeding, consistency of stool and diarrhea compared to animals fed with the control diet and had a similar effect to that of sulfasalazine. On day 5 of DSS treatment, only 25 % of rats treated with sulfasalazine and 33% of rats treated with the extract made from the equivalent of 4% dried Z. aromaticum were occult blood positive compared to 100 % of those untreated animals. The DAI was 0.5 for sulfasalazine group and 0.4 for 4% ZA group compared to 1.4 of the control AIN rats.

There was a significant reduction on PGE-2 concentrations and TXB-2 concentrations in the colon of rats treated with the extract prepared from 4% dried Z. aromaticum to be lower than in untreated animals however this was not significant. The myeloperoxidase activity in rats treated with the extract made from 4% Z. aromaticum, however did not differ from untreated

rats. The crypt score and other histological scores of rats given diet containing extract prepared from 4% Z. aromaticum were also not significantly different from those of untreated rats. It was apparent from this study that treatment with 2% DSS for five days resulted in severely damage to colonic mucosa of about 190 g weighed rats. Iba et al. (2003) reported that DSS is very toxic and damages the colonic mucosa of the animals. Reepithelialization, restoration of goblet cells and crypt recovery were observed at day 5, 10 and 20 after cessation of the DSS challenge. In this present study the recovery period was only three days and this may have been insufficient time for the extract of Z. aromaticum to significantly heal the colonic damage induced by DSS.

The extract prepared from the equivalent of 8% dried rhizome of Z. aromaticum regressed the development of ulcerative colitis induced by DSS on day 3 with 11% compared to 67% of rats of AIN group but the number of rats with positive blood in their faeces was higher than those treated with sulfasalazine or 4% ZA groups on day 4 and 5. The TXB-2 content was slightly decreased and PGE-2 content was significantly reduced compared to untreated animals. The crypt score of rats treated with the extract from equivalent of 8% rhizome of Z. aromaticum was slightly but not significantly decreased compared to other groups. The crypt scores of rats fed with diet containing extract made of 8% Z. aromaticum were similar to those of rats treated with sulfasalazine. The myeloperoxidase activity in the inflamed colon of rats treated with this extract also did not differ from that of untreated rats. The amount of extract prepared from 8% Z. aromaticum in the diet seemed to be slightly too much and create unpalatable taste for rats since the rats from this group had significantly less food intake than the rats from other groups. However there was no sign of any growth retardation compared to rats treated with sulfasalazine and/or extract made from 4% Z. aromaticum indicating that the amount of extract made from 8% of Z. aromaticum in the diet was not toxic. The degree of inflammation such as eicosanoid productions and crypt score were less in rats fed with diet containing extract from 8% Z. aromaticum compared to those of untreated animals and slightly

less or the same as those of rats treated with extract of 4% Z. aromaticum or sulfasalazine. Significantly less food intake by rats in 8% ZA group may lead to escalate the DAI compared to those of treated animals (ie sulfasalazine and 4% extract of Z. aromaticum). Ulcerative colitis is unknown etiology and is suggested as a result of interactions between hereditary, environmental and immunologic factors. Clinical studies suggest that nutrition is an important adjuvant to medical therapy for IBD especially in young children (Duerksen et al.,1998). Components in the diet such as protein, fibre are very important in generating a balanced environment in the colon, in order to prevent inflammation and also to boost immunosuppressive pathways and therefore suppressing ongoing inflammation (Beattie et al., 1998).

Unlike extracts of Z. aromaticum, sulfasalazine at a dose of 0.05% did no have an effect on the level of TBX-2 and PGE-2 may indicate that they work through different mechanisms.

Zerumbone, a sesquiterpenoid compound, has been isolated from Z. aromaticum (Chapter 4) and shown to have anticancer activity in vitro and in vivo studies (Chapter 5). The antiinflammatory activity of extract of Z. aromaticum in this study was partly due to zerumbone. The antiinflammatory activity of zerumbone has been reported by other researchers. Tanaka et al (2001) reported that zerumbone, which was isolated from Z zerumbet, has decreased PGE_2 and PGD_2 content in colonic mucosa of rats and in cell culture studies (Murakami et al., 2002).

The activities of NSAIDs to inhibit COX-1 and COX-2 are suggested to create gastrointestinal and renal adverse effects (Simon, 1999). This major limitation concerning the use of classical NSAIDs for treatment of IBD and prevention of colorectal cancer has resulted in the development of highly selective COX-2 inhibitors such as celecoxib and rofecoxib (Turini and DuBois, 2002). COX-2 inhibitors consequently spare COX-1 activity for maintaining mucosal integrity and renal blood flow (Warrer et al., 1999) however their safety has not been

clearly established (Wolfe et al., 1999) and there was a claim for longterm use of these drugs to generate cardiac problems (Mukherjee et al., 2001). Agents especially from natural origin have been investigated for their antiinflammatory activities in favour of more gentle and possible less side effects on targeted and other organs (Sautebin, 2000). Like other members of the ginger family for example *C. longa* (turmeric) and its bioactive compound curcumin (Goel et al., 2001), zerumbone has been found to inhibit COX-2 but not COX-1 (Tanaka et al., 2001 and Murakami et al., 2002). *Z. officinale*, which contains a number of pungent active ingredients and highly sesquiterpene hydrocarbons inhibited platelet aggregation and TXB-2 production *in vitro* (Srivastava and Mustafa, 1989). Thomson et al. (2002) reported that extract of ginger reduced TXB-2 and PGE-2 production and lower serum cholesterol and triglyceride levels. [6]-gingerol, an active compound from *Z. officinale* has been reported to inhibit gastric lesions by 54.5% on ethanol-induced gastric lesions in rats (Yamahara et al., 1988)

Inhibition of nitric oxide (NO), which is known as one of mediators of inflammation, has been used as molecular targets for antiinflammatory therapy (Sautebin, 2000). Zerumbone has been reported to inhibit inducible nitric oxide synthase (iNOS) expression in combine lipopolysaccharide- and interferon-γ-stimulated protein expression model in cell culture (Murakami et al., 2002).

In addition zerumbone also has been reported to inhibit the release of tumor necrosis factor (TNF)-α in cell culture studies (Murakami et al., 2002). TNF-α is a pro-inflammatory cytokine which has been shown to be one of the most significant factors participating in the inflammatory process of patients with inflammatory bowel disease (Louis, 2001).

The DSS colitis model, reported to resemble human inflammatory bowel disease in terms of the prolonged colonic inflammation that is induced, has served as a useful animal model to investigate the efficacy of agents (Krawisz et al.,1984). Although the exact pathological mechanisms of DSS-induced colitis has hot been clearly elucidated, colitis can

occur through the defect in bacterial phagocytosis due to accumulation of H2S in lamina propria macrophage and direct epithelial injury leading to early crypt shortening and erosions (Cooper et al., 1993). Only a few studies on *Z. aromaticum* and its active compound, zerumbone have been conducted possibly be due to the availability of the species only in certain regions therefore further studies are needed to elucidate the mechanisms of zerumbone and other compounds within the extracts of *Z. aromaticum*, especially using DSS colitis model to improve understanding of their pharmacological actions and on carcinogenesis associated inflammatory bowel disease.

6.5. *Summary of experiment*

Extract prepared from the equivalent of 4% dried rhizome *Z. aromaticum* reduced the development of DSS-induced colitis with suppression of diarrhea and rectal bleeding equivalent to sulfasalazine at a dose of 0.05%. Extracts of 4% and 8% *Z. aromaticum* decreased PGE-2 and TBX content. Three days was insufficient to recover mucosal damage induced by DSS. Extract prepared from the equivalent of 8% of dried rhizome of *Z. aromaticum* in the diet inhibited feed intake and created an unpalatable taste. Stool consistency and incidence of rectal bleeding were useful parameters to evaluate the efficacy of the extract. Myeloperoxidase assay was not found useful measure in this study. Further studies are needed to investigate the mechanisms of antiinflammatory of extract of *Z. aromaticum*.

