Preclinical *in vitro* and *in vivo* effects of purified and synthetic bioactive compounds from marine mollusc *Dicathais orbita* on colorectal cancer: Cancer prevention and toxicity study

By

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Abstract

Marine indole-based compounds, including precursors and isomers of the ancient purple dye, Tyrian purple, are known for their biological activity. In particular, the precursors 6-bromoisatin and tyrindoleninone from the Australian whelk, *Dicathais orbita* are compounds that have gained specific interest over the past few years for their anticancer effects in several cancer cell lines. Previous *in vivo* studies in mice by administration of *D. orbita* extract has indicated the potential for these bioactive compounds to prevent colon cancer, but with possible idiosyncratic liver toxicity. Therefore, purification of the most likely bioactive compounds (tyrindoleninone and 6-bromoisatin) from *D. orbita* could be helpful to enhance the anticancer properties and potentially reduce the toxicity associated with the crude extract. Synthetic 6-bromoisatin is commercially available, so testing the pure synthetic compound will also help confirm any activity associated with this compound. Tyrindoleninone is a compound which can be easily oxidized to other components. Therefore, stabilizing it by using antioxidants might be beneficial to increase its bioactive effects. The objective of this project was to optimize the purification of tyrindoleninone and 6-bromoisatin and examine the effects of these compounds, along with crude extract from *D. orbita*, on colorectal cancer *in vitro* and *in vivo*. The toxicity of these compounds and extracts was also assessed *in vivo* to establish the safety of these compounds in the body system.

To optimize the purification of tyrindoleninone and 6-bromoisatin, initially the compounds were separated from the extract using thin layer chromatography (TLC) using a gradient of hexane, dichloromethane and methanol. Then flash chromatography was used to purify these compounds using the most effective solvent

system from TLC. The purified compounds were analysed using liquid chromatography/mass spectrometry (LC/MS) to confirm their identity and purity. The chemical composition of crude extracts from egg masses and hypobranchial glands were also compared by LC/MS and found to contain a very similar percent composition of the main brominated compounds. In order to inhibit the degradation of tyrindoleninone, a fraction containing tyrindoleninone and tyrindolenine was exposed to oxygen overnight in the presence of two antioxidants, Vitamin A and Vitamin E, and then reanalysed by LC/MS. The synergic anti-proliferative effect of tyrindoleninone with the most effective antioxidant was then tested on HT29 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium bromide (MTT) assay. The antioxidant experiment showed that 0.1 % Vitamin E was the most effective antioxidant for inhibiting tyrindoleninone degradation, but it failed to increase the cytotoxic effect of tyrindoleninone on HT29 cells and in fact appeared to provide some protection against the cytotoxic properties of tyrindoleninone.

In the next *in vitro* experiments, an egg mass extract was used for purification of the bioactive compounds with the optimised flash silica chromatography method. Bioassay guided fractionation was performed to identify the compounds with the greatest antiproliferative effects against colon cancer cells. The identity of the main bioactive compounds was confirmed by LC/MS, GC/MS and NMR as tyrindoleninone (>99% purity) and 6-bromoisatin (90% purity). These compounds were then tested for cytotoxic, apoptotic or necrotic effects using MTT, caspase 3/7 and membrane integrity assays respectively, on HT29 and Caco2 cells. The apoptotic effects of the bioactive compounds were confirmed by flow cytometry using Annexin-V-FITC and PI staining. Cell cycle analysis was also performed on HT29 cells treated with the

most bioactive compound. The 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay showed that semi-purified 6-bromoisatin inhibited the viability of both cell lines (IC_{50}= 100 μM). The fraction containing 6-bromoisatin activated caspase-3 and -7 enzymes in Caco2 and HT29 cells at approximately 100 μM (0.025 mg/mL) and 200 μM (0.05 mg/mL) respectively, much lower concentrations than those required to cause LDH release and necrosis (~1000 to ~2000 μM). Flow cytometry showed that semi-purified 6-bromoisatin (~200 μM) induced 77.6% apoptosis in HT29 cells. Cell cycle analysis showed the accumulation of 25.7% of HT29 cells treated with semi-purified 6-bromoisatin (~100 μM) in G2/M phase. The other compound, tyrindoleninone, was also found to inhibit the proliferation of Caco2 cells (IC_{50}= 98 μM) and HT29 (IC_{50}= 390 μM). Caspase-3 and -7 activity significantly increased only in HT29 cells treated with 195 μM (0.05 mg/mL) tyrindoleninone. LDH was released in both cell lines treated with high concentrations of tyrindoleninone.

In an *in vivo* trial, the effects of the purified tyrindoleninone and semi-purified 6-bromoisatin, along with the crude extract were tested for prevention of colorectal cancer in a two week mouse trial to determine whether these compounds can enhance the acute apoptotic response to genotoxic carcinogens (AARGC). The anti-proliferative effects of the extract and purified/semi-purified compounds were also tested by immunohistological examination using Ki-67 antibody. To evaluate any possible toxicity of the compounds, mouse general health, behavior, body weight and liver weight were assessed. Liver damage was also tested using histopathology and also biochemistry by measuring liver enzymes (ALT, AST and ALP) in the serum. Some other biochemical and also hematological blood tests were performed to

evaluate any other toxicity or side effects in blood and kidney. Semi-purified 6-bromoisatin (0.05 mg/g) was found to be the most bioactive compound in the crude extract capable of enhancing the apoptotic index in distal colon of mice. Tyrindoleninone did not increase the apoptotic index significantly. Semi-purified 6-bromoisatin did not show any toxic effect on liver, as indicated by no significant difference in the liver enzymes in comparison to the controls. In contrast, tyrindoleninone caused an increase in AST level compared to the saline control and also caused a reduction in red blood cell counts.

In my last experiment, pure synthetic 6-bromoisatin was tested for *in vitro* anticancer activity and prevention of the colorectal cancer using the same *in vivo* model. Administration of pure synthetic 6-bromoisatin to the mice, confirmed the results from the semi-purified 6-bromoisatin, with a significant increase in apoptosis at 0.05 mg/g, without any sign of toxicity in the liver or blood cells. However, a decrease in the potassium levels in the blood indicated the possibility of a diuretic effect associated with synthetic 6-bromoisatin.

This research confirmed the anticancer effects of 6-bromoisatin against two colorectal cancer cell lines *in vitro*, as well as the potential cancer preventative effects *in vivo* based on the ability to induce apoptosis in DNA damaged cells. This supports the potential development of this molluscan extract or natural 6-bromoisatin as a nutraceutical for chemoprevention of colorectal cancer. In addition, synthetic 6-bromoisatin is a promising lead for further pharmaceutical development for prevention of this disease. However, future studies in longer term animal models are required to confirm that the early stage prevention of tumors by apoptosis in DNA damaged cells

by 6-bromoistain does prevent the formation of actual tumors at the later developmental stages.

Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree of diploma in any university; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Babak Esmaeelian

Preface

Parts of the work presented in this thesis have been submitted for publication, or are currently in preparation for submission for publication. In this thesis, Chapters 3-5 are in manuscript format and hence there is some necessary repetition in the methods and introduction sections. Chapters 2, 3 and 5 are in the format more typical for chemistry journals (e.g. *Marine Drugs*) with combined results and discussion, whereas Chapter 4 is in the format more typical for cancer journals (e.g. *Cancer Letters*), with separate results and discussion sections. All references are listed at the end of the thesis to eliminate repetition,

Manuscripts for publication:

Chapter 3. Esmaeelian, B, Benkendorff, K, Johnston, MR and Abbott, CA "Purified brominated indole derivatives from *Dicathais orbita* induce apoptosis and cell cycle arrest in colorectal cancer cell lines" This chapter has been published as a research paper in Marine Drugs on 11 October 2013.

Chapter 5. Esmaeelian, B, Benkendorff, K, Le Leu, R and Abbott, CA "Bromoisatin found in muricid mollusc extracts inhibits colon cancer cell proliferation and induces apoptosis, preventing early stage tumor formation in a colorectal cancer rodent model" This chapter has been published as a research paper in Marine Drugs on 24 December 2014.

Chapter 2. Esmaeelian, B, Abbott, CA and Benkendorff, K "Optimizing the purification of tyrindoleninone and 6-bromoisatin and testing the proliferative effect of stabilized tryindoleninone on HT29 cells" A part of this chapter (The optimization of purification) is under preparation to *BMC Complementary Medicine* but the other part (Stabilization of tyrindoleninone) will go to another Journal.

Chapter 4. Esmaeelian, B, Benkendorff, K, Le Leu, RK and Abbott, CA "Brominated indoles from a marine mollusc extract prevent early stage colon cancer formation *in vivo*" Under preparation for submission to *Cancer Letters*

Conference presentations:

Esmaeelian, B, Benkendorff, K and Abbott, CA, "Preclinical testing of purified Muricid mollusc extract in rodent models for colorectal cancer", Molluscs 2009 Conference, Brisbane, Qld, Australia, November 25- 27, 2009, Poster

Esmaeelian, B, Benkendorff, K and Abbott, CA "Anti-cancer activity of the purified bioactive compounds from Australian muricid mollusk on colorectal cancer cell lines" The 13th International Symposium on Marine Natural Products, Phuket, Thailand, October 17-22, 2010, Poster & Oral presentation

Esmaeelian, B, Abbott, CA, Benkendorff, K, Le Leu, RK and Johnston, MR "*In vitro* and preclinical testing of purified muricid mollusc extract for colorectal cancer, Postgraduate Conference, School of Biological Sciences, Flinders University, Adelaide, Australia, June 2012, Oral presentation

Esmaeelian, B, Benkendorff, K, Le Leu, RK and Abbott, CA "The preclinical effects of purified mollusk extracts in a short term mouse model for early-stage colon cancer" Eleventh Annual AACR International Conference on Frontiers in Cancer Prevention Research, Anaheim, California, October 16-19, 2012, Poster

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Abbreviations

AARGC	acute apoptotic response to genotoxic carcinogens
ACF	aberrant crypt foci
ACS	American Cancer Society
AIF	apoptosis inducing factor
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AOM	azoxymethane
ANOVA	analysis of variance
APC	adenomatous polyposis coli
AST	asparate aminotransferase
CDK	cyclin dependent kinase
CE	crude extract
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
COX2	cyclo-oxygenase-2
CRC	colorectal cancer
DAPI	4',6-diamidino-2-phenylindole
DCM	dichloromethane
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EC	European Commission
EGFR	epidermal growth factor receptor
EM	egg mass
EMEA	European Medicines Evaluation Agency
FDA	Food and Drug Administration
FOBT	fecal occult blood test
GC/MS	gas chromatography–mass spectrometry
GSK-3	glycogen synthase kinase-3
HCAs	heterocyclic amines
Hct	hemoglobin, hematocrit
HG	hypobranchial gland
HPLC	high-performance liquid chromatography

HX	hexane
IARC	International Agency for Research on Cancer
IBD	inflammatory bowel disease
LC/MS	liquid chromatography–mass spectrometry
LDH	lactate dehydrogenase
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MMR	mismatch repair
MSI	microsatellite instability
MTT	3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	nuclear magnetic resonance
NCI	National Cancer Institute
NSAIDs	non-steroidal anti-inflammatory drugs
PAHs	polycyclic aromatic hydrocarbons
PBS	phosphate buffered saline
PI	propidium iodide
SEER	surveillance, epidemiology and end results
SRB	sulphorhodimine B reagent
Str	staurosporine
TLC	thin-layer chromatography
TNF	tumour necrosis factor
TNM	tumor node metastasis
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
TYR	tyrindoleninone
VEGF	vascular endothelial growth fact

1. General introduction

1.1 Colorectal cancer

Colorectal (CRC) often called colon or bowel cancer, is the third most common cancer worldwide (McLeod et al., 2009, Jemal et al., 2011) and the second leading cause of cancer-related death in the United States (Chan and Giovannucci, 2010). About 1.2 million new incidences of CRC (9.7% of all cancers excl. non-melanoma skin cancers) and 608,000 deaths have been reported globally in 2008 (Ferlay et al., 2010). The highest incidence rates of CRC have been reported in Australia and New Zealand, North America and Europe, while the lowest rates found in South-Central and Asia Africa (Jemal et al., 2011). More than two-third of the CRC cases are in the colon and less than one-third occur in the rectum (ACS, 2013). The tumor usually starts as a noncancerous polyp which grows in the colon or rectum and can become cancerous. Adenomas or adenomatous polyps are one type of these polyps that have the potential to develop into cancer (Levine and Ahnen, 2006). It is estimated that one-third to one-half of all people develop one or more adenomas during their life (Schatzkin et al., 1994, Bond, 2000); however, just less than 10% of adenomas become cancerous (Levine and Ahnen, 2006). More than approximately 95% of colorectal cancers are adenocarcinomas, but there are several less common kinds of tumors that may also develop in the colon and rectum, such as carcinoid tumors, gastrointestinal stromal tumors and lymphomas (Stewart et al., 2006, Cascinu and Jelic, 2009). Once the cancer forms, it can invade the colon wall and rectum and also penetrate lymph vessels or blood (ACS, 2013). It usually takes about 10-15 years for CRC to develop (Kelloff et al., 2004). Lifetime risk for being diagnosed with CRC has been reported about 5% in both males and females (ACS, 2013).

1.2 Biology of colorectal cancer

CRC develops from the progressive accumulation of mutations in tumor suppressor genes and oncogenes that activate pathways for CRC initiation and progression in a multistep process (Migheli and Migliore, 2012). To date, four main molecular pathways have been identified in association with CRC carcinogenesis, including chromosomal instability pathway (CIN), CpG island methylator phenotype pathway (CIMP), the microsatellite instability pathway (MSI) and the serrated pathway (Figure 1.1); (Harrison and Benziger, 2011).

