POTENTIAL ANTICANCER ACTIVITY OF IN RHIZOMES OF GINGER SPECIES (ZINGIBERACEAE FAMILY)

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Department of Medicine

Department of Horticulture and Viticulture and Oenology

The University of Adelaide

South Australia

By

CHANDRA KIRANA MAgSc.

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TABLE OF CONTENTS

CHAPTER 2. GENERAL MATERIALS AND METHODS

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 chernopreventative 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.