CHAPTER 7

GENERAL DISCUSSION

Despite advances in medical research on various chemotherapeutic treatments for colorectal cancers in the Western world, mortality rates with colorectal cancer have not changed significantly in the past 20 years (Langham and Boyle, 1998). Differing population study data suggest that this cancer is expressed in some societies at only a fraction of that applying for example in the US, Western Europe and Australia and dietary/lifestyle factors probably account for this difference. Dietary and herbal components offer one possible means for reducing expression of colorectal cancer in societies. Epidemiological studies have reported that the use of herbal medications has increased substantially over the past several years (Bernstein and Grasso, 2001). Clinical and animal studies to investigate the role of dietary components offering prevention or risk reducing strategies are being conducted, with attention focused on identifying naturally occurring substances capable of inhibiting, retarding or reversing the process of multistage carcinogenesis.

Use of members of the Zingiberaceae family as medicine and food has been a part of life of Asians for centuries. Some members of the ginger family have been shown to possess significant antioxidant and anti-inflammatory activity (Surh et al., 1999) and have also potential anticancer activity (Surh, 2002). However, compared to *C. longa* and *Z. officinale*, which have been used worldwide, use of other members of the Zingiberaceae family have been confined to SE Asia and have not been extensively investigated.

In this study, initially the ethanol extracts of rhizomes of eleven Indonesian species of the ginger family were screened for their inhibitory activity on the growth of colon and breast cancer cell lines. They were *Amomum cardamomum, Curcuma aeruginosa, C. longa, C. mangga, C. xanthorrhiza, Kaempferia galanga, K. rotunda, Boesenbergia pandurata* or *K. pandurata, Zingiber officinale, Z. aromaticum* and *Z. cassumunar.* These most popular species are used for medicine

and/or food. The extracts of eight species showed very strong inhibitory activity against the growth of HT-29 colon and MCF-7 breast cancer cells with the IC_{50} ranging from 11.8 ± 1.0 to 84.9 ± 8.3 µg/ml. The US National Cancer Institute (NCI) reports suggest ≤ 250 µg/ml is a cut-off point in terms of activity. At the same concentrations, however, the extracts of these species were non-toxic to skin fibroblasts. The extracts of *Z. aromaticum* and *B. pandurata* showed inhibitory activity similar to the extract of *C. longa* which has been reported to have potential anticancer activity in *in vitro* and *in vivo* studies. Currently curcumin, which has been identified as the active compound of this species, is undergoing clinical trials against colon cancer in the US (Greenwald et al, 2001) and the UK (Sharma et al., 2001) and provided a useful positive control. The two species above were of particular interest and were studied further. Curcumin was not found in the extract of either *Z. aromaticum* or *B. pandurata*.

The extract of *Z. aromaticum* contains an active sesquiterpenoid compound, zerumbone. Zerumbone inhibited cell proliferation by arresting cells in G0/G1 and G2 phase and induced apoptosis *in vitro*. Mori et al. (1999) proposed that cell proliferation has an essential role in carcinogenesis, and that therefore control of cell proliferation is important for cancer prevention. In addition apoptosis removes DNA-damaged cells without causing inflammation in surrounding tissue, and is therefore considered to be one of the key targets for chemopreventive agents (Lapoczynski et al., 2001). Murakami et al. (2002) isolated zerumbone from *Z. zerumbet* and showed that zerumbone had antioxidant activitiy by inhibiting superoxide anion (O_2^-) formation, increasing phase II enzymes activity, had an antiproliferative effect and also induced apoptosis *in vitro*.

The anticancer potential of ethanol extracts of *Z. aromaticum* and *B. pandurata* was further investigated using aberrant crypt foci (ACF) induced by azoxymethane (AOM) in rats in a 13 week experiment. A diet containing an ethanol extract (0.34%) made from the equivalent of 4% by weight of dried rhizome of *Z. aromaticum* significantly decreased ACF formation, especially the large ACFs (3 - 4 or more aberrant crypt (AC)/focus). These large ACFs showed a greater rate of cell proliferation and

a higher degree of dysplasia and are considered to provide better predictors of colon cancer (Uchida et.al., 1997) and Jenab et al., 2001).

Tanaka et al. (2001) reported that a diet containing zerumbone at 500 ppm inhibited the formation of total ACF by 46% in AOM-induced rats in a 5 week experiment. In the study, run for thirteen weeks (long term study), however, it was found that AOM produced larger ACFs than the short term study (five weeks) which had mainly small ACFs (1-2 AC/focus). Diets containing extract of *Z. aromaticum* with zerumbone at a concentration of 300 ppm did not affect small ACF numbers (1-2 AC/focus). This study shows that zerumbone was effective at inhibiting in AOM-induced ACF-formation at a lower concentration than that shown by Tanaka et al (2001) when run over a longer period (thirteen weeks). The colon cancer inhibitory activity of zerumbone appeared at the initiation stage as a blocking agent and during promotion and/or progression as a suppressing agent by inhibiting cell proliferation and/or inducing apoptosis, thereby impacting on the developmental stage of colorectal cancer. Tanaka et al. (2001) reported that zerumbone inhibited the development of carcinogen-induced ACF by inducing of phase II detoxification enzymes and inhibiting cell proliferation of colonic crypts.

The ethanol extract (0.34%) of *Z. aromaticum* in the diet which containing 300 ppm of zerumbone had a similar effect in reducing the formation of ACF to diet containing ethanol extract (0.86%) of *C. longa* and diet containing standard curcumin at 2000 ppm. Curcumin at 2000 ppm has been reported to effectively inhibit AOM-induced ACF formation (Rao et al., 1993; Rao et al., 1999) and reduce tumor incidence and multiplicity of diethylnitrosamine (DEN)-induced hepatocarcinogenesis (Chuang et al., 2000). The concentration of curcumin in the extract of *C. longa* containing diet was assayed at 300 ppm. At this concentration it showed an effect similar to pure curcumin at 2000 ppm, which could be explained by the presence of other active components in the extract of *C. longa* such as demethoxycurcumin and bisdemethoxycurcumin and some other essential oils (He et al., 1998). Demethoxycurcumin was found to have the same or greater antiinflammatory

activity (Huang et al., 1995; Ruby et al., 1995). The present study also has shown that compounds in *C. longa* other than curcumin could be effective as anticancer agentss. For example, extracts of *C. xanthorrhiza* had a very similar concentration of curcumin to the extract of *C. longa*, but a much lower concentration of demethoxycurcumin and no bisdemethoxycurcumin and was significantly less active in inhibiting cancer cell growth than extracts of *C. longa*.

This study also found that the extract (0.34%) made of 4% dried rhizome of *Z. aromaticum* in the diet also had antiinflammatory activity, as shown by suppression of clinical signs of ulcerative colitis such as dysentery and diarrhea in the DSS-induced ulcerative colitis rodent model. It was similar in efficacy to the therapeutic agent, sulfasalazine. Murakami et al (2002) reported that zerumbone inhibited pro-inflammatory mediators including inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF-α) and cyclooxygenase-2 *in vitro*. Inflammation is produced by excessive level of reactive oxygen species (ROS) which react with various bio-molecules including lipid, protein, lipoprotein and DNA in the gut and which may thereby cause DNA damage to colon crypt maternal and/or epithelial cells (Nordberg and Arner, 2001). By this means it can contribute to gene mutation and cancer development (Oshima and Bartsh, 1994). Antioxidants can scavenge free radicals generated by oxidative stress, and may thereby have beneficial effects in preventing or reducing inflammation and DNA mutation, thereby inhibiting the initiation and progression of cancer (Krishnan et al., 2000). In addition detoxifying enzymes (phase-2 enzymes) protect normal cells from a variety of endogenous toxins and xenobiotics in the gut (Atay et a., 2000).

The *Z. aromaticum* extract suppressed the formation of TXB-2 and PGE-2 in the colonic mucosa. These eicosanoids are modulators of inflammation and involved in pathogenesis of ulcerative colitis (Vilaseca et al., 1990). Tanaka et al., (2001) also reported that zerumbone reduced the concentration of PGE-2 in the colon mucosa in an AOM-induced ACF study.