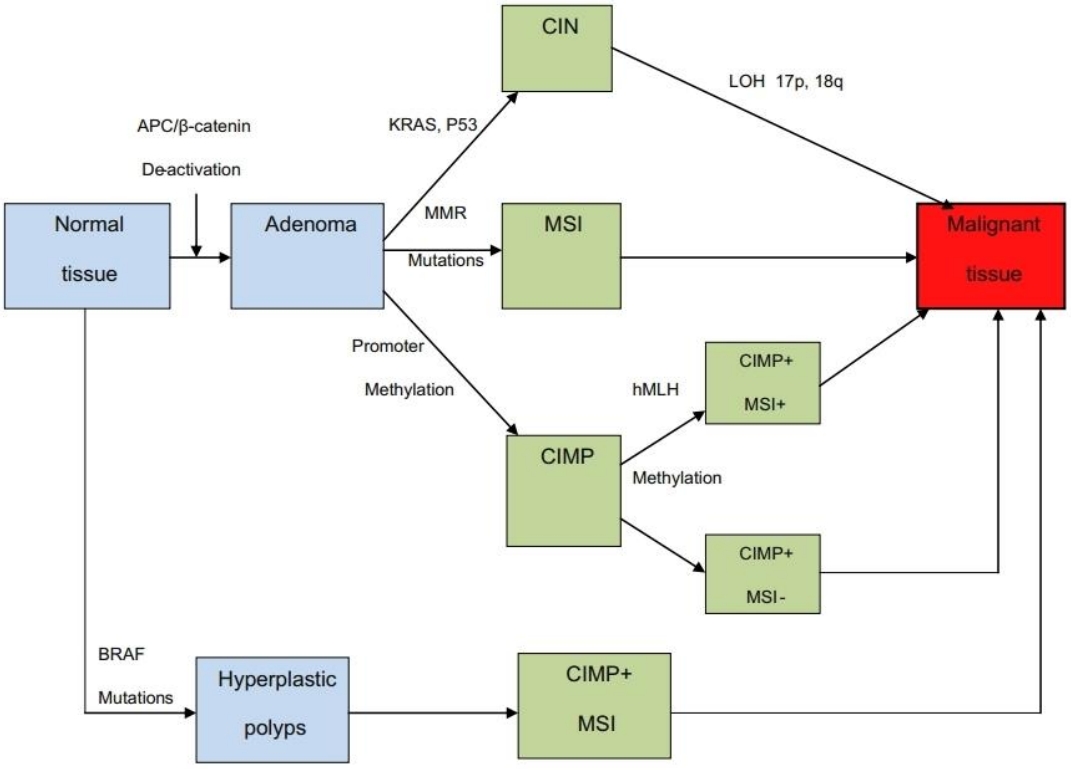

Figure 1.1 Adenocarcinoma progression model for colorectal cancer by Harrison and Benziger (2011). CIN = Chromosomal Instability pathway; CIMP = CpG Island Methylator Phenotype pathway; MSI = Microsatellite Instability pathway; MMR = Mismatch Repair system. BRAF is a member of the RAF family of serine/threonine kinases. Mutation in BRAF leads to activation of the cellular proliferation (Minoo et al., 2007) which is associated with aberrant genomic methylation in CRC (Nagasaka et al., 2008). Mutation in APC and P53 which are tumour suppressor genes and KRAS, which is an oncogene, are also linked to CRC.

The CIN pathway is the most common genetic aberration, occurring in 80-85% of CRC (Grady and Carethers, 2008). It is associated with a mutation in the adenomatous polyposis coli (APC) gene (a tumour suppressor gene) and KRAS gene (a proto-oncogene) (Harrison and Benziger, 2011). In this pathway, mutations of the APC gene predispose adenoma formation. Larger adenomas and early carcinoma formation involves mutation of KRAS and loss of chromosome 18q with SMAD4 protein that is downstream of transforming growth factor-β (TGFβ), followed by mutations of P53 in the carcinoma (Walther et al., 2009). KRAS mutations have been found in over 50% of the CRC cases. This demonstrates the important role of this gene in the pathogenesis of CRC (Yuen et al., 2002). The KRAS gene plays an important role in cellular proliferation by intracellular signal transduction and extracellular signal integration (Harrison and Benziger, 2011).

The CIMP pathway is the second most common pathway (15% of all sporadic CRC) in colorectal carcinogenesis (Boland et al., 2009) and is associated with hypermethylation of the respective promoters that result in silencing of tumour suppressor genes (Kim and Kim, 2010). The *KRAS* gene has also been shown to have a prominent role in the CIMP pathway (Jiang et al., 2009). The MSI pathway is associated with the mismatch repair (MMR) system that corrects DNA polymerase errors during replication, so this pathway relates to any mutation in the MMR system that impairs its efficacy (Vilar and Gruber, 2010, Shah et al., 2010). Finally, the serrated pathway is a distinct pathway to CRC carcinogenesis that has been recognized recently (Sheffer et al., 2009). This pathway explains the progression of serrated polyps (sessile serrated adenomas and traditional serrated adenomas) to CRC (Leggett and Whitehall, 2010).

There are several other biochemical pathways that act in conjunction with the above mentioned pathways that can predispose people to the development of cancer (Harrison and Benziger, 2011). Mutation of the P53 gene is one of the most common predispositions that has been recognized in approximately 50% of all CRC (Kern et al., 2002) and occurs with high frequencies in some other cancers as well (Harrison and Benziger, 2011). P53 is a tumor suppressor that is triggered in cellular stress conditions such as hypoxia, DNA damage, depletion of nucleosides and oncogenes (Molchadsky et al., 2010). The other pathways that are involved with development of CRC include the TGF-β pathway (Harrison and Benziger, 2011), the EGFR pathway (Roda et al., 2007) and loss of heterozygosity (LOH) of 18q (Popat and Houlston, 2005).

Inflammation has also been shown to have an important role in CRC carcinogenesis. Chronic inflammation can often predispose people to the development of cancer (Terzić et al., 2010, Harrison and Benziger, 2011). This has been evidenced by increased incidence of CRC in patients suffering from inflammatory bowel disease (IBD) (Harpaz and Polydorides, 2010, Harrison and Benziger, 2011). This process involves a complex interplay of various inflammatory factors, such as cyclo-oxygenase-2 (COX2), TNF-α, NF-κB and toll like receptors (TLR) (Zisman and Rubin, 2008). The role of COX2 is highlighted in studies showing that genetically modified mice deficient in the APC gene had a lower rate of polyp formation after treatment with a COX2 inhibitor (Kitamura et al., 2004). Activation of NF-kB has been linked to the expression of anti-apoptotic factors (Bcl-2 family members) and various inflammatory cytokines and also COX2 (Harrison and Benziger, 2011). Activation of TNF-a has also been associated with the activation of inflammatory

responses via NF-kB or activation of caspase-8 (Balkwill, 2006). People who are predisposed to CRC via these pathways, genetic mutation of P53 and other genes, or due to IBD, could benefit from functional foods or nutracueticals that reduce the risk of CRC developing.

1.3 Treatment and prognosis of colorectal cancer

The treatment and prognosis of CRC often depends on its stage. Staging is a process that shows whether the cancer has spread and how far and how widespread it may be at the time of diagnosis. The two most usual systems using for staging colorectal cancer are: 1) the Tumor Node Metastasis (TNM) system, which is used more often in clinical studies; and 2) the Surveillance, Epidemiology and End Results (SEER) summary staging system, which is used to statistically describe and analyze tumor registry data. The SEER summary staging system shows the spread of the cancer in relation to the wall of the colon and rectum or other tissues next to them and also other distant organs. This system includes four stages (ACS, 2013):

1) In situ: the cancer has not invaded into the wall of colon or rectum
2) Local: the cancer has invaded into the wall of colon or rectum but not through the nearby tissues
3) Regional: the cancer has invaded into the wall of colon or rectum and also in to the nearby tissue such as lymph nodes
4) Distant: the cancer has spread to the distant organs such as the liver, lung, peritoneum and ovaries

Surgery, chemotherapy, radiation therapy and targeted therapies, called monoclonal antibodies, are the four main types of treatment for colorectal cancer. Polypectomy

(polyp removal) at the in situ stage, and surgery during the localized stage of CRC are the standard treatments and might be the only treatment needed for early cancers. The 5-year survival rate for people in these two stages of CRC are 90%, but only 39% of colorectal cancers are diagnosed at early stages because most people with early colon cancer do not have any symptoms of the disease (ACS, 2013). If the cancer is in the regional stage and has spread to nearby lymph nodes, adjuvant chemotherapy and radiation therapy are usually recommended with surgery to lower the risk of recurrence. The 5-years survival rate decreases to 70% at this stage (ACS, 2013). For cancer in the distant stage that has spread to other organs, the aim of surgery is usually to prevent colon blockage and other local complications, though surgery is not recommended in all cases. Patients with CRC at the distant stage might be given radiation, chemotherapy and targeted monoclonal antibody therapies, in combination or alone, to prolong life and relieve the complications (ACS, 2013). The 5-years survival rate drops to only 12% at this stage (ACS, 2013).

As discussed, surgery is the main treatment for colon cancer and segmental resection is performed in most operations. However, 40-60% of stage II patients who are not typically eligible for adjuvant chemotherapy and undergo resection, die because of metastasis (Liefers et al., 1998). Some side effects of surgery include blood clots in the legs, bleeding from the surgery, damage to nearby organs during the operation, infection and adhesions that can cause the bowel to become blocked (Obrand and Gordon, 1997, Benson et al., 2004), temporary or permanent colostomy, constipation or diarrhea, sexual dysfunction in men and the possibility of fatigue for a long term (ACS, 2013).

Adjuvant chemotherapy (after surgery) and/or neoadjuvant chemotherapy (before surgery) can increase the survival rate for CRC patients by reducing the risk of recurrence and metastasis because it may eliminate a small number of cancer cells that may have escaped from the primary tumor and settled in other organs (ACS, 2013). Fluorouracil (5-FU) is one of the most common chemotherapies used after, or before surgery, and in metastatic CRC as part of the treatment (de Gramont et al., 2000, ACS, 2013) and provides a 78% three year survival rate (André et al., 2004). The combination of 5-FU, irinotecan and leucovorin (FOLFIRI) has been used as the first-line chemotherapy regime for metastatic colorectal cancer (Van Cutsem et al., 2011). The addition of cetuximab (targeting monoclonal antibody) to FOLFIRI in patients with KRAS wild-type disease was shown to significantly increase the survival rate (Van Cutsem et al., 2011). Oxaliplatin is another drug that is often used after surgery or for the treatment of metastatic CRC (ACS, 2013). The first-line oxaliplatin-based chemotherapy regime in patients with metastatic CRC are 5-FU/folinic acid plus oxaliplatin (FOLFOX-4) and capecitabine plus oxaliplatin (XELOX), (Saltz et al., 2008). The addition of bevacizumab to oxaliplatin-based chemotherapy in patients with metastatic colorectal cancer significantly improved the median progression-free survival (Saltz et al., 2008).

Although chemotherapy drugs can kill cancer cells, they may also damage some normal cells. The type of chemotherapy drugs, length of treatment and their dosage are the main factors that determine the severity of side effect associated with these drugs (ACS, 2013). For example, 5-FU and leucovorin, as a standard chemotherapy for metastatic colorectal, has many serious side effects reported, such as myelosuppression, mucositis (Elting et al., 2003, Benson et al., 2004, Peterson et al.,

2013), peripheral sensory neuropathy (Cassidy et al., 2008), pulmonary embolus, myocardial infarction and death (Rothenberg et al., 2001). There are some other side effects associated with current chemotherapeutic treatment regimes, such as neutropenia leading to infection (Hoff et al., 2001), bleeding or bruising after minor cuts or injuries (due to a shortage of blood platelets); (Benson et al., 2004), fatigue (due to low red blood cell counts); (Cassidy et al., 2008), myocardial infarction, pulmonary embolus, stroke and death (Rothenberg et al., 2001).

Although 5-FU and leucovorin are relatively inexpensive, many of the anti-cancer drugs used in chemotherapy are very expensive. It has been reported that adding combinatorial therapy drugs such as oxaliplatin to leucovorin/5-FU can significantly improve the 6-year overall survival rate in the adjuvant treatment of stage II or III colon cancer (André et al., 2009), but 5-FU or leucovorin, with a combination that includes oxaliplatin or irinotecan, costs at least $20,000 to $30,000 for 6 months treatment (Lines, 2008). Cheaper combinatorial therapies could be tested using known anticancer compounds that are no longer under patent protection by pharmaceutical companies.

Similar to chemotherapy, radiation therapy alone or in combination with chemotherapy can be also used with surgery to lower the risk of recurrence and increase the survival rate for CRC patients (ACS, 2013). However, radiation therapy is more effective for patients with rectal cancer than colon cancer (Glimelius et al., 2003). In addition, there are some side effects associated with radiotherapy such as nausea, sexual problems, diarrhea, fatigue, bladder irritation and rectal irritation. Bladder or rectal irritation can be a permanent side effect (ACS, 2013).

Symptoms of CRC usually appear with more advanced disease and most of patients with CRC often remain undiagnosed until they present with metastasis and micrometastases (Beart Jr et al., 1990, Brown and DuBois, 2005), thus increasing global mortality from CRC, which is approximately 50% of incidence (IARC, 2008). Therefore, due to the morbidity and poor prognosis of the cancer therapy regimes, along with the large economic burden colorectal cancer presents, prevention of this disease is an important priority (IARC, 2008).

1.4 Prevention of colorectal cancer

1.4.1 Screening tests:

Although the exact cause of most colorectal cancer is not known, it is possible to prevent many colorectal cancers. Therefore a significant body of research is focused on identifying causes and new ways to prevent colorectal cancer. For the early detection of cancer, screening or testing can be done to find any abnormalities, like the detection of polyps that might eventually become cancer, before any signs and symptoms of disease (ACS, 2013). These polyps can be removed and thus prevent the development of cancer.