Cyclooxygenase (COX) catalyzes the initial step in the metabolism of arachidonic acid to various prostaglandins (PGs) and thromboxanes (TXs) (Peskar, 2001). COX-1 functions as a

"housekeeping" enzyme whereas COX-2 produced prostaglandins which are involved in certain pathophysiological reactions such as inflammation and cancer (Kawai et al., 2002). COX-2 activation is associated with inhibition of apoptosis and the immune response; it may also play a role in angiogenesis (Eschwege et al., 2001). NSAIDs have been shown to have beneficial effects in prevention of colorectal cancer in animal and human studies (Kawai et al., 2002). Corpet and Tache (2002) in reviewing ACF inhibiting agents listed zerumbone and curcumin as moderately effective chemopreventive agents. In their summary, COX-2 inhibitors such as celecoxib and piroxicam and classic NSAIDs such as aspirin and sulindac were more effective in preventing colon cancer. However, the activities of classical NSAIDs to inhibit both COX-1 and COX-2 create gastrointestinal bleeding and adverse renal effects (Simon, 1999). COX-2 inhibitors spare COX-1 activity for maintaining mucosal integrity and renal blood flow. However to date their safety has not been clearly established (Wolfe et al., 1999), and there may be cardiac problems for long term users of this drug (Mukherjee et al., 2001). The antiinflammatory and anticancer activity of curcumin (Goel et al., 2001) and zerumbone (Tanaka et al., 2001 and Murakami et al., 2002) were associated with the ability to inhibit COX-2 but not COX-1 activity.

A diet containing an extract (0.58%) prepared from the equivalent of 4% (w/w) of dried rhizome of *B. pandurata* did not have an effect on AOM-induced colon cancer in rats. Panduratin A, a chalcone derivative was isolated from *B. pandurata*. Panduratin A caused cell cycle arrest at G0/G1 and induced apoptosis in *in vitro* studies. Panduratin-A has been reported also to have strong topical antiinflammatory activity in the TPA-induced ear edema model in rats (Tuchinda et al., 2002). Panduratin A also has antimutagenic activity due to its inhibition of phase I enzymes (Trakoontivakorn et al., 2001). The cancer cell culture studies also revealed that panduratin-A was more active in inhibiting the growth of MCF-7 cells (IC_{50}= 9 µM) than HT-29 cells (IC_{50}= 16 µM) and CaCo-2 cells (IC_{50}= 17 µM). Although not possible within the timeframe of this thesis the potential for testing in a breast cancer model would be relevant to follow up this *in vitro* observation. Other active compounds

in the extract of *B. pandurata* have been shown also to have pharmacological activity, for example cardamonin has been reported to exhibit antitumor activity in the Epstein-Barr virus activation assay (Murakami et al., 1993) and pinocembrin and pinostrobin which activated phase 2 enzymes (Fahey et al., 2002).

Components of some natural herbs and spices such as those seen in ginger species show moderate effects relative to some synthetic drugs (Corpet and Tache, 2002). They are however comparable to a number of other food related components which have attracted interest with regard to cancer preventing strategies. They have as well been shown to have other health benefits. It has been reported, for example, that daily intake of 200 mg of extract of *C. longa* by healthy subjects had no reported side effects but decreased risk of cardiovascular problems (Miquel et al (2002). Other Zingiberaceae such as common ginger (*Z. officinale*) has been shown to have anticancer activity via gingerol and paradol in cell culture and animal studies (Katiyar et al., 1996 and Park et al., 1998) are antiinflammatory, antithrombotic and plasma cholesterol lowering agents (Thompson et al., 2002).

Future Work:

This thesis has shown that extracts of *Zingiber aromaticum* and its bioactive compound, zerumbone have significant potential as anticancer and antiinflammatory agents. Extract of *Boesenbergia pandurata* and the active compounds, panduratin A showed potential anticancer activity in *in vitro* studies. There have however been only limited number of studies on *Z. aromaticum* and *B. pandurata* which could be due to their limited availability of these species. Further work to assess bioavailability of active compounds would be desirable, to add understanding to their likely efficacy in cancer prevention.

More cellular and biochemical studies are needed to further elucidate the anticancer mechanisms of extracts of *Z. aromaticum* and zerumbone. A model study such as inflammatory bowel disease (IBD) with repeated application of chemicals to induce inflammation which lead to tumors in

the colon could be carried out, to investigate the chemoprevention capacity *Z. aromaticum* as well as to understand the mechanisms of colon cancer associated with IBD.

Extracts of *B. pandurata* which contain panduratin A showed more inhibitory activity in breast cancer cells than colon cancer cells. A different model of cancer (eg mammary) would be useful to evaluate further the anticancer potential, to understand the relationship between the extract, the active compound and its potential against cancer as well as the mechanisms of their anticancer activity.

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APPENDIX A

The chromatograms of HPLC of standard curcumin and ethanol extracts (10 mg/ml) of ginger species at 254 nm
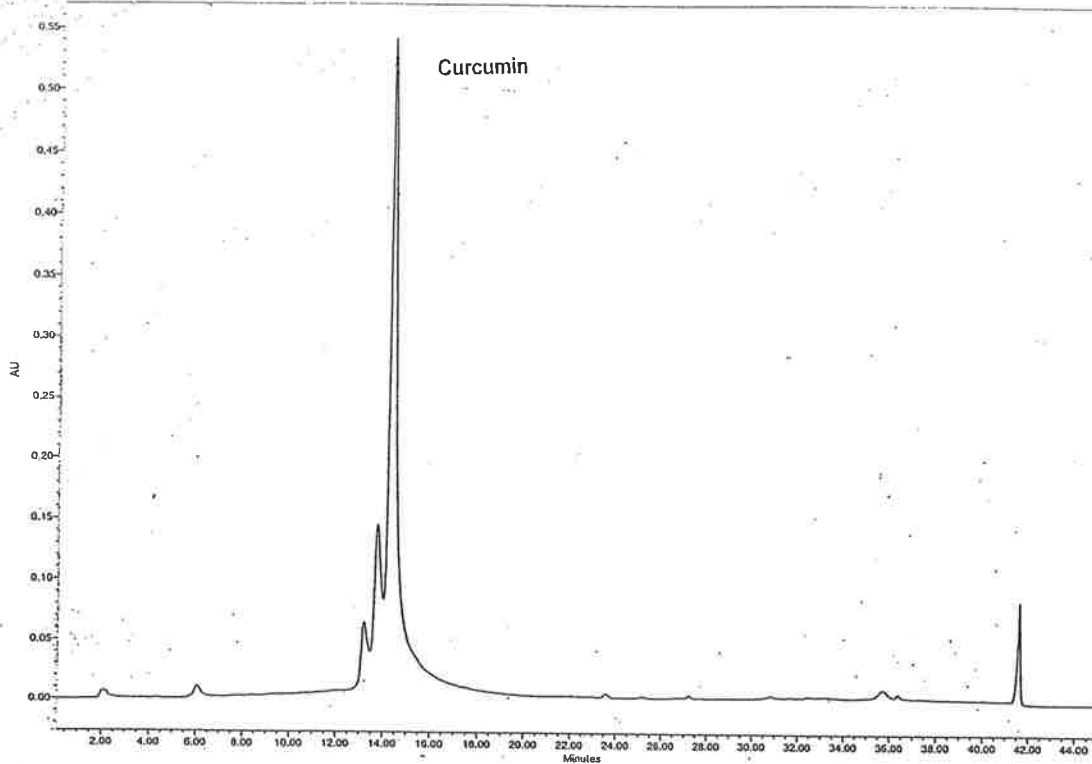
Chromatogram of standard curcumin
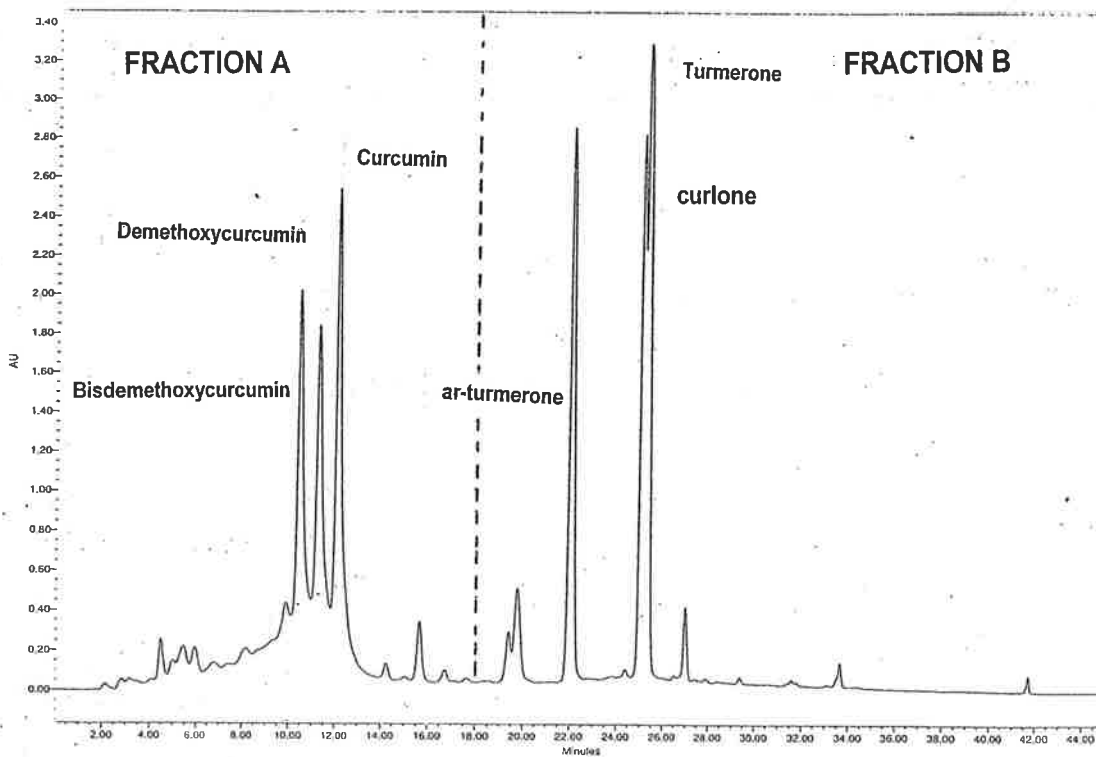
Chromatogram of ethanolic extract of *C. longa*. These compounds were on the bases of retention times (He et al., 1998).
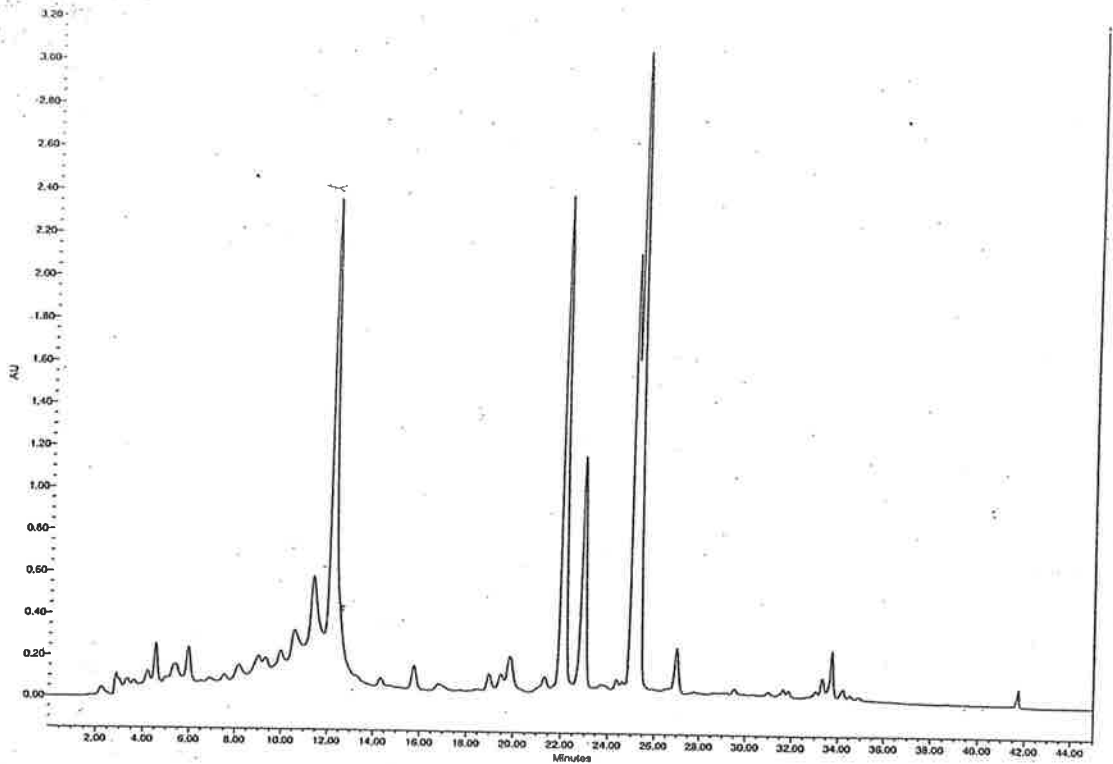
Ethanol extract of *C. xanthorrhiza*
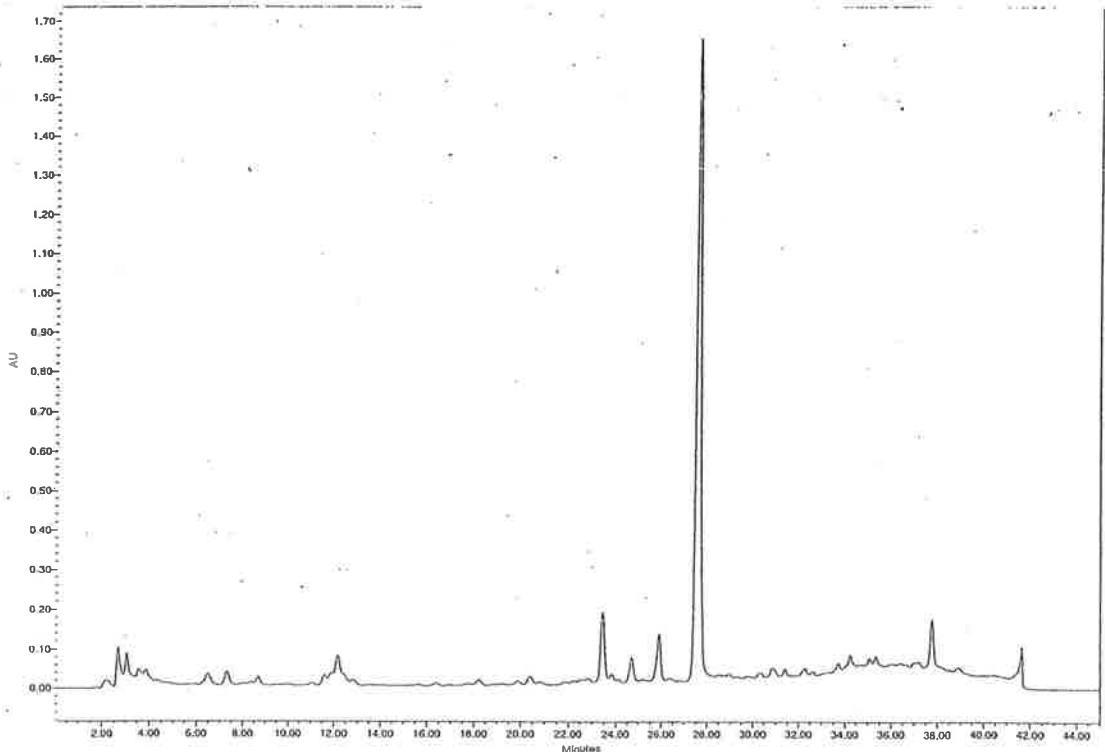
Ethanol extract of *C. mangga*