There are two groups of screening tests for colorectal cancer. In the first group there are two tests that primarily detect cancer (stool tests) as follow (ACS, 2013):

a) Fecal occult blood test (FOBT): Blood vessels at the surface of colorectal tumors or some large polyps can be damaged by the passage of faeces, so hidden blood in stool can be determined by this test (ACS, 2013).
b) Stool DNA test: DNA mutations often affect certain genes in colorectal cancer cells (such as the APC gene, p53 tumor suppressor gene and K-ras oncogene).

Studies are testing new ways to find the polyps and colorectal cancers at earlier stage by screening DNA mutations in the cells found in stool samples (Diehl et al., 2008).

Although some polyps may be detected by these tests, the potential for detecting adenomatous polyps are incidental and cannot be the aim of these tests (ACS, 2013). In the second group, there are some tests that detect both precancerous growths (adenomatous polyps) and cancer as follow:

a) Flexible sigmoidoscopy: The rectum and part of the colon can be viewed by this method to detect any abnormality. If a polyp or tumor is observed, the patient can be further examined by colonoscopy.
b) Colonoscopy: This is a longer version of a sigmoidoscope and allows the doctor to monitor the lining of the rectum and the entire colon and remove the polyps if present (ACS, 2013). Colonoscopy can prevent approximately 65% of colorectal cancer incidents (Brenner et al., 2007). The rescreening interval for colonoscopy is the longest among other tests and it is not required to be repeated for 10 years if the exam is normal (Levin et al., 2008).
c) Barium enema with air contrast: Barium sulphate is used to fill and open up the colon and it spreads throughout the colon. Then an x-ray picture can be used to determine large polyps (ACS, 2013).
d) Computed tomographic colonography (CTC): This is also called virtual colonoscopy. In this method the colon is opened by a flexible tube that allow air or carbon dioxide be inserted to the colon. Then a special x-ray machine is used to CT scan the colon and rectum. Patients are referred to colonoscopy if any polyp is observed (Butterworth et al., 2006, Johnson et al., 2008).

For the early detection of cancer, screening tests can be helpful to find abnormalities in the bowel (Cappell, 2007). However, there are some limitations and there can be complications with the testing procedures. Indeed, stool tests are useful to detect colorectal cancer, but they are not reliable for finding premalignant colonic polyps that might eventually become cancer (ACS, 2013). Among the other mentioned tests, colonoscopy has been shown as a most sensitive method to detect adenomatous polyps or CRC by preventing approximately 65% of colorectal cancer incidents (Rockey et al., 2005, Brenner et al., 2007). However, colonoscopy has a higher risk of complications when compared to other tests, such as bowel tears or bleeding, especially when specialists remove a polyp (Levin et al., 2008). In addition, screening tests are not always successful due to some problems such as ineffective implementation of protocols by physicians, under-referral for screening, patient fears about screening tests and test costs (Cappell, 2007).

1.4.2 Impact of life style in colorectal cancer prevention

Lifestyle alteration is an important way to lower the risk of developing colorectal cancer. CRC is initiated via the alteration of genes in a multistep process and is classified into hereditary and sporadic cases (Benito and Díaz-Rubio, 2006). More than 95% of colorectal cancer cases are sporadic, without a significant hereditary risk (Watson and Collins, 2011). A few non-modifiable hereditary factors increase the risk of CRC, such as the family history of colorectal cancer (Butterworth et al., 2006), chronic inflammatory bowel disease (Bernstein et al., 2001) and diabetes (Huxley et al., 2009). So CRC is usually preventable because it is mainly associated with environmental factors (Lynch and de la Chapelle, 2003, Gingras and Béliveau, 2011).

Lifestyle, especially in western populations (physical inactivity, smoking and drinking, high caloric intake and obesity), is an important factor in the etiology of CRC (Le Marchand et al., 1997). Therefore, changes in lifestyle to reduce the risk of developing CRC is recommended (Cappell, 2007). Eating plenty of vegetables, fruits and whole grain food can reduce the intake of high-fat foods like red and processed meat (Courtney et al., 2004, Cappell, 2007). Studies have shown some potential mutagens are present in meat cooked diets, such as heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) (Cross and Sinha, 2004). These carcinogens produce different types of DNA-adducts, which can cause damage and mutation in DNA resulting in the formation of aberrant crypt foci (ACF), mucosal cell clusters, polyps, adenomas or colonic polyps, and ultimately adenocarcinomas (Al-Saleh et al., 2008, Cappell, 2007).

Some studies suggest taking daily calcium, Vitamin D and folic acid to reduce colorectal cancer risk (Park et al., 2007). Another recent study showed that a high magnesium diet may also reduce colorectal cancer risk in Japanese men (Ma et al., 2010). There are some studies that show long-term and regular use of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen and naproxen is associated with the lower risk of colorectal cancer (Giardiello et al., 1995, Rothwell et al., 2010). Cyclooxygenase-2 (COX-2) inhibitors such as celecoxib are also useful (Steinbach et al., 2000). However, NSAIDs can cause serious side effects such as gastrointestinal bleeding and selective COX-2 inhibitors can cause heart attack, so NSAIDs or selective COX-2 inhibitors are not advised as CRC chemopreventatives, due to uncertainties about their potential toxicity, appropriate dose and effectiveness (ACS, 2013).

Some other risk factors such as physical inactivity (Kirkegaard et al., 2010), alcohol consumption (Ferrari et al., 2007), smoking and obesity (Huxley et al., 2009) have been shown to increase the risk of CRC. Physical activity for 45 minutes on 5 days per week and decrease in smoking can lower the risk for colorectal cancer (Soerjomataram et al., 2007, ACS, 2013). Nevertheless, implementing global life style alteration is inherently difficult (Courtney et al., 2004). For example, the trend towards diets that are too high in calories and fat in the last few decades have increased the susceptibility to CRC (Shike, 1999). It is believed that overall mortality from cancer is unlikely to be changed statistically unless there is trend towards increased use of scientifically substantiated natural products as a new chemopreventatives (Reddy et al., 2003).

1.5 Natural products benefits and their anticancer properties

An important strategy for the development of new medicines owes its success to the discovery of bioactive natural products. In recent decades many of the new antibiotics and new antitumor drugs approved by the US Food and Drug Administration (FDA), or comparable entities in other countries, were natural products or derived from natural products (Harvey, 2000, Esmaeelian et al., 2007, Newman and Cragg, 2012). Natural products have been used as traditional anticancer medicines for thousands of years (Rajamanickam and Agarwal, 2008). They are usually low toxic, cost effective and socially acceptable alternatives to pharmaceutical chemopreventatives (Manson et al., 2005). Of the 183 antitumor agents approved world-wide from the beginning of formal chemotherapy (World War one mustard gas) until middle of June 2012, 87 compounds (48 % of total) are natural products or derived from natural products (Newman and Giddings, 2013). For examples, some current anticancer natural product drugs approved in 2012 include homoharringtonine (a cephalotaxine alkaloid) with the name

of Omacetaxine, approved by FDA to treat chronic myeloid leukemia (Newman and Cragg, 2012) and another drug with the name of mitoxantrone (a derivative of an anthraquinone), approved by the European Medicines Evaluation Agency (EMEA), for treatment of B cell lymphomas (Newman and Giddings, 2013). Results from a clinical trial revealed that 12 months treatment with garlic extract significantly reduced the number and size of colonic adenomas in patients with CRC (Tanaka et al., 2006, Rajamanickam and Agarwal, 2008).

Polygenic cancer drug resistance has been identified in conventional chemotherapy over the past decade, which limits the utility of the associated drugs (Al-Lazikani et al., 2012). One strategy to resolve this challenge could be the replacement of the conventional chemotherapy with combinatorial targeted therapy (Al-Lazikani et al., 2012). The last decade has seen a renaissance in applying combinatorial chemistry to natural product libraries for the development of novel multi-target therapies (Boldi, 2010). For example, the inhibiting effects of the natural products geldanamycin and radicicol has been combined with the purine scaffoldon the molecular chaperone HSP90 for a novel targeted cancer treatment reviewed by Workman et al. (2007).

One of the benefits of the natural compounds in crude organic extracts, such as typically used nutraceuticals, could be the synergic effect of some bioactive compounds that increase each other's effectiveness. This synergic effect has been shown in several extracts with various activities, including anticancer and antimicrobial activities (Bankole et al., 2008, Chen et al., 2010, Machana et al., 2012). However, some potential toxicity, especially on liver and kidney, have been reported in association with certain compounds in some crude extracts (Huang and Sun, 2010, Nascimento et al., 2012). This toxicity can be eliminated by isolating or fractionating

the bioactive compounds from the crude extracts, although possibly limiting the potential synergic interaction of the purified compounds with others. Quality control for developing nutraceuticals and pharmaceuticals is an important issue that requires identification of the main bioactive components and efficacy studies using the purified compounds.

1.5.1 Marine natural products and molluscs as a potential source of anticancer compounds

One of the major sources for natural products comes from marine environment, due to the vast biodiversity in this environment it is expected that there are still a great variety of potential bioactive compounds in organisms that are yet to be discovered or chemically screened for novel secondary metabolites (Blunt et al., 2013). The ocean covers about 70% of the earth and the biodiversity of marine organisms and their associated natural products is much greater than the terrestrial environment (Haefner, 2003). Marine natural products play a major role in the discovery and development of new drugs (Nuijen et al., 2000). Over 7000 marine natural products and 2500 different metabolites have been isolated from species such as algae, bryozoans, sponges, tunicates and molluscs (Jimeno et al., 2004).

In 1950, some of the first bioactive compounds, such as spongouridine, spongothymidine and c-nucleosides, were discovered from marine resources (Nuijen et al., 2000, Newman and Cragg, 2007). 1-Beta-d-arabinofuranosylcytosine (Ara-C), a synthetic analogue derived from the structure of these compounds, showed anti-proliferation activity against acute myeloid leukaemia (Jimeno et al., 2004). Ara-C is also called cytosine arabinoside because it combines an arabinose sugar with a cytosine base. This compound is incorporated into human DNA due to its similarity

with human cytosine deoxyribose (deoxycytidine) which kills cancer cells (Groothuis et al., 2000). Ara-C is used for the clinical treatment of leukaemia nowadays (Jimeno et al., 2004).

Some of these marine drugs are in various stages of preclinical or clinical trials, whilst others are in their infancy of development (Chin et al., 2006). Simmons et al. (2005) have reviewed marine natural products in the various stages of anticancer preclinical or clinical trials. For example, Aplidine is a compound isolated from the marine tunicate *Aplidium albicans* and demonstrates *in vivo* angiogenesis effects in human ovarian cancer by arresting the cell cycle at G1 (Taraboletti et al., 2004). Aplidine is a cyclic depsipeptide, a cyclic peptide, with a very similar chemical structure to didemnin B (Celli et al., 2004). Phase1 clinical trials have shown that Aplidin has anticancer activity on pre-treated solid tumours and lymphoma (Jimeno et al., 2004, Chin et al., 2006). Some other marine natural products with established mechanism of action have been reviewed by Sarfaraj et al. (2012). In addition, marine natural products and related compounds as anticancer agents with their clinical status (Five molecules approved and 11 molecules currently on the clinical pipeline for anticancer chemotherapy) have been comprehensively reviewed in a current review by Petit and Biard (2013), (Table 1.1). For example, Ecteinascidin-743 is anti-cancer compound derived from a tunicate (sea squirt, Phlyum Chordata). This alkaloid down-regulates p-glycoprotein in cell membranes and is in phase II clinical trials for prostate cancer and osteosarcoma and phase III for metastatic ovarian cancer and breast cancer (Carter and Keam, 2007, Kim, 2013). The FDA and European Commission (EC) granted orphan drug status to trabectedin for ovarian cancer and soft tissue sarcomas in April 2005 (Kim, 2013).