127
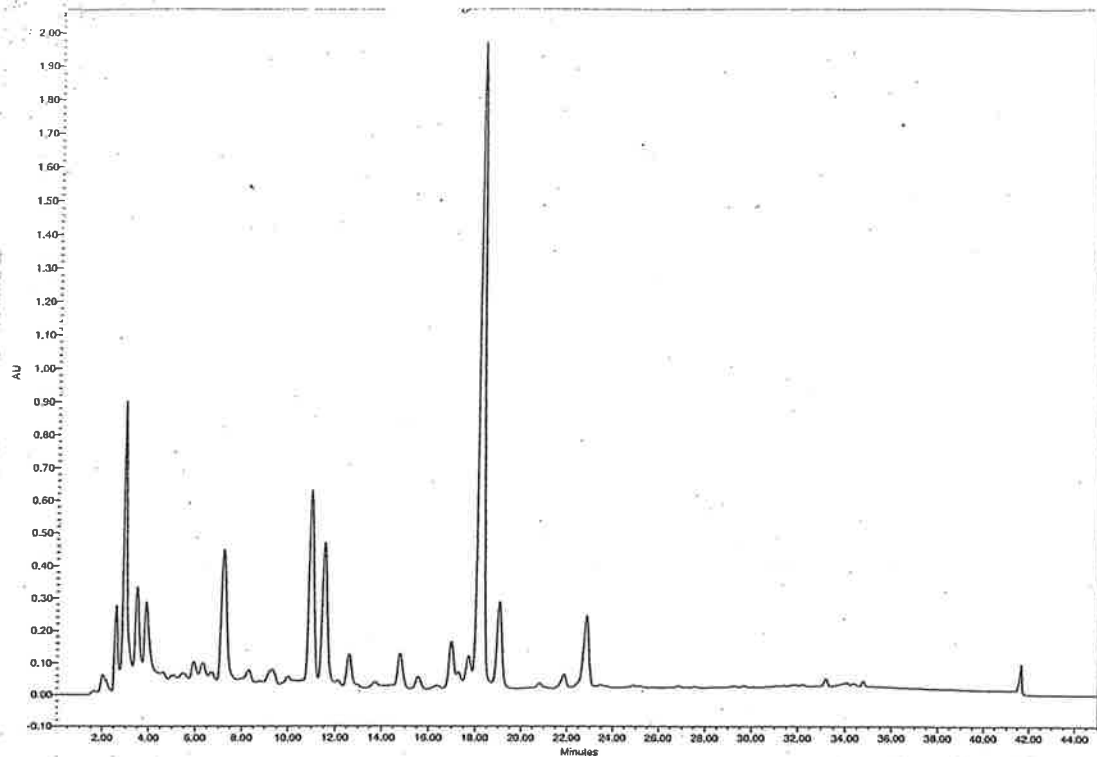
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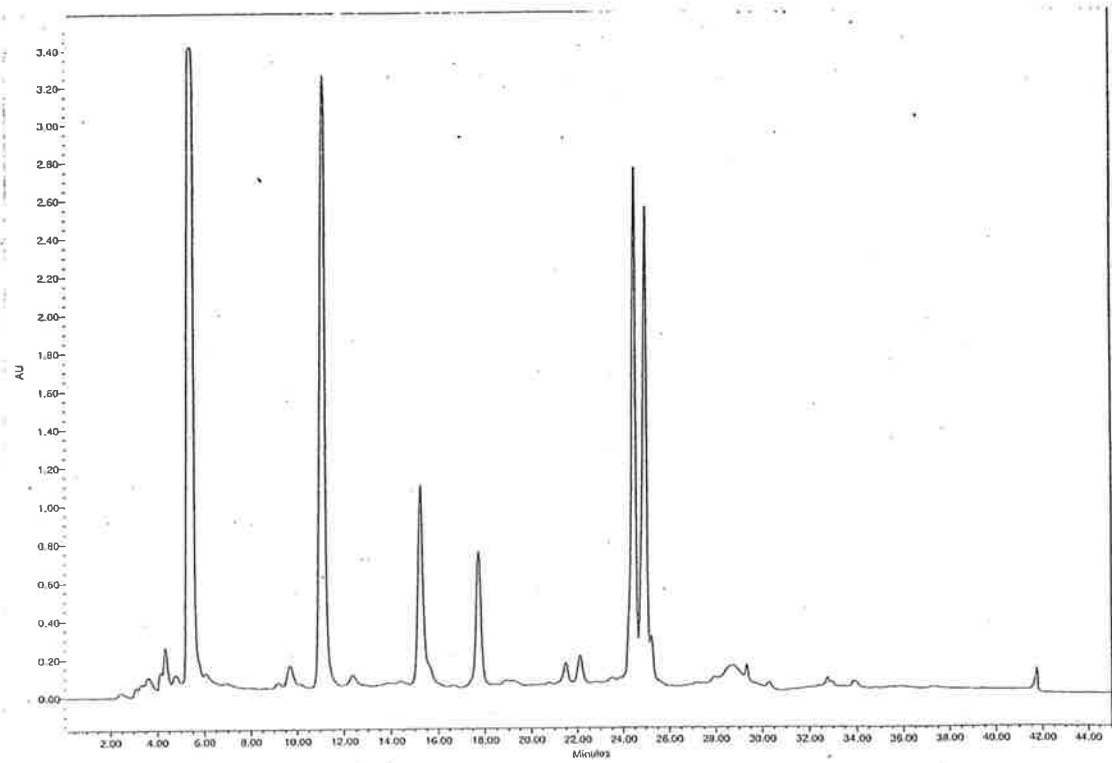
Ethanol extract of *Z. cassumunar*

128
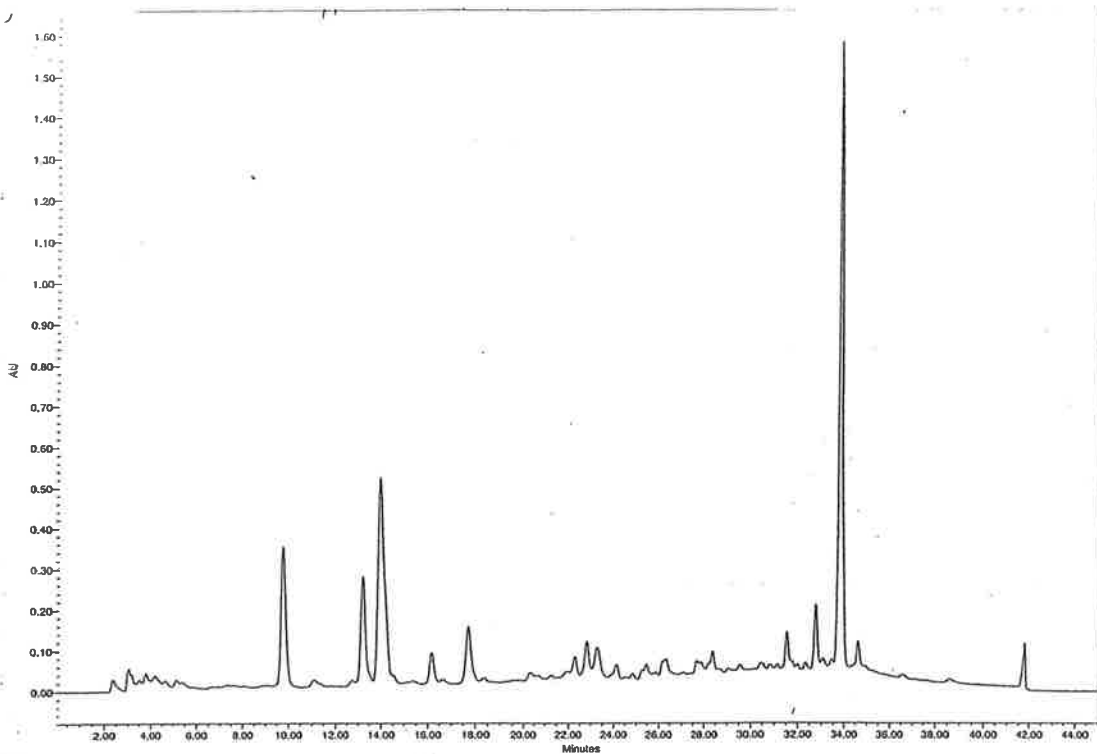
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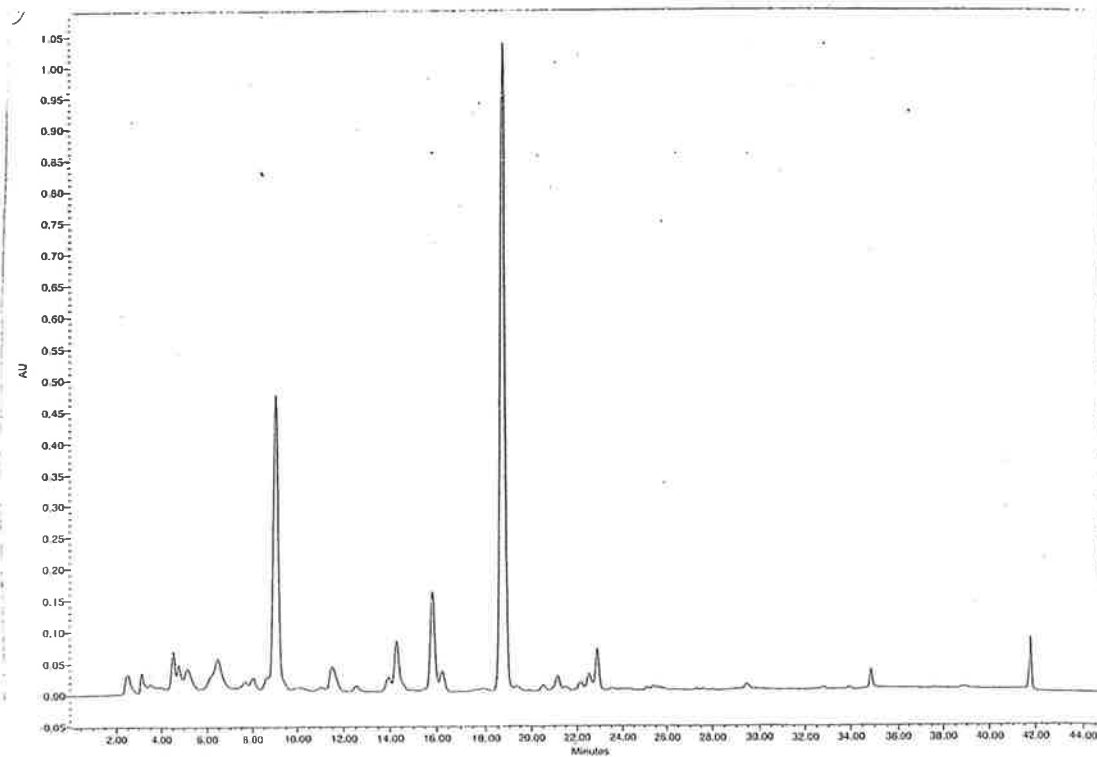
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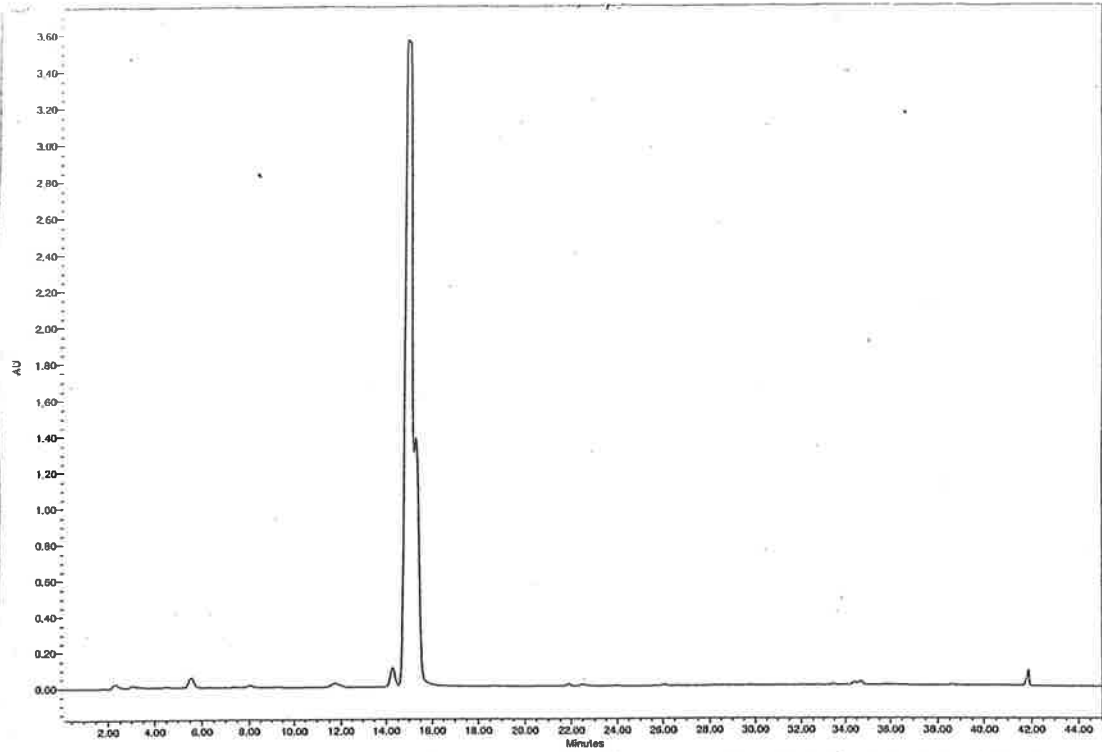
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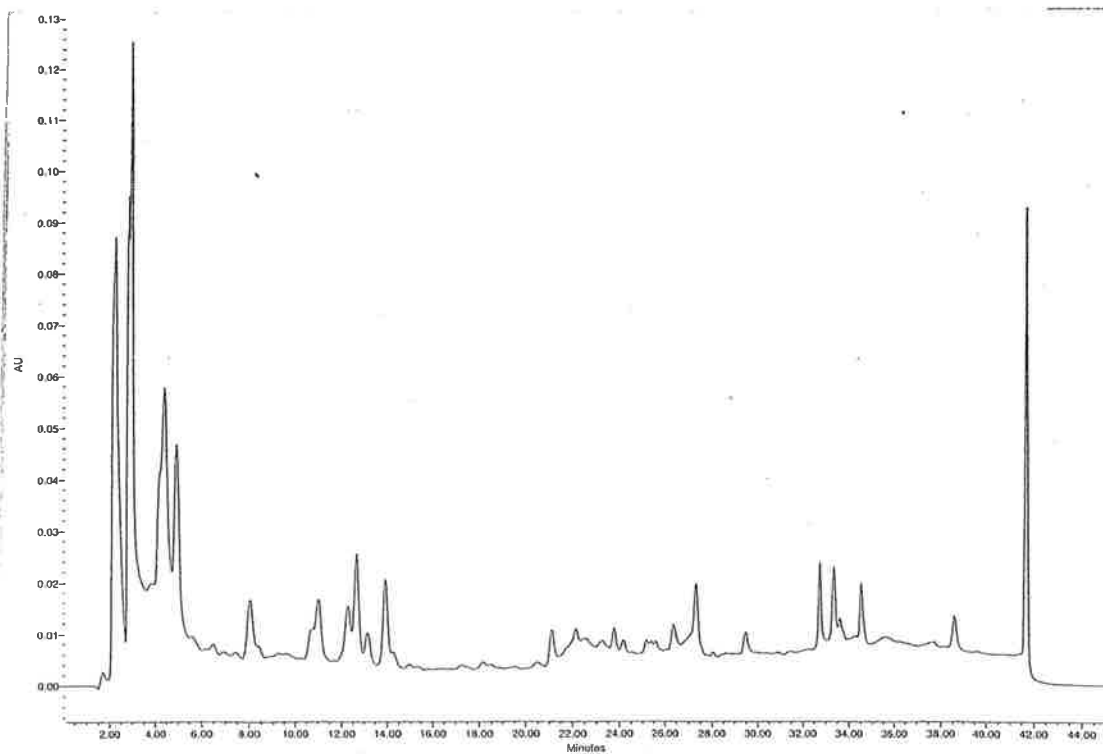
Ethanol extract of *A. cardamomum*