Table 1.1 Status of marine-derived natural products in anticancer clinical trials, adapted from Petit and Biard (2013)

Compound name	**Chemical Class**	**Source Organism**	**Production**	**Clinical status**
Cytarabine (Ara-C)	Nucleoside	Sponge, *Thetya crypta*	Synthesis, analogue of spongothymidine	Approved, Cytosar-U® for leukemia; Depocyte® for lymphomatous meningitis Phase I to IV in combination therapies
Trabectedin (ET-743)	Tetrahydrois-quinolone alkaloid	Tunicate, *Ecteinascidia turbinata*	Hemisynthesis	Approved in EU, Yondelis® for sarcoma Phase I to III for various cancers such as ovarian etc.
Eribulin (E-7389)	Macrolide	Sponge, *Halichondria okadai*	Synthesis, analogue of halichondrin B	Approved, Halaven® for breast cancer Phase I to III for various cancers such as NSCLC etc.
Brentuximab vendotin (SGN-35, MMAE)	Peptide	Mollusc, *Dolabella auricularia*	Synthesis, analogue of dolastatin10, antibody- drug conjugate	Approved in USA, Adcentris® for Hodgkin Lymphoma and sALCL
Keyhole Limpet Hemocyanin (KLH)	Glycoprotein	Mollusc, *Megathura crenulata*	Extraction	Approved, Immucothel® for bladder cancer Phase I to III for various cancers such as myeloma, lymphoma, etc.
Plinabulin (NPI-2358, KPU-2)	Diketo-piperazine	Fungus, *Aspergillus ustus*	Synthesis, analogue of halimide	Phase II
Plitidepsin (dehydrodidemnin B)	Cyclic depsipeptide	Tunicate, *Tridemnum solidum*	Synthesis	Phase I to II, alone, Aplidin® Phase I in combination
Elisidepsin (PM02734)	Depsipeptide	Mollusc, *Elysia rufescens* and his diet green alga *Bryospis* sp.	Synthesis, analogue of kahalalide F	Phase I to II, Irvalec®
PM00104	Tetrahydroiso - quinolone alkaloid	Nudibranch, *Joruna funebris*	Synthesis, inspired by jorumycin, renieramycins, ET-743	Phase I to II, Zalypsis®
PM01183 (lurbinectedin)	Alkaloid	Tunicate, *Ecteinascdia tubinata*	Synthesis, analogue of ET-743	Phase I to II
ILX-651 (Tasidotin, Synthadotin)	Peptide	Mollusc, *Dolabella auricularia*	Synthesis, analogue of dolastatin 15	Phase I to II
TZT-1027 (auristatin PE, soblidotin)	Peptide	Mollusc, *Dolabella auricularia*	Synthesis, analogue of dolastatin 10	Phase I to II
MMAE/MMAE+ antibody: PSMA-ADC, ASG-5ME, CR011-vcMMAE, BAY79-4620, SGN-35, Glembatumumab vedotin CDX-011	Peptide	Mollusc, *Dolabella auricularia*	Synthesis, analogue of dolastatin 10, antibody- drug conjugate	Phase I to II
MMAF + antibody: AGS-16M8F, SGN-75	Peptide	Mollusc, *Dolabella auricularia*	Synthesis, analogue of dolastatin 10, antibody- drug conjugate	Phase I
Salinosporamide A (NPI-0052)	β-lactone γ-lactam	Bacterium, *Salinospora tropica*	Synthesis	Phase I
PM060184	Unspecified	Sponge	Synthesis	Phase I

The gastropod class of molluscs includes the largest diversity of species (about 80% of living molluscs) ranging from an estimated 40,000 to over 100,000 species (Bieler, 1992). Some potential pharmaceuticals are being developed from the bioactive compounds of several species of mollusc (Chin et al., 2006, Benkendorff, 2010). The linear peptides derived from *Dollabella auricularia*, a shell-less mollusc, have demonstrated anti-neoplastic, antibacterial and fungicidal properties, as well as anti-tumour effects on breast and liver cancer (Chin et al., 2006). Of the anticancer compounds derived from marine molluscs, Dolastatin-10 and kahalalide F are under phase II of clinical trials for the treatment of cancer, as reported by Simmons et al. (2005). The bioactive compound Kahalaide F derived from *Elysia rufescens*, a shell-less marine mollusc, shows promising anti-tumour activity against breast, melanoma, hepatoma and pancreatic carcinomas (Janmaat et al., 2006, Chin et al., 2006). Considering the huge species diversity of molluscs and the fact that natural products from only <1% of molluscan species have been tested (Benkendorff, 2010), there is potential for further new drug development using molluscs as a source of bioactive compounds.

1.5.2 Muricidae molluscs as a source of bioactive compounds

The family of Muricidae includes a large group of predatory snails (whelks) that live in many parts of the world. *Dicathais orbita* is one of the larger species of muricid, which can be found in the southern regions of Australia, New Zealand, Norfolk Island and Howe Island (Tan, 2003). It has been investigated due to a number of bioactive compounds and pigments produced in its tissues (Baker and Duke, 1973, Benkendorff et al., 2000, Benkendorff et al., 2001, Benkendorff et al., 2004, Westley et al., 2010, Benkendorff et al., 2011, Edwards et al., 2012, Benkendorff, 2013).

The first compound identified from the Muricidae was a brominated indole derivative known as Tyrian purple (6,6'-dibromoindigo); (Friedlaender, 1907); (**6,** Figure 1.2). This compound is stored as a choline ester precursor salt of tyrindoxyl sulphate (**1,** Figure 1.2) in the hypobranchial gland and egg masses of Muricidae marine molluscs and produced after a series of oxidative, enzymatic and photochemical reactions (Baker, 1974, Benkendorff et al., 2000, Cooksey, 2001, Baker and Duke, 1976, Westley et al., 2006). The purple secretion from the Muricidae is also the source of "Murex purpurea" a homeopathic remedy used for the treatment of gynaecological disorders, including cancer (Dunham, 1864, Benkendorff et al., 2011). Tyrian purple has not revealed any notable biological activity itself, largely due to its insolubility in water and most organic solvents (Benkendorff 2013); however, some of its precursors such as tyrindoleninone (**3,** Figure 1.2) and the oxidation product 6-bromoisatin (**7,** Figure 1.2) have shown good anticancer effects on different human cancer cell lines (Vine et al., 2007a, Benkendorff et al., 2011, Edwards et al., 2012). For example, tyrindoleninone has been reported for its cytotoxic effect on U937 human lymphoma cells (Vine et al., 2007a). Furthermore, an extract containing this compound significantly reduced cell viability and induced apoptosis in Jurkat cells (Benkendorff et al., 2011) and female reproductive cancer cells (Edwards et al., 2012). Studies on synthetic 6-bromoisatin, reduced the cell viability of lymphoma U937 and colorectal HCT-116 cell lines (Vine et al., 2007a) and purified extracts containing 6-bromoistain showed anti-proliferative and apoptotic effects on female reproductive cancer cells (Edwards et al., 2012). Moreover, it has been shown that the minor dye pigment of Tyrian purple 6,6'-dibromoindirubin (**8,** Figure 1.2) is an inhibitor of protein kinase and efficiently inhibits cell proliferation by selectively targeting glycogen synthase kinase-3 (GSK-3); (Meijer et al., 2003, Leclerc et al., 2001). All known brominated

indole metabolites from *D. orbita* and their generation in the mollusc are shown in Figure 1.2.

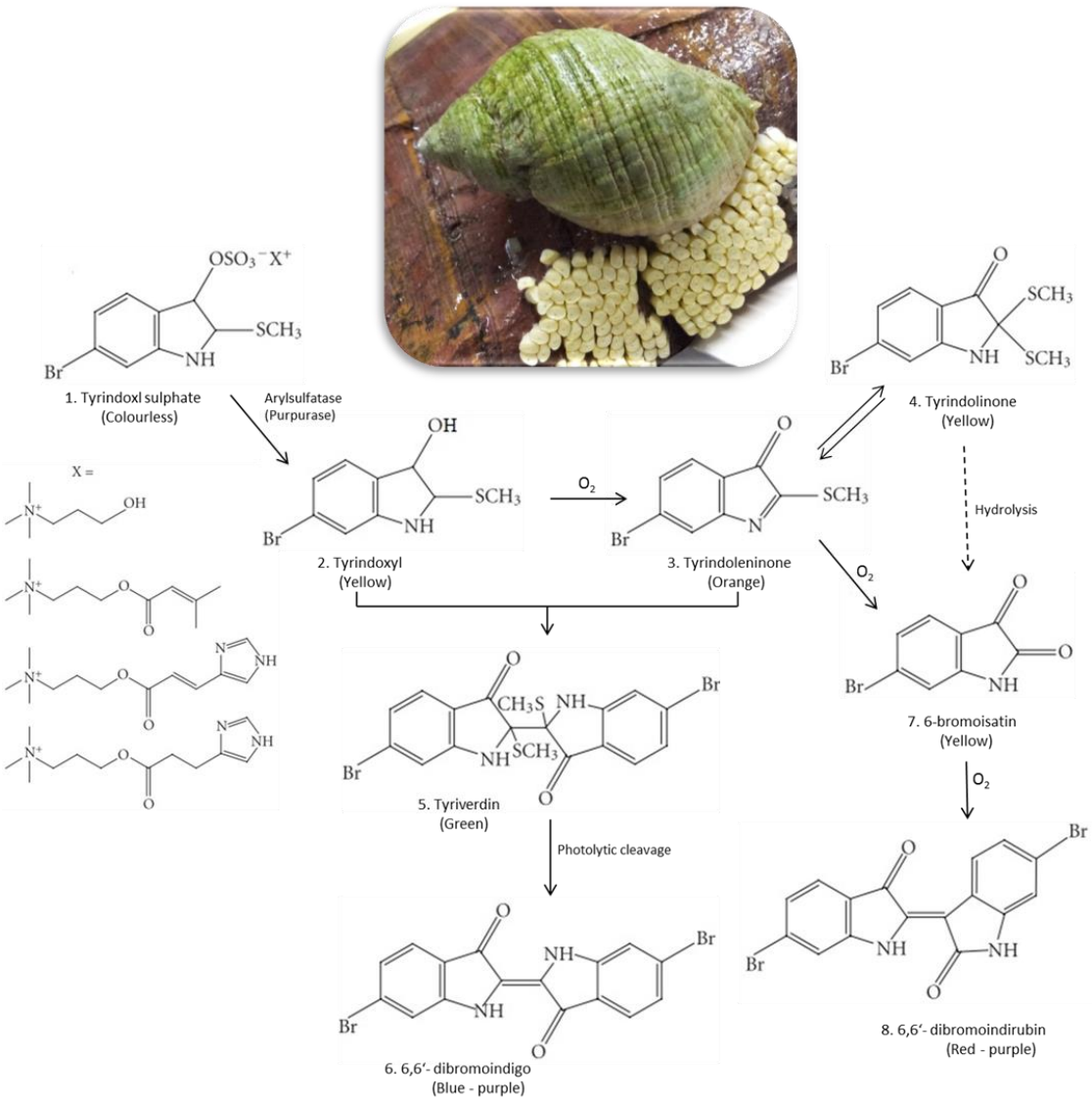

Figure 1.2 *Dicathais orbita*, the source of biologically active secondary metabolites, adapted from Westley and Benkendorff (2008).

Crude extracts from *D. orbita* have also been shown to have some *in vitro* anticancer activity inducing apoptosis in Jurkat cell lines and these extracts appear to have more anti-proliferation activity toward cancer cells in comparison to human untransformed mononuclear cells (Vine, 2002, Benkendorff et al., 2011). The crude extract from this mollusc has also been tested for *in vivo* efficacy by Westley et al. (2010) using a short-term rodent model for the prevention of colorectal cancer. The results from this study revealed that the extract induced apoptosis in the colon in response to genotoxic damage (Westley et al., 2010). However, a four week toxicity study in mice revealed the potential for idiosyncratic liver toxicity associated with the oxidized extract (Westley et al., 2013).

The crude extract from *D. orbita* used in the *in vivo* study contained significant amounts of brominated indoles, dominated by tyrindoleninone and 6-bromoisatin (Westley et al., 2010). So purification of the most likely bioactive compounds (tyrindoleninone and 6-bromoisatin) is required to confirm the active factor(s), enhance the *in vivo* anticancer activity and potentially reduce the idiosyncratic toxicity associated with the crude extract (Westley et al., 2013). As some isatin derivatives have been shown to be cytotoxic on some normal human cells (Vine et al., 2007a), purification of 6-bromoisatin and use of the pure synthetic compound will help establish and potential side effects associated with this compound.

1.6 Preclinical studies for anticancer drug development

In vitro testing of the purified compounds on colorectal cancer cell lines will help to identify the most bioactive compound and the mode of cell death before commencing preclinical studies in animal models. Bioassay guided fractionation is a useful method

for screening the bioactivity of all compounds in the crude extract, identifying the most active components, as well as assessing the possible toxic effects (e.g. necrosis) corresponding with any compound or fraction.

1.6.1 Colon cancer cell lines

Colorectal epithelial cells are constantly exposed to mixtures of different compounds including carcinogens or protective compounds, which can counteract carcinogens effects (Patil and Jadhav, 2013). Epithelial cells can be isolated from colon tissue and used in primary culture for some studies, such as for testing the effects of tumor promoters on these cells, but they are not suitable for assessing the effect of chemopreventive compounds (Patil and Jadhav, 2013). For this aim, colon carcinoma cell lines have to be used (Patil and Jadhav, 2013). The HT-29 cell line was obtained from a Caucasian woman with the primary tumor of adenocarcinoma (Friedman et al., 1989). HT29 cell line is included in the National Cancer Institute 60 cell lines for *in vitro* screening (Shoemaker, 2006). The Caco2 cell line is derived from heterogeneous human epithelial colorectal adenocarcinoma cells, and was developed by Sloan-Kettering Institute for cancer research (Fogh, 1975).

1.6.2 Cell viability assays

Cell viability can be determined using metabolic cell proliferations assays, such as the tetrazolium salt, 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Tetrazolium salt is actively absorbed into cells and is reduced to yield a formazan product in living cells, from a mitochondrial dependent reaction (Cory et al., 1991). As formazan cannot pass through the cell membrane, it accumulates within the cell. By adding a suitable solvent (e.g. isopropanol) to the cells, formazan is

solubilized and liberated and can be measured colorimetrically (Mosmann, 1983). This ability of living cells to reduce MTT is considered a suitable indicator for cell viability because it depends on mitochondrial integrity and activity (Mosmann, 1983). The assay has been used in several studies to measure the viability of a variety of cell lines including HT-29 and Caco2 cells (Carmichael et al., 1988, Frade et al., 2007).

1.6.3 Apoptosis or programmed cell death versus necrosis

Apoptosis or programmed cell death is a particular mode of cell death that eliminates unwanted cells characterized by specific changes in nucleus and cytoplasm (Compton and Cidlowski, 1992, Hengartner, 2000). Apoptosis is a normal and vital cellular process involved in cell turnover, embryonic development, functioning of the immune system etc (Elmore, 2007). However, dysregulation of apoptosis is a factor in many human diseases, such as autoimmune disorders, neurodegenerative diseases, ischemic damage and many types of cancer (Fischer and Schulze-Osthoff, 2005, Elmore, 2007). In contrast, necrosis is an alternative process leading to cell death, usually caused by acute injury to the cell (Jin and El-Deiry, 2005a). In this kind of cell death, injury can destroy the membrane integrity, which leads to release of cytoplasmic constituents to the surrounding area (Jin and El-Deiry, 2005a). Unlike apoptosis, necrosis is a degenerative process which can trigger an inflammatory response *in vivo* (Columbano, 1995, Zong and Thompson, 2006).