130

APPENDIX B

^{13}C NMR data of zerumbone and panduratin
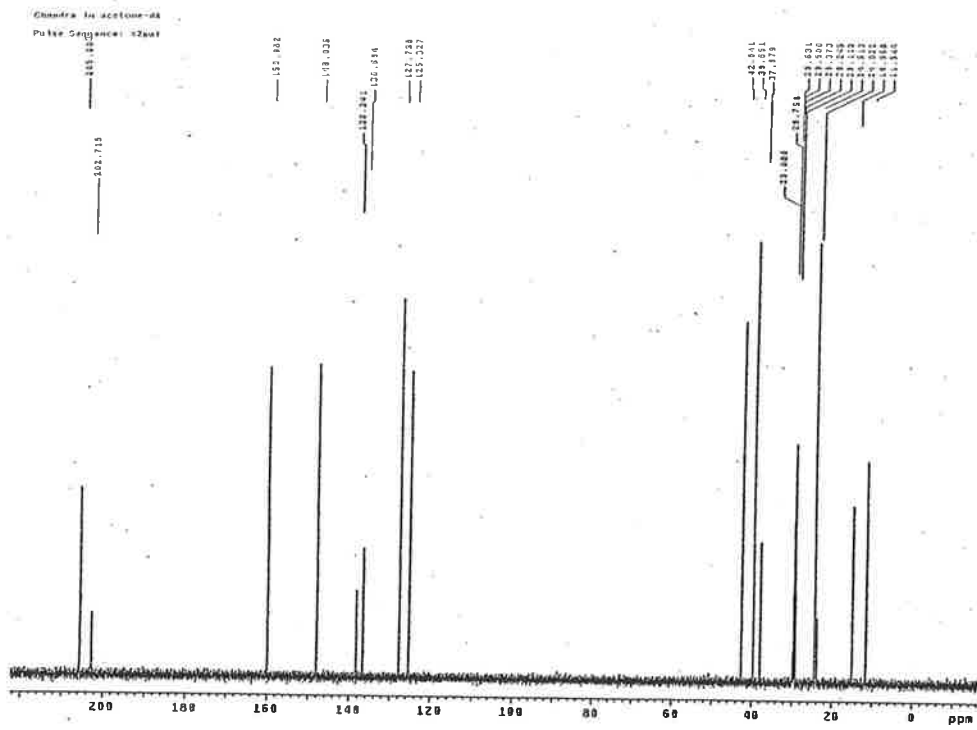
^{13}C NMR data of zerumbone
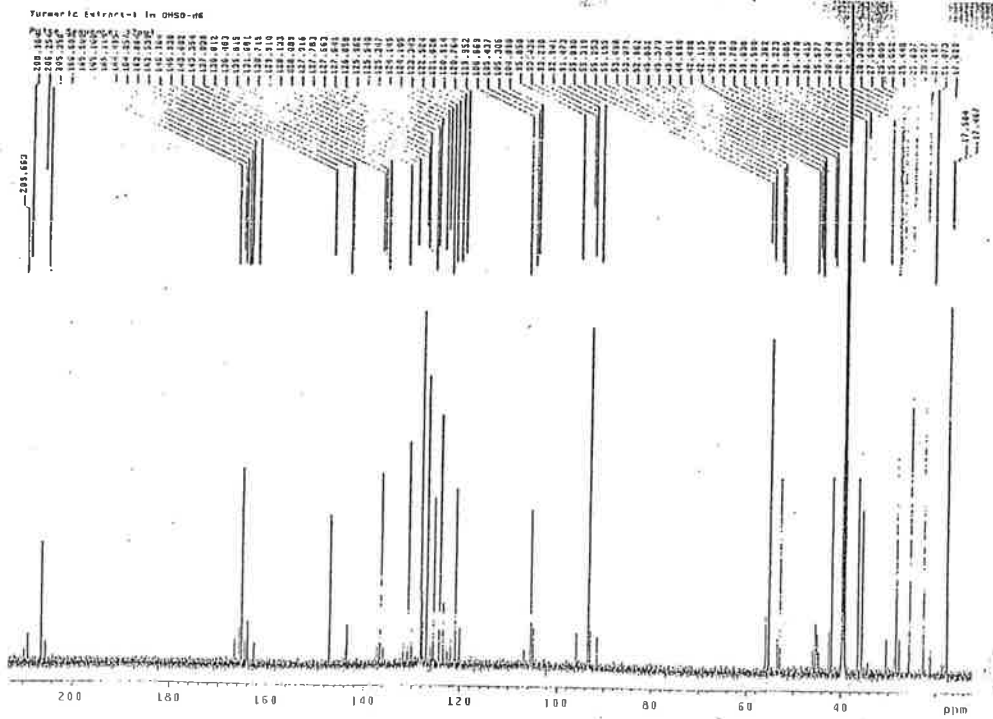
^{13}C NMR data of panduratin

APPENDIX C

The chromatograms of HPLC of curcumin and ethanol extracts of ginger species in the diet
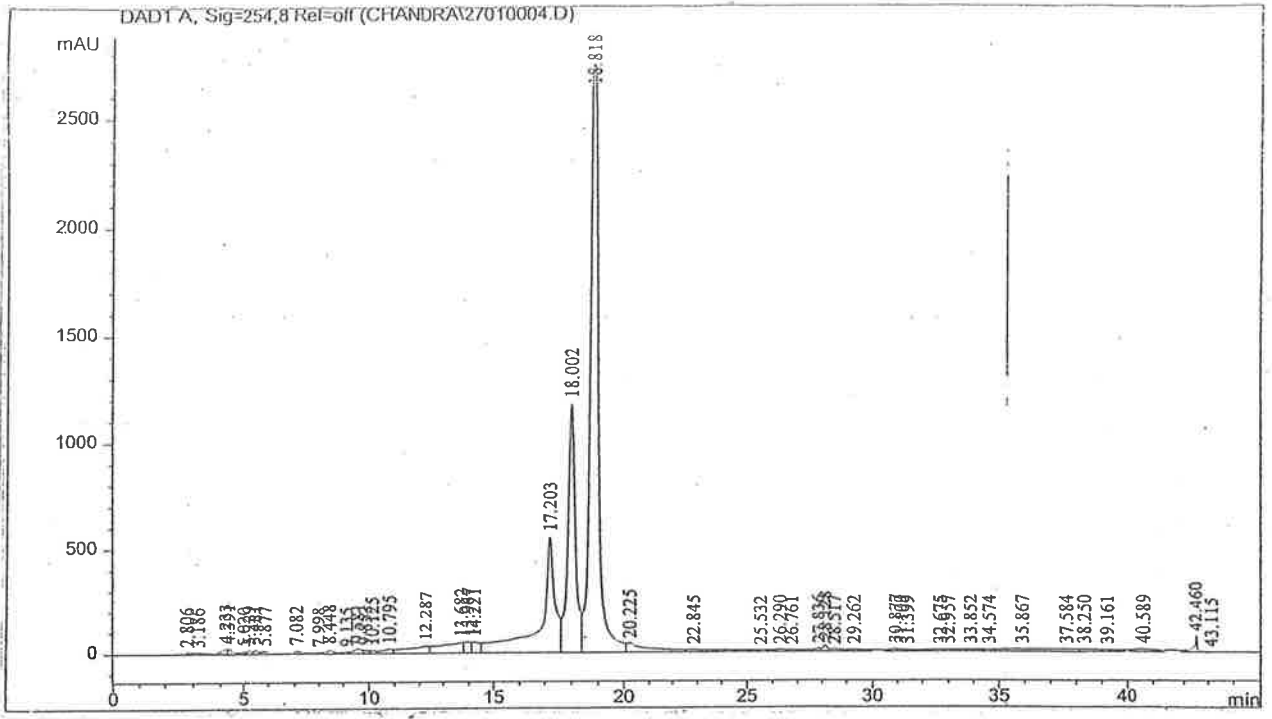
The chromatogram of a diet containing curcumin
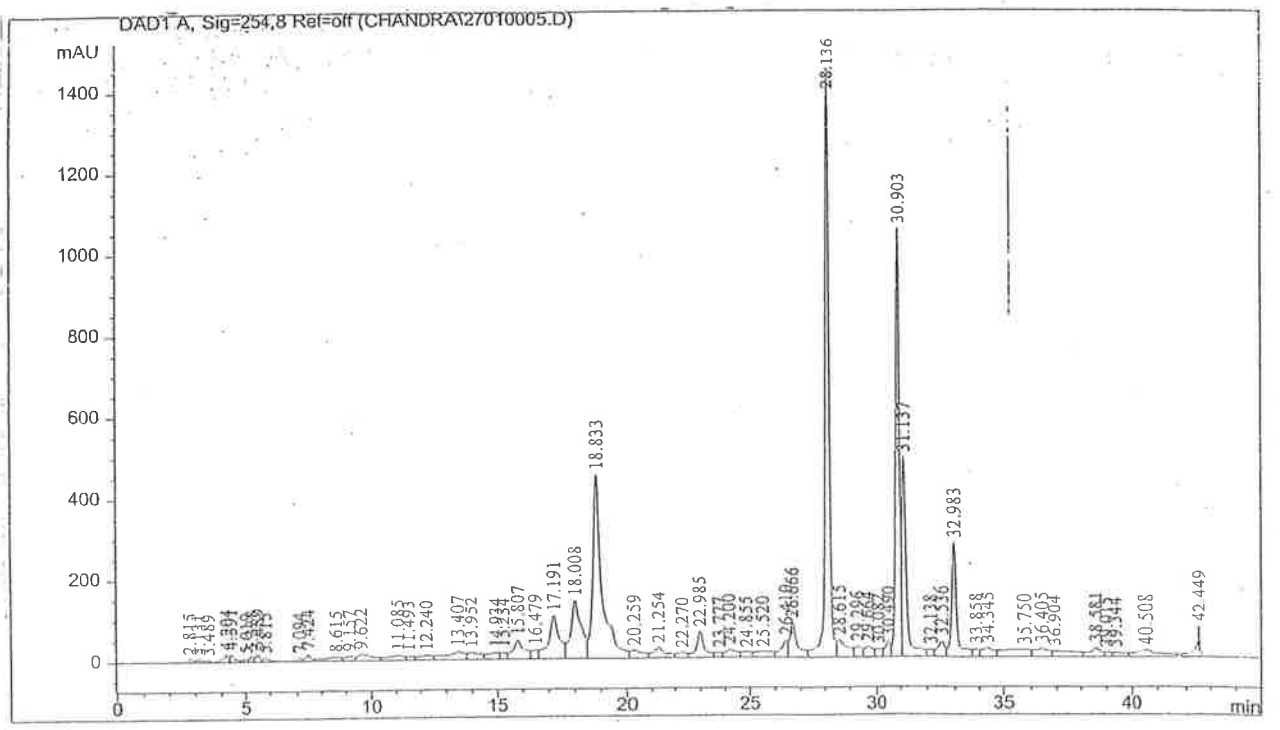
The chromatogram of a diet containing ethanol extracts of *C. longa*