The morphological changes in apoptosis can be identified by light microscopy (Häcker, 2000). For example, chromatin condensation and cell shrinkage are characteristic features in the early stages of apoptosis that are visible by light microscopy (Kerr et al., 1972). Histopathological studies on tissues using with

hematoxylin and eosin staining, can also reveal apoptotic cells under a light microscope, with single cells or a small cluster of cells with dense purple nuclear chromatin fragments and dark eosinophilic cytoplasm (Elmore, 2007). Therefore, light microscopy is a good way to visually confirm the presence of apoptotic cells.

Apoptosis or programmed cell death proceeds via a very complex mechanism, which involves an energy-dependent cascade (Fischer and Schulze-Osthoff, 2005, Elmore, 2007). Two main apoptotic pathways have been identified by researchers: the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway (Elmore, 2007) (Figure 1.3). Both of these pathways are linked with cysteine proteases or caspases (Igney and Krammer, 2002). There are ten main types of caspases that are categorized into initiator caspases (2, 8, 9, and 10), the effector caspases (3, 6, 7) and the inflammatory caspases (1, 4, 5) (Rai et al., 2005, Elmore, 2007). Activation of caspase 9 is involved in the intrinsic pathway that results from the mitochondrial release of cytochrome c. The initiator caspase 8 is associated with the extrinsic apoptosis pathway, that is activated by the cell surface death receptors and ligands (Nicholson, 1999, Elmore, 2007). These activated initiator caspases then cleave the effector pro-caspases to form the caspase effector enzymes (Nicholson, 1999, Elmore, 2007). However, the intrinsic and extrinsic pathways converge on the same terminal by the cleavage of caspase-3 (Figure 1.3) and this results in DNA fragmentation and formation of apoptotic bodies (Elmore, 2007). In addition to intrinsic and extrinsic pathways, there is a third pathway called perforin/granzyme pathway which is associated with cytotoxic T-cells (Figure 1.3) and can induce apoptosis via granzyme B or granzyme A (Martinvalet et al., 2005). Similar to intrinsic and extrinsic pathways, the granzyme B pathway converges on the caspase-3 terminal, whereas granzyme A

pathway activates caspase-independent apoptosis by single stranded DNA damage (Martinvalet et al., 2005).

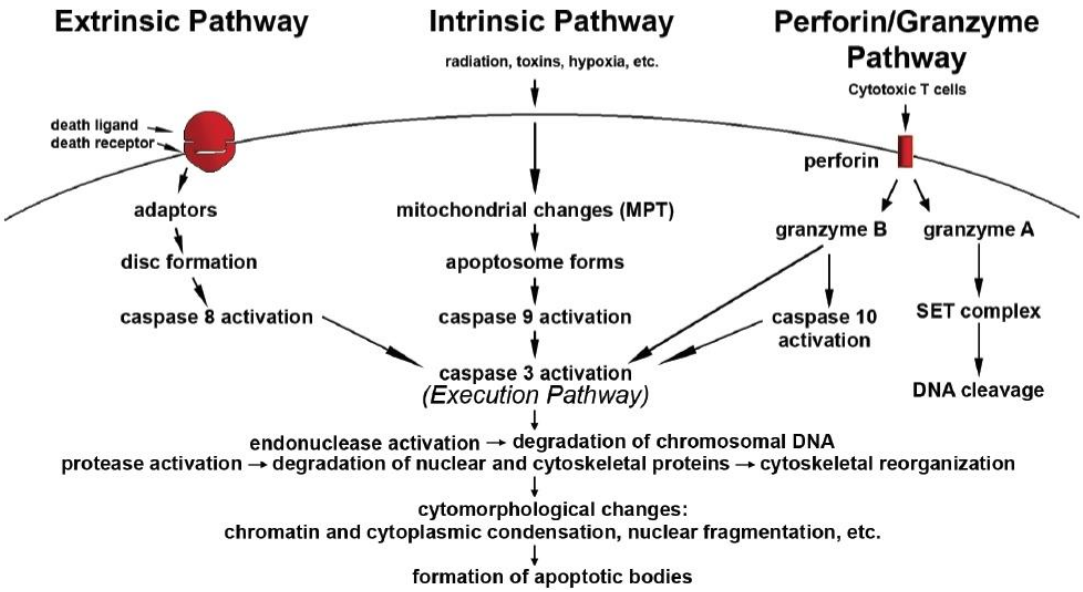

Figure 1.3 Three apoptosis mechanism including two main intrinsic and extrinsic pathways with the third perforin/granzyme pathway (Elmore, 2007).

1.6.4. Flow cytometry for detection of apoptosis and cell cycle analysis

Flow cytometry is a laser based technology for cell counting and sorting, protein engineering and biomarker detection that allows analysis of the chemical and physical characteristics of cells simultaneously (Nunez, 2001). Translocation of phosphatidylserine from the inside of the membrane to the external cell surface is one of the plasma membrane alterations in the cell surface that occur at the early stages of apoptosis (Vermes et al., 1995). This phosphatidylserine is detectable by flow cytometry by staining apoptotic cells using Annexin V (Ca2+ phospholipid binding protein), which has a high affinity to bind with phosphatidylserine. This translocation of the phosphatidylserine to the cell surface can also occur in necrotic cells, but the cell membrane in necrotic cells lose integrity and become leaky, while the cell membrane during the early stages of apoptosis remains intact (Vermes et al., 1995, Lecoeur et al., 2001). These two types of cell death can therefore be distinguished by using a dye exclusion test to identify the integrity of the cell membrane. Propidium iodide (PI) is a membrane impermeant dye that binds to double stranded DNA and is generally excluded from viable cells (Glander and Schaller, 1999). So PI used along with Annexin V can distinguish apoptotic cells (FITC+/PI-) from necrotic cells (FITC+/PI+). The intact cells (FITC-/PI-) remain unstained by Annexin V and PI (Vermes et al., 1995). The Annexin V assay is sensitive and offers the ability to detect early phases of apoptosis before cells lose their membrane integrity. It also allows to identify the relationship between the kinetics of apoptosis and cell cycle (Vermes et al., 1995).

Flow cytometry is also used to determine the distribution of the cells in the different stages of cell cycle (Darzynkiewicz et al., 2001). The cell cycle consists of a mitotic

(M) phase where the cell divides in two, and interphase which is a long period of cell development containing three phases: Gap 1 (G1), Synthesis (S) and Gap 2 (G2). In G1 the cell grows in size. In S phase the cell duplicates its DNA and in G2 phase the cells checks that all its DNA has been correctly copied (O'Connor, 2008). The cells can be stained by fluorescent dyes such as PI or 4',6-diamidino-2-phenylindole (DAPI) for staging the cells based on DNA content, but these dyes should be used in fixed cells or detergent-permeabilized cells. For live cells, the membrane-permeable stain, Hoechst 33242 can be used (Darzynkiewicz et al., 2001). Flow cytometry is useful to assess whether bioactive compounds arrest the cancer cells at a particular stage of the cell cycle. This can help us to understand more about the mechanism of action of the drug.

1.6.5. *In vivo* models for colon cancer prevention

In vitro studies can provide a lot of good information at the initial stages of research for drug development. However, there are some limitations for working with cells because the cells are treated outside the body system, which is not their normal environment. In pharmaceutical discovery programs, properties of a drug cannot be considered independent of the system that the drug needs to be tested in (Lipinski and Hopkins, 2004). Binding of the compound to isolated recombinant proteins or pharmacological toleration and detoxification of the agents by the liver are some examples that can affect the ultimate efficacy of the drugs (Lipinski and Hopkins, 2004). Therefore, *in vivo* studies are required in addition to *in vitro* screening, to investigate the overall and ultimate effects of the drug in the body system (Hodgson, 2001, Davis and Riley, 2004).

The two most common carcinogen-induced animal models that are widely used to study the anticancer activity of natural and synthetic compounds are the dimethyhydrazine (DMH) and azoxymethane (AOM) models. The genetic model, APCmin mice is also considered as a promising model for CRC preventive studies (Corpet and Pierre, 2005, Femia and Caderni, 2008, Perše and Cerar, 2010). Enhancing the acute apoptotic response to genotoxic carcinogens (AARGC) has been recently developed as a model for chemopreventative research (Hong et al., 1999, Reddy et al., 2000, Hu et al., 2002, Le Leu et al., 2002, Le Leu et al., 2003, Westley et al., 2010) with the aim of identifying the efficacy of natural products for reducing the incidence of CRC by inducing apoptosis of damaged colon cells. The alkylating genotoxic carcinogen AOM induces O^6-meG DNA-adduct formation (Hirose et al., 1996) and genetic mutation in distal colonic epithelium within 6-8 h (Hu et al., 2002). This model has been mainly used to test acute apoptotic response of dietary components, such as starches and oils (Hong et al., 1999, Le Leu et al., 2002, Le Leu et al., 2003). In a study by Westley et al. (2010) this model was also successfully used to investigate the chemopreventative effects of crude extract from *D. orbita* in four week mouse trial. Therefore, this model has been selected as a useful animal model due to its time-saving, cost effectiveness and reliability to test the purified bioactive compounds from Muricid extracts and/or the synthetic compounds for prevention of CRC.

1.6.6 Toxicity testing

Drug safety profiles are an important issue for new drug development and approval. *In vivo* safety can be investigated by toxicity testing through animal trials before shifting to clinical trials in humans (Nicholson et al., 2002). The liver is the most

important organ in the body that is influenced by drug toxicity due to its function of detoxifying the blood, such that many drugs accumulate in the liver. Histopathological analysis by staining with haematoxylin and eosin (H&E) under a light microscope can be used to indicate toxicity induced by cytotoxic or antimitotic drugs, which can cause liver damage such as micro and macrovesicular steatosis, congestion, haemorrhage, necrosis, etc (Greaves, 2007, Ferrell, 2010). The activities of liver enzymes, such as asparate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in the serum are also considered indicators for hepatotoxicity because in the injured or damaged liver cells, these enzymes leak higher than the normal amount, into the bloodstream (Hewawasam et al., 2004). Kidney function can also be measured by biochemical indicators, such as creatinine, total protein and urea concentrations in serum to evaluate drug toxicity in this organ (Brzoska et al., 2003).

The concentration of other serum biochemical parameters, such as sodium, potassium and the sodium/potassium ratio (Na/K) are useful for identifying whether the drug can upset the balance of these electrolytes in the serum. The balance of electrolytes is likely to be impacted if the drug has some side effects such as vomiting or diarrhea (Oliver et al., 1957, Gennari, 2002). Hematology factors such as red blood count, hemoglobin, hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) can be measured to identify whether the drug has any side effect on red blood cells or haemoglobin resulting in blood disorders, such as anemia (Pellock, 1987). White cell count including neutrophil, lymphocyte and monocyte numbers are factors of immune system that could indicate possible inflammatory responses or immune-suppressive

activities, and thus are useful indicators for further possible side effects of the drug (Veenman and Gavish, 2006).

1.7 Aims

This project aims to identify and characterize the bioactive factors in *D. orbita* extract for potential use in the treatment or prevention of colorectal cancer. Previous work has demonstrated that extracts from this snail inhibit the proliferation of cancer cell lines and testing in preliminary animal models support the efficacy of the crude mollusc extract for preventing colorectal cancer. This project will build upon the previous work by purifying the anticancer agents from the mollusc, with the hope of increasing the activity in a two week AOM-induced rodent model and testing for potential toxic side-effects. We hypothesize that by purifying the bioactive compounds and administering them to the mice by oral gavage, we will be able to reduce the toxic side effects and increase the efficacy of muricid extracts for the prevention of colon cancer. The specific objectives of this project are to:

1. Optimize methods for purification of the bioactive compounds tyrindoleninone and 6-bromoisatin on a larger scale to supply *in-vitro* assays and *in-vivo* preclinical rodent trials (Chapter 2)
2. Test the ability of antioxidants (Vitamin E and Vitamin A) to prevent the degradation of tyrindoleninone to 6-bromoisatin (Chapter 2)
3. Bioassay guided fractionation of crude extract and assessment of the *in vitro* cytotoxic effect of all fractions to identify the most bioactive compound against colon cancer cells (Caco-2 & HT29); (Chapter 3).

4. Determine whether the compounds effectively cause apoptosis or necrosis in colon cancer cells *in vitro* (Chapter 3)
5. Establish *in vivo* efficacy of the purified compounds in a two week AOM-induced rodent model for the prevention of colorectal cancer (Chapter 4)
6. *In vitro* and *in vivo* testing of synthetic 6-bromoisatin for comparison with the purified fraction (Chapter 5)
7. Histopathological evaluation of the liver after treatment with the purified and synthetic bioactive compounds and crude extract (Chapters 4 & 5)
8. Measure the haematological and biochemical factors (including liver enzymes) to identify any toxicity associated with these compounds in the blood and liver (Chapter 4 & 5)

1.8 Thesis structure

Chapter 2: Optimizing the purification of tyrindoleninone and 6-bromoisatin and testing the proliferative effect of Vitamin E stabilized tryindoleninone on HT29 cells

This is a method development chapter that underpins all subsequent chapters in the thesis. It outlines the optimization of a method for purifying tyrindoleninone and 6-bromoisatin from the organic extracts of *D. orbita*. It also describes some preliminary experiments aimed at testing whether the extracts and tyrindoleninone in particular can be stabilized with antioxidants Vit E and Vit A in the presence of oxygen. Tyrindoleninone was then tested *in vitro*, with and without Vit E, to identify whether Vit E can increase the effect of this compound in cell culture conditions.

Chapter 3: Purified brominated indole derivatives from *Dicathais orbita* induce apoptosis and cell cycle arrest in colorectal cancer cell lines

The chapter is presented as a manuscript that has been provisionally accepted in the journal Marine Drugs. It presents the results of the bioassay guided fractionation and *in vitro* cytotoxicity of fractions from a chloroform extract of *D. orbita* egg masses. The most bioactive fractions were then analysed by LC/MS, GC/MS and NMR to confirm the identity and purity of the compounds. The purified and semi-purified bioactive compounds were then tested for their anti-proliferation and apoptotic effects, in comparison to the crude extract. The effect of the most bioactive compound on the cell cycle of HT29 cells was also assessed by flow cytometric analysis.

Chapter 4: Brominated indoles from a marine mollusc extract prevent early stage colon cancer formation *in vivo*

This chapter presents the preclinical two week *in vivo* testing to demonstrate the efficacy of the crude *D. orbita* extract and purified brominated indole derivatives for enhancing AARGC and preventing or alleviating tumor formation in the colon. The possible toxic effects of the purified compounds were also assessed by histopathological evaluation of the liver, as well as the hematologic and biochemical measurements of the blood.

Chapter 5: Bromoisatin found in muricid mollusc extracts inhibits colon cancer cell proliferation and induces apoptosis, preventing early stage tumor formation in a colorectal cancer rodent model

In this chapter synthetic 6-bromoisatin has been tested for its anti-proliferative and apoptotic effects on HT29 cells *in vitro*. The cell morphology of apoptotic cells was also studied by light microscopy. The synthetic compound was then tested using the *in vivo* rodent model for colon cancer prevention, to confirm whether this compound alone could enhance AARGC. Measurements of hematologic and biochemical factors (including liver enzymes) in the blood were undertaken to determine any potential side effects associated with the daily oral ingestion of 6-bromoisatin for 2 weeks.

Chapter 6: Final discussion and conclusion

This chapter provides a synthesis of the main outcomes from all the experiments undertaken in this thesis, and provides recommendations for future research.

2. Optimizing the purification of tyrindoleninone and 6-bromoisatin and testing the proliferative effect of Vitamin E stabilized tryindoleninone on HT29 cells

2.1 Introduction

The Australian whelk, *Dicathais orbita* (Muricidae), is a novel source of brominated indoles with bioactive properties (Benkendorff, 2013). Extracts from the egg masses contain compounds with demonstrated anticancer properties against a range of cell lines (Vine et al., 2007a, Benkendorff et al., 2011, Edwards et al., 2012, Benkendorff, 2013). Crude extracts from the hypobranchial glands of this mollusc have been tested *in vivo* using short-term rodent models for toxicity and efficacy in the prevention of colorectal cancer (Westley et al., 2010). The hypobranchial glands were used in this *in vivo* study due to the difficulty in obtaining enough egg masses to support an *in vivo* study and chemical analysis revealed a similar composition of brominated indoles. However, the percent composition of these two sources has never been directly compared using the same extraction and analysis procedures. A further important limitation of the preclinical models was the lack of purification in the extracts, such that the bioactive compounds responsible for initiating apoptosis in the DNA damaged colon cells *in vivo* remain unidentified.

Previous methods to purify the bioactive brominated indoles from *D. orbita* have mostly focused on silica chromatography. Benkendorff *et al.* (2000) used a flash silica column with 10% methanol in dichloromethane (DCM) followed by radial chromatography on a chromatotron using 9:1 light petroleum to DCM, followed by 100% DCM and then 10% methanol in DCM. However, several fractions contained a mixture of compounds and repeated separation on the chromatotron was required to isolate tyrindoleninone and tyrindolinone (Benkendorff, personal communication). In a detailed honours research project, Bogdanovic (2007) attempted to optimise the separation of compounds from *D. orbita* egg mass extracts using HPLC. The final

separation method used a gradient of toluene, hexane and acetone, but not all compounds eluted from the column. Tyrindoleninone was only recovered in low yields in a mixture with tyrindolinone using a normal phase preparative silica column (Bogdanovic, 2007). Eichinger (2008) then attempted to purify tyrindoleninone by reverse phase HPLC on a preparative hydro - C_{18} column using a gradient of acetonitrile and water, with and without 0.1% formic acid. Despite obtaining a pure peak for tyrindoleninone on the analytical reverse phase column, after scaling up to the preparative column this pure peak was not recovered and tyrindoleninone only eluted in a mixture that included tryindoxyl, tyrindolinone and 6-bromoistain, with a yield of just 0.77% of the original extract (Eichinger, 2008). Edwards *et al*. (2012) then returned to a flash silica column chromatography method to purify the bioactive compounds from *D. orbita* egg extracts. Using a solvent system with increasing polarity from 100% DCM, 5% methanol in DCM and 10% methanol in DCM, semi-purified fractions of tyrindoleninone and 6-bromoistain were obtained with yields of 3.22% and 2.18% respectively. Minor trace contaminants (<1%) were detected by LC/MS for both of these semi-purified fractions (Edwards et al., 2012). Consequently, further optimization of the separation procedure is required to confirm the activity of the main brominated compounds and to obtain higher yields as required for *in vivo* testing.

Semi-purified fractions containing tyrindoleninone from *D. orbita* show anticancer effects against a range of cancer cell lines *in vitro* (Benkendorff et al., 2011, Edwards et al., 2012), However, this compound can be easily degraded to the other compounds, such as tyriverdin and 6-bromoisatin in the presence of oxygen and light (Figure 1.2), (Westley and Benkendorff, 2008). This chemical degradation has also been noted

when the extract from *D. orbita* is exposed to simulated gastric fluid (Westley et al., 2010). The use of antioxidants such as Vitamin E and A, which have been added into a range of food products (Sies and Stahl, 1995) may help prevent the oxidation of tyrindoleninone. These natural antioxidants have been also used for the stabilization of some foods (Ltiliger, 1989).

The aim of this chapter was to optimize the purification of the bioactive compounds tyrindoleninone and 6-bromoisatin from the crude extracts of egg masses and hypobranchial glands and test the antioxidant effects of Vitamin E and Vitamin A to identify whether these antioxidants can prevent degradation of tyrindoleninone. Furthermore, the synergistic effect of the antioxidants with tyrindoleninone will be investigated *in vitro* to ascertain possible effects on the anticancer activity.

2.2 Material and methods

2.2.1 Extraction and separation of compounds by thin-layer chromatography (TLC)

D. orbita were collected from a brood stock held in the recirculating aquarium in the School of Biological Sciences, Flinders University, South Australia (originally collected from intertidal reefs in the coast south of Adelaide) and approximately 10g of dissected hypobranchial glands was soaked in chloroform and methanol (1:1, v/v), filtered then transferred to a separating funnel. The chloroform layer was separated and dried on a rotary evaporator according to the method previously described by Westley et al. (2010). Thin layer chromatography (TLC) aluminium sheets 20 × 20 cm silica gel 60 F_{254} (Merck) were used to optimise the separation of the main coloured compounds in the extract. TLC plates were spotted with the crude extract dissolved in a small amount of dichloromethane. Twelve different mixtures of solvents were

trialled, including hexane (HX), dichloromethane (DCM) and methanol, with a gradient method from a low polarity to a high polarity: 1) 100% HX; 2) DCM/ HX (1:9 v/v); 3) DCM/ HX (1:8 v/v); 4) DCM/ HX (1:7 v/v); 5) DCM/ HX (1:6 v/v); 6) DCM/ HX (1:5 v/v); 7) DCM/ HX (1:4 v/v); 8) DCM/ HX (1:3 v/v); 9) DCM/ HX (1:2 v/v); 10) DCM/ HX (1:1 v/v); 11) 100% DCM; 12) Methanol/ DCM (1:9 v/v).

Chloroform extracts from the egg masses (20 g) of *D. orbita* were also prepared according to the above method. The crude chloroform extracts from the egg masses and hypobranchial glands were analysed using liquid chromatography/mass spectrometry (LC/MS), according to the methods of Westley and Benkendorff (2008) to enable direct comparison of the extract composition.

2.2.2 Flash chromatography and chemical analysis

Based on the TLC results, a new flash column chromatography method was developed, focusing on the separation of tyrindolenine, tyrindolinone and 6-bromoisatin. The chloroform extract (250 mg) from the hypobranchial glands was loaded on a flash column (24 mm diameter) containing 20 g silica gel (Sigma Aldrich, 100 mesh) mixed with HX as a stationary phase and pressurized with nitrogen gas. In the first step, 100 mL HX was used to wash any fat, such as fatty acids/esters out of the column. Then, 50 mL DCM/ HX (4:1, v/v/) was used to purify the red/orange layer (tyrindoleninone) as fraction 1. The yellow layer (tyrindolinone) following just after the red/orange layer was purified with 200 mL DCM/ HX (3:1, v/v/) as fraction 2. The column was then washed with 200 mL DCM before purifying the last yellow layer (6-bromoisatin) with 15 mL DCM/ methanol (9:1, v/v) as fraction 3. The solvent were evaporated from the fractions using rotary evaporation at 40°C. To confirm the

identity and purity of the compounds, all three fractions were analysed using liquid chromatography (Waters Alliance) coupled with Quattro micro™ mass spectrometer (LC/MS) with a Hydro-RP C18 column (250 × 4.6 mm×4 µm) and parallel UV/Vis diode-array detection at 300 and 600 nm according to the method established by Westley and Benkendorff (2008). This method was repeated using extracts from the egg masses for Chapter 3.

2.2.3 Antioxidant assay

In order to prevent tyrindoleninone degradation, an assay was developed using two antioxidants Vitamin E (α-Tocopherol synthetic, ≥96% HPLC; Sigma-Aldrich) and Vitamin A (Retinol synthetic, ≥95% HPLC; Sigma-Aldrich) and tested their effects on the semi-purified fraction containing tyrindoleninone and tyrindolinone. To do this, Vitamin E (final concentration 0.1%) or Vitamin A (final concentrations of 5%, 1% and 0.1%) was dissolved along with the same amount of semi-purified fraction (2 mg) in dimethylsulphoxide (DMSO; final concentration of 2%) before dissolving all of them in 980 µl milli-Q water. For example, for preparing a solution containing 0.1% Vitamin E, 2 mg Vitamin E and 2 mg of the semi-purified fraction was dissolved in 20µl DMSO, then the DMSO containing these compounds was dissolved in 980 µl milli-Q water. As Vitamin A was more soluble in water than Vitamin E, higher concentrations of Vitamin A were tested. Vitamin E at greater than 0.1 % concentration did not dissolve in water using 2% DMSO. However, the effects of the high concentrations of Vitamin E (5%), could be tested by dissolving it first in 1 mL of 100% DMSO containing semi-purified fraction (2 mg). Two positive controls were added to this assay. In one, the semi-purified fraction (2 mg) was dissolved in DMSO (final concentration of 2%) and then solubilized in 1 mL milli-Q water without adding

any antioxidant. In the other one, the semi-purified fraction (2 mg) was dissolved in 1 mL of 100% DMSO. All of these extracts were left in a fume hood, with exposure to atmospheric oxygen, overnight and then re-analyzed by LC/MS to identify any change in composition of the compounds. A sample of the semi-purified fraction without the addition of antioxidant, or exposure to oxygen, was also analysed by LC/MS as a negative control.

2.2.4 Cell viability assay

The most protective antioxidant (1% Vitamin E) on tyrindoleninone from the antioxidant assay was selected to investigate whether Vitamin E can influence the anti-proliferative effects of tyrindoleninone on HT29 and Caco2 cells by reducing its degradation. To measure the viability of the cells, the MTT assay (Mosmann, 1983) was performed with and without addition of 0.1 % Vitamin E. The semi-purified extract containing both tyrindoleninone and tyrindolinone was used as the treatment.

HT29 and Caco2 cells were grown to 70% confluence and seeded into 96-well plates (Costar®) (2×10^4 cells in 100 µl media/ well). The cells were incubated for 48 h before treatment. Semi-purified tyrindoleninone and tyrindolinone was dissolved in DMSO (final concentration of 2%), diluted in media and added to the cell culture. The cells were treated for 12 h with a range of 0.01 mg/mL to 0.5 mg/mL semi-purified compounds with and without the addition of the 0.1% Vitamin E to determine IC_{50} for cell viability. This experiment was repeated on three separate occasions and each treatment was performed in triplicate (n = 3).

2.3 Results and discussion

2.3.1 TLC separation

The different solvent systems tested in TLC varied in their ability to separate the coloured compounds in the chloroform extract from the hypobranchial glands. No compounds were separated from the crude extract using the least polar solvent systems, from 100% HX, up until HX/ DCM (4:1, v/v), which only just separated the first spot with R_f value of 0.02 (Figure 2.1 a). By increasing the polarity of the solvent using HX/ DCM (3:1, v/v), two spots were separated and the first spot migrated further to the R_f value of 0.07 (Figure 2.1 b). Improved separation of these two compounds was observed with the use of HX/ DCM (1:1, v/v) (Figure 2.1 c). With this solvent system, compound 1 with red/orange colour under visible light was detected at R_f = 0.15. The second compound, with a yellow colour, was observed at R_f value = 0.07 (Figure 2.1 c). The third compound (yellow colour) was separated more effectively using 100% DCM (R_f = 0.06; Figure 2.1 d) and went up to R_f value of 0.7 by adding 10% methanol to DCM (Figure 2.1 e).