132
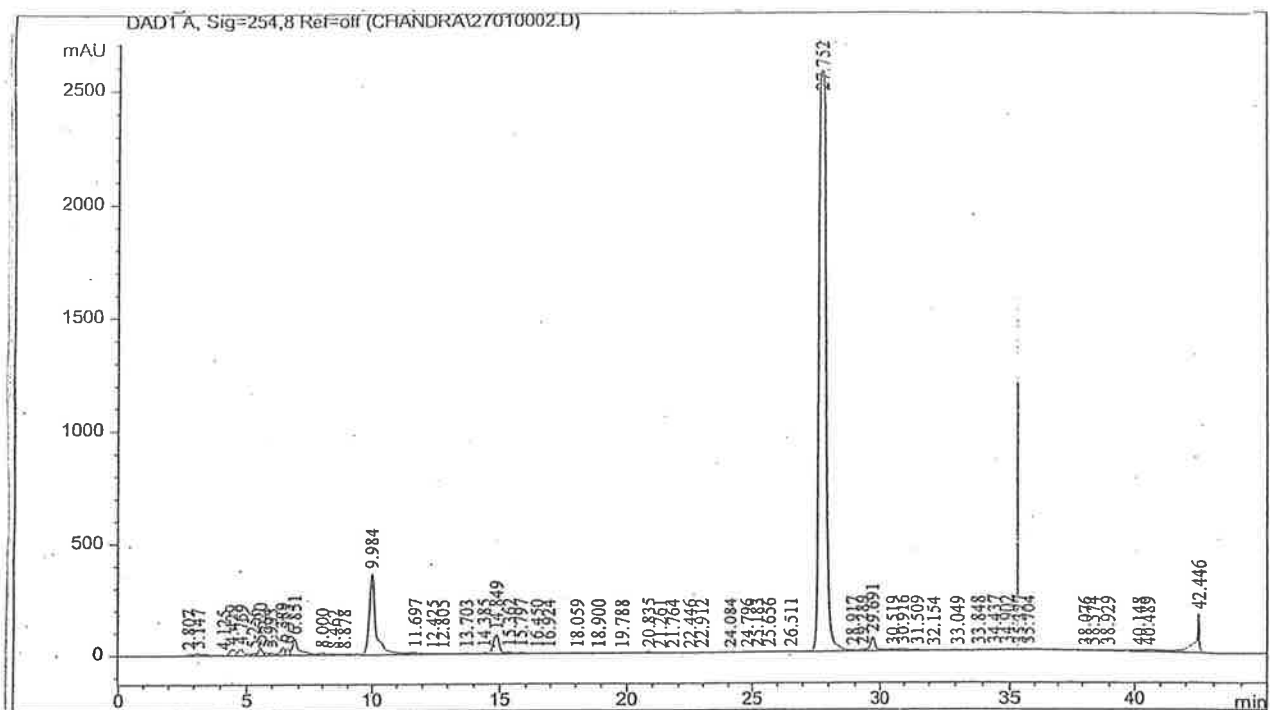
The chromatogram of a diet containing ethanol extract of *Z. aromaticum*
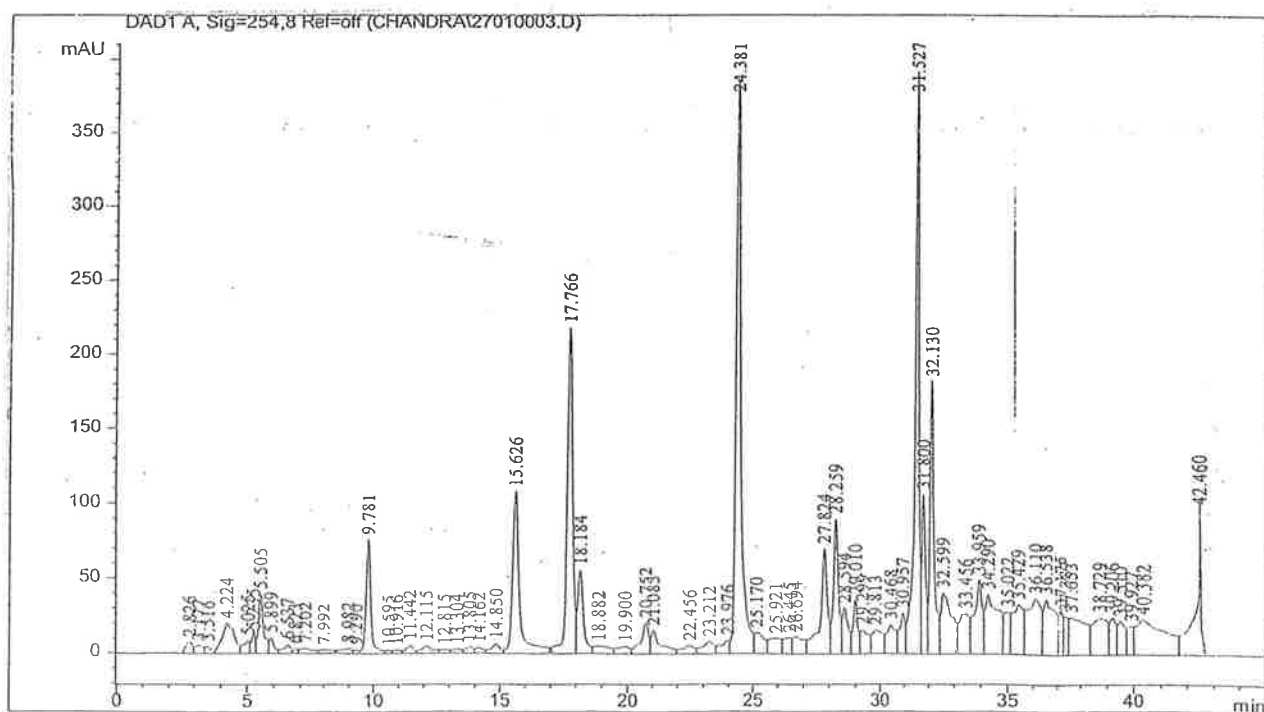
The chromatogram of a diet containing ethanol extract of *B. pandurata*

APPENDIX D

Published paper:
Screening for antitumor activity of 11 species of Indonesian Zingiberaceae using MCF-7 and HT-29 cancer cells (Pharmaceutical Biology, In Press)

Antitumor activity of extract of *Zingiber aromaticum* and its bioactive sesquiterpenoid zerumbone (Nutrition and Cancer, In Press)