Previous research has revealed that the dominant compounds in *D. orbita* egg mass extracts are coloured and can be separated by TLC (Benkendorff et al., 2000). For example, tyrindoleninone (orange colour) with R_f = 0.5 and tyrindolinone (yellow colour) with R_f = 0.4 have been separated by TLC (Silicagel 60) using light petroleum/ DCM (1:1, v/v) as the solvent (Benkendorff et al., 2000). Comparison of the compound colours and R_f values in Benkendorff et al. (2000), suggests that the compound 1 in our TLC analysis matches tyrindolinone and compound 2 matches with tyrindoleninone. Benkendorff (2013) reports that these two compounds have a very similar LogP value of 2.999 for tyrindolinone and 2.889 for tyrindoleninone which

explains why they are difficult to separate based on polarity. The higher R_f value of tyrindoleninone and tyrindolinone with light petroleum/ DCM (1:1, v/v) in Benkendorff et al. (2000), in comparison to the R_f value of these compounds using HX/ DCM (1:1, v/v) in our study would be due to the lower polarity of HX compared to light petroleum.

In addition to these compounds, Benkendorff et al. (2000) also detected a yellow polar compound corresponding to 6-bromoisatin using TLC with light petroleum/ DCM (R_f = 0.05). This compound was not effectively separated using HX/ DCM (Figure 2.1 a-c), but just emerged using 100% DCM and was clearly separated using 10% methanol in DCM with R_f value of 0.7 (Figure 2.1 e). The R_f value for 6-bromoistain was confirmed at R_f 0.7 using 10% methanol in DCM, in comparison to a synthetic standard for 6-bromoistatin (TCI AMERICA; purity of >97.0% GC); (Figure 2.1 f). Compound 3 in our TLC was also consistent with other reports of 6-bromoisatin, that has been previously separated from the hypobranchial glands of muricids (Baker, 1974, Clark and Cooksey, 1997). 6-bromoisatin is a much more polar compound with a Log P of 1.615 (Benkendorff, 2013), which is consistent with its good separation from the other brominated compounds in the *D. orbita* extract.

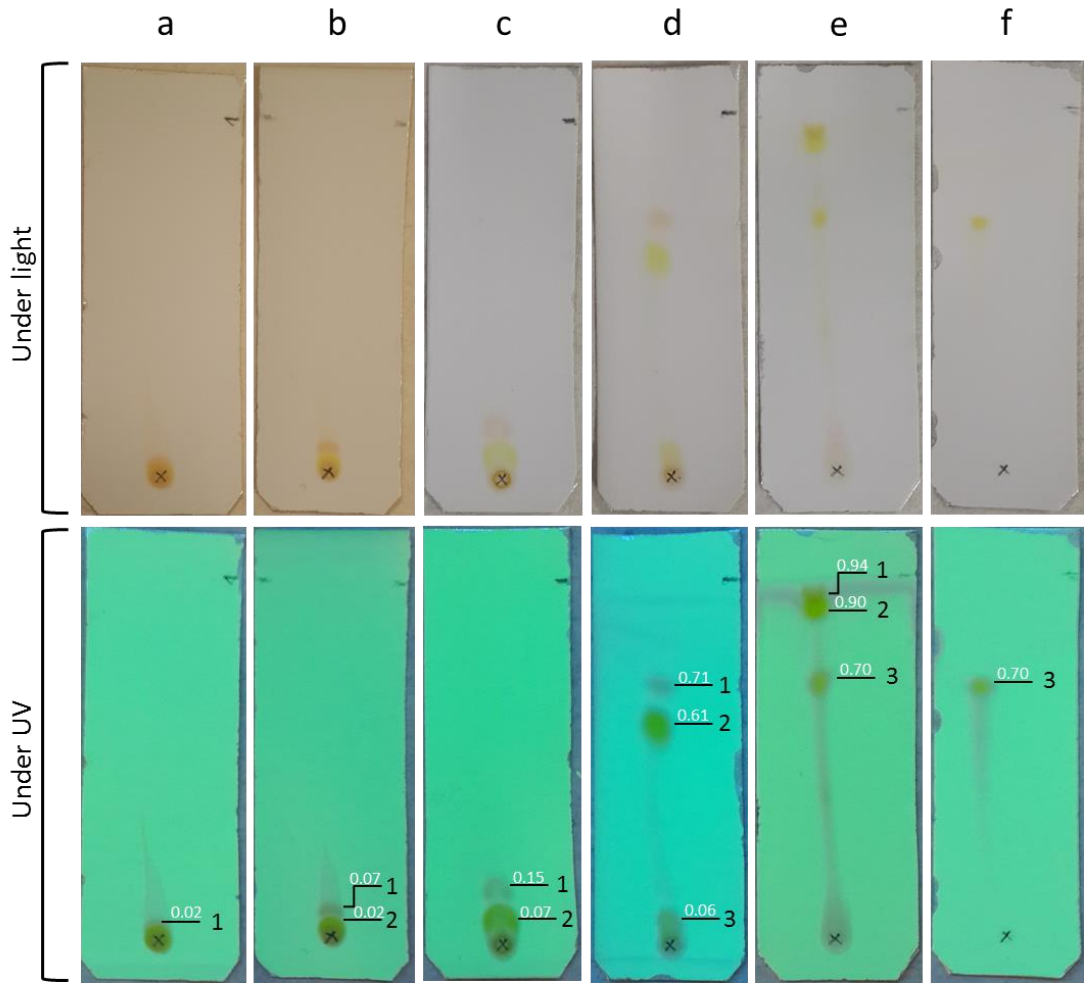

Figure 2.1 Thin layer chromatography of the crude extract from the hypobranchial gland of *Dicathais orbita* using different solvent systems from low to high polarity: a) hexane/ dichloromethane (4:1, v/v); b) hexane/ dichloromethane (3:1, v/v); c) hexane/ dichloromethane (1:1, v/v); d) 100% dichloromethane; e) dichloromethane/ methanol (9:1, v/v). Synthetic 6-bromoisatin was also tested as a control: f) dichloromethane/ methanol (9:1, v/v). Three visible and colourful compounds were visualised under the light and UV: 1= tyrindoleninone; 2= tyrindolinone and 3= 6-bromoisatin. The R_f value of the compounds in different solvent have been calculated as the proportion of the distance moved from the baseline, relative to the solvent front.

2.3.2 LC/MS analysis

Based on the result from the TLC analyses, the same solvent systems were used to isolate tyrindoleninone, tyrindolinone and 6-bromoisatin from the hypobranchial gland extract by flash column chromatography. After evaporating the solvents from the collected fractions, the yields from 250 mg crude extract were 4.1 mg from fraction 1 (1.64%), 28.5 mg from fraction 2 (11.4%) and 23 mg from fraction 3 (9.2%). LC/MS analysis of the silica column fractions confirmed the identity of the compounds predicted by TLC. The first fraction with an orange colour revealed a single dominant peak at t_R 11.18 min (Figure 2.2 a), with a corresponding molecular weight for tyrindoleninone, with major ions at *m/z* 255, 257. The second fraction with yellow colour revealed two dominant peaks corresponding to tyrindoleninone at t_R 11.18 min and tyrindolinone at t_R 9.46 min (Figure 2.2 b), with the major ions in ESI-MS at *m/z* 302, 304. The last fraction showed a dominant peak at 6.4 min attributed to the molecular mass of 6-bromoisatin, with major ions in ESI-MS at *m/z* 224, 226 (Figure 2.2 c) and several other minor peaks. These results are consistent with the LC/MS results reported by Edwards *et al* (2012) for tyrindoleninone (t_R 11.32 min) and 6-bromoisatin (t_R 6.45 min). Separation of the crude extract of the egg mass using the same silica chromatography method produced the same results for the purification and identification of tyrindoleninone, tyrindolinone and 6-bromoistain (Chapter 3).

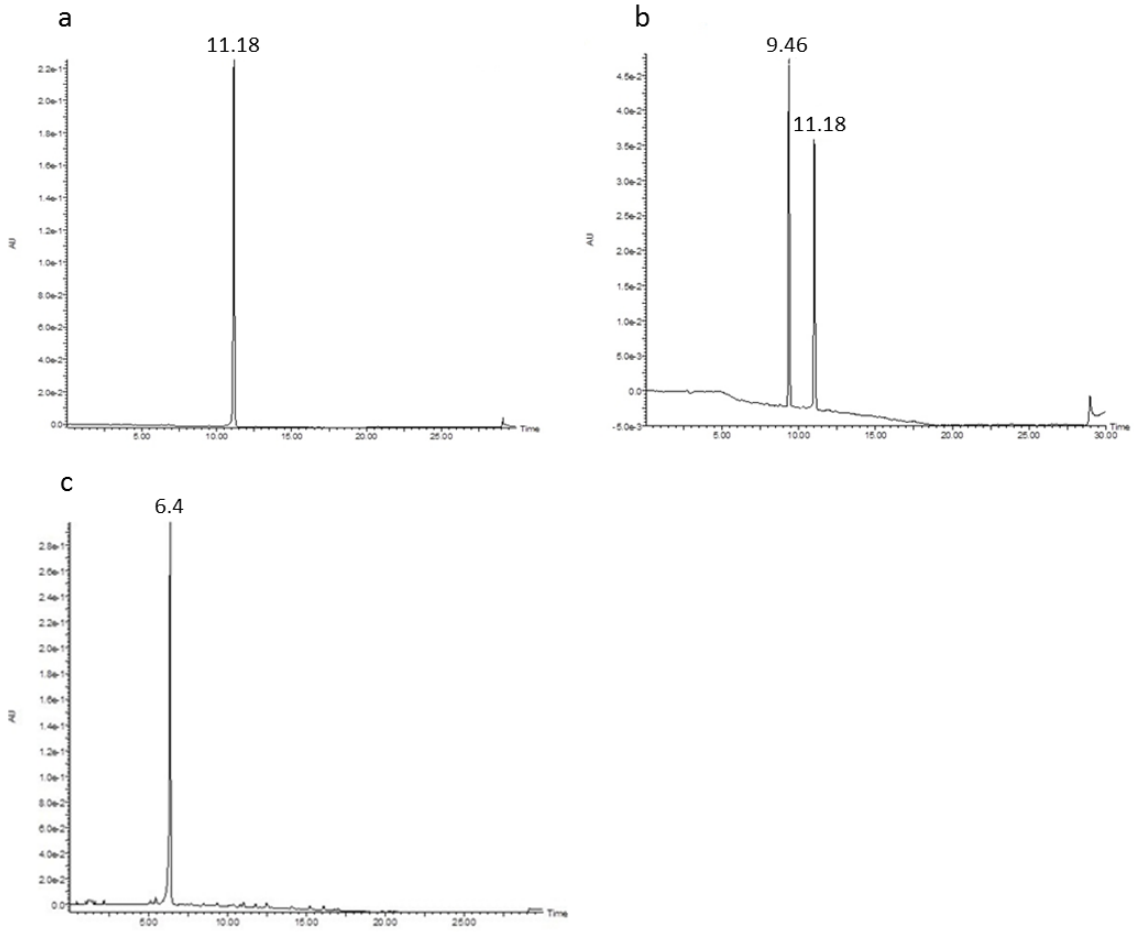

Figure 2.2 Liquid chromatography- mass spectrometry (UV/Vis diode-array detection at 300 and 600 nm) analysis of purified/semi-purified fraction from hypobranchial gland extract of *D. orbita*: a) Fraction 1 (tyrindoleninone); b) Fraction 2 (tyrindoleninone and tyrindolinone); c) fraction 3 (6-bromoisatin).

LCMS analysis of the crude chloroform extract from hypobranchial gland and the egg mass showed the same dominate compounds with a similar percent of composition for the brominated compounds (Table 2.1). A slightly higher proportion of tyrindoxyl sulphate in the egg masses, relative to the hypobranchial gland could be due to higher concentrations of arylsulphatase in the hypobranchial gland (Baker and Sutherland, 1968, Westley and Benkendorff, 2008). The higher concentration of tyrindoleninone in the egg mass extract relative to 6-bromoisatin and tyriverdin in the hypobranchical gland could also indicate more oxidation in the hypobranchial gland extracts. Nevertheless, both of these tissues provide a good source of the main brominated compounds previously reported to have anticancer activity (Vine et al., 2007a, Edwards et al., 2012). The major compounds in our extracts are consistent with previous reports of these compounds in *D. orbita* extracts by Benkendorff et al. (2011).

Table 2.1 Retention time and mass values of major compounds in the crude chloroform extract from the hypobranchial gland (HG) and the egg mass (EM) of *Dicathais orbita* by LCMS analysis. Percent composition of the major compounds was calculated from the sum of integrated peak area from all compounds in the extracts.

Major compounds	**Retention time** (min)	**Mass values** (*m/z*)	**Percent composition**	
			HG	EM
6-bromoisatin	6.4	224, 226	33.4	29.7
Tyrindolinone	9.46	302, 304	25.1	19.1
Tyrindoleninone	11.8	255, 257	21.3	26.7
Tyrindoxyl sulphate	8.58	336, 338	5.8	12.5
Tyriverdin	11.90	511, 513, 515	8.9	4.5
Unknown			5.5	7.5

2.3.3 Antioxidant effects on extract stability

The LCMS results from the antioxidant study are shown in Figure 2.3 and the percent composition of the compounds from the chromatograms have been summarised in Table 2.2. Exposure of the semi-purified tyrindoleninone and tyrindolinone to oxygen, without adding any antioxidant, results in a 50% reduction in tyrindolinone concentration after 12 h (Figure 2.3 a & b), as it is transformed to tyrindoleninone and 6-bromoisatin. By adding 0.1% Vitamin A, conversion of tyrindolinone to tyridoleninone was reduced (Figure 2.3 c), but tyrindolinone was still converted to 6-bromoisatin, with up to 20% increase compared to the untreated control (Table 2.2). With the addition of more Vitamin A (1% and 5%), tyridoleninone was stabilised,

whereas tyrindoleninone started to oxidise to 6-bromoisatin (Figure 2.3 d & e). In contrast, Vitamin E was found to stabilize tyrindoleninone more than tyrindolinone (Figure 2.3 f & g). For example, by adding 0.1 % Vitamin E to the semi-purified extract, tyrindoleninone was stabilized and the production of 6-bromoisatin was reduced by 6.8%, in comparison with 24.1% 6-bromoisatin in the control positive (Table 2.2). So Vitamin E with the concentration of 0.1% was found the most protective antioxidant to inhibit the degradation of tyrindoleninone.

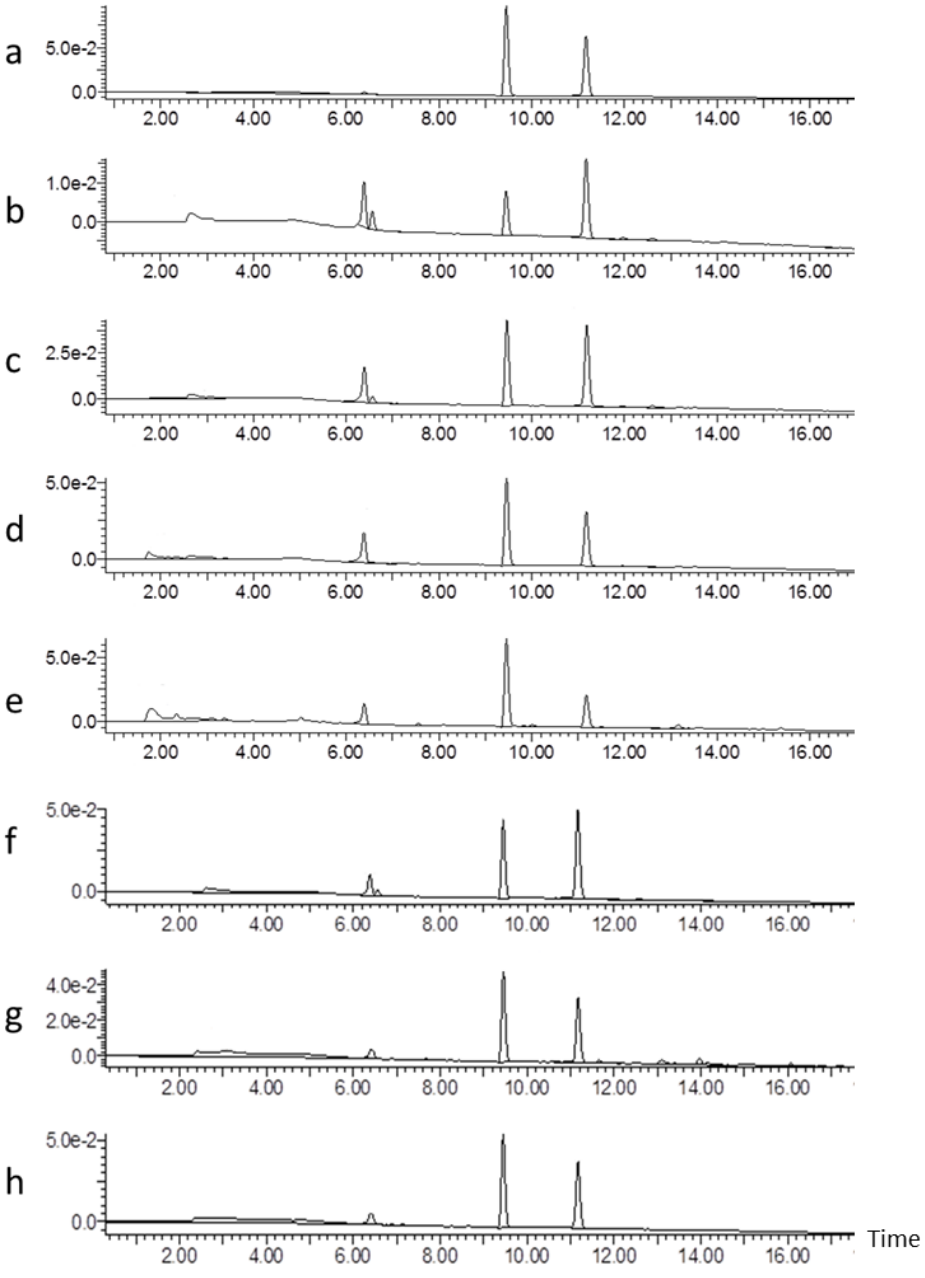

Figure 2.3. LCMS analysis of the semi-purified tyrindoleninone and tyrindolinone from *D. orbita* after 12 h oxygen exposure in the presence of different concentrations of Vitamin A and Vitamin E dissolved in DMSO: a) Untreated control in 2% DMSO); b) Positive control in 2% DMSO; c) 0.1 % Vitamin A in 2% DMSO; d) 1% Vitamin A in 2% DMSO; e) 5% Vitamin A in 2% DMSO; f) 0.1% Vitamin E in 2% DMSO; g) 5% Vitamin E in 100% DMSO; h) Positive Control in 100% DMSO.

Table 2.2 Percent composition (relative to the sum of brominated compounds) of semi-purified tyridoleninone and tyrindolinone from *D. orbita* after exposure to oxygen in the presence of antioxidants Vitamin A and Vitamin E dissolved in DMSO.

Sample	**DMSO Conc.**	**Oxygen exposure**	**6-bromoisatin**	**Tyrindolinone**	**Tyrindoleninone**
Retention time			6.4 min	9.46 min	11.18 min
Untreated control	2%	No	0	57.4	42.6
Positive control	2%	Yes	24.1	25.4	50.5
0.1 % Vitamin A	2%	Yes	20.5	38.2	41.3
1% Vitamin A	2%	Yes	19.4	47.3	33.3
5% Vitamin A	2%	Yes	15.9	59.6	24.5
0.1% Vitamin E	2%	Yes	6.8	39.9	53.3
5% Vitamin E	100%	Yes	7.7	51.2	41.1
Positive Control	100%	Yes	8.4	51.0	40.6

2.3.4 Synergistic effects of Vitamin E on cell viability

MTT assays showed that adding 0.1% Vitamin E to the DMSO control and semi-purified fraction containing tyrindoleninone and tyrindolinone did not increase the cytotoxic effect of tyrindoleninone. Conversely, 0.1% Vit E significantly increased the cell viability of both HT29 and Caco2 cells (Figure 2.4). This increase of cell proliferation in the presence of Vit E in DMSO, relative to the DMSO control without Vit E, was more prevalent in Caco2 cells ($p= 0.002$) than HT29 cells ($p= 0.04$). In treatment groups, the IC50 for Caco2 cells treated with semi-purified tyrindoleninone and tyrindolinone was 0.035 mg/mL without Vit E, while in combination with 0.1% Vitamin E, the IC50 increased to >0.5 mg/L (Figure 2.4 b). Similarly, in HT29 cells, the semi-purified extract decreased cell viability with an IC50 = 0.08 mg/mL in the absence of Vit E, while in the presence of 0.1% Vitamin E, the IC50 increased to 0.27

mg/mL (Figure 2.4 a). This suggests a potential protective effect of Vitamin E on colon cancer cell lines against tyrindoleninone or tyrindolinone.

Although, the negligible effect of Vitamin E has been shown on the proliferation of some cells such as the human fibroblast (WI-38) cell line (Packer and Smith, 1977), some other studies have shown different effects of Vitamin E on cell proliferation depending on cell line. For example, Vitamin E inhibited the proliferation of mammary cancer cells (Sylvester and Shah, 2005) and human mastocytoma cell line (HMC-1); (Kempná et al., 2004). In contrast, the proliferative effect of Vitamin E (α-Tocopherol) has been shown on oral carcinoma cells (Odukoya et al., 1986). In our study, Vitamin E itself increased the proliferation of both HT29 and Caco2 cells suggesting a proliferative effect of Vitamin E on these colorectal cancer cell lines. On the other hand, our studies also showed the protective effect of Vitamin E against cytotoxic effect of tyrindoleninone in both cells. This effect of Vitamin E in this study is consistent with a study by Kuroda (1975) that showed the protective effect of Vitamin E from the cytotoxic effect of bisulfite on cervical carcinoma (HeLa) cells. Bisulfite with a concentration of 10^{-5}M had a moderate cytotoxic effect on HeLa cells by reducing colony-forming activity of the cells to one-third of untreated control, while addition of Vitamin E (10^{-6}M) resulted in almost complete recovery of the colony-forming activity of the cells treated with bisulfite compared to control (Kuroda, 1975). Consequently, Vitamin E shows complex interactions with cells and has the potential to mask the effects of anticancer agents when used in *in vitro* screening assays.

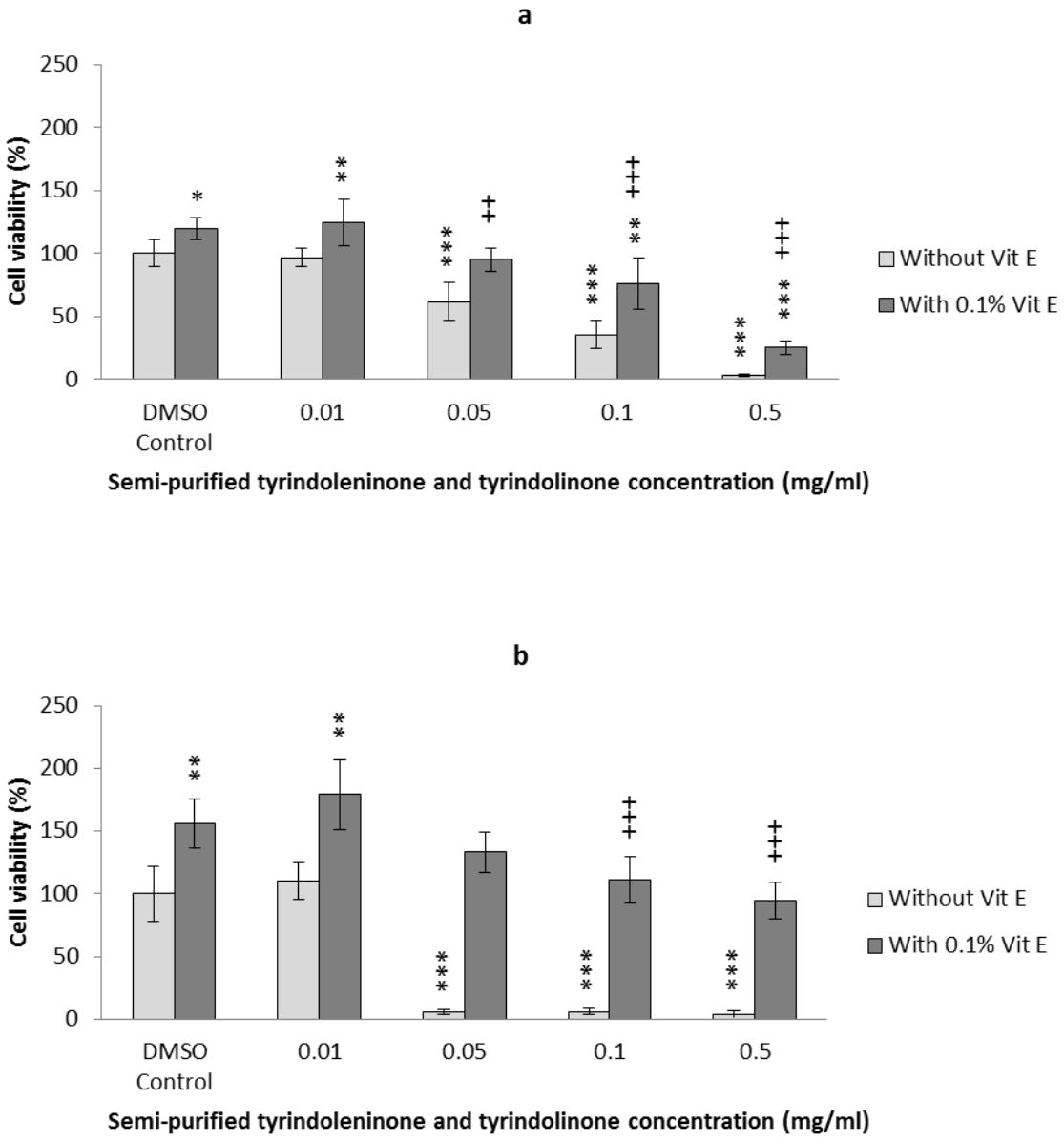

Figure 2.4 Cell viability of HT29 cells (a) and Caco2 cells (b) treated with different concentrations of semi-purified fraction containing tyrindoleninone and tyrindolinone (0.01 to 0.5 mg/mL) with and without the presence of 0.1% Vitamin E. Significant difference between each group and the DMSO control (without Vit E) are shown as $p \leq 0.05$ (*); $p \leq 0.01$ (**) and $p \leq 0.001$ (***). Significant difference between treatment groups (with 0.1% Vit E) and DMSO control (with 0.1% Vit E) are shown as $p \leq 0.01$ (++) and $p \leq 0.001$ (+++).

2.4 Conclusion

Comparison of the hypobranchial gland and egg mass extracts from *D. orbita* reveals that these tissues are equally a good source of bioactive brominated indoles. Testing the different concentrations of solvents by TLC, in order to separate the bioactive compounds tyrindoleninone and 6-bromoisatin, facilitated the development of an appropriate solvent system for purification by flash silica chromatography. LC/MS analysis confirmed the relative purity of the isolated compounds. The antioxidant Vitamin E was found to help stabilise tyrindoleninone in the semi-purified extract, while Vitamin A showed more protective effects on tyrindolinone. Therefore, Vitamin E was selected to test for potential synergistic effects on the bioactivity of the fraction containing tyrindoleninone. The cell viability results showed that Vitamin E actually reduced, rather than increased the anti-proliferative effect of the semi-purified tyrindoleninone and tyrindolinone on colorectal cancer cell lines.

The results of this Chapter provide a basis for the methodological approach used in subsequent chapters of this thesis. In Chapter 3, extracts from the egg masses have been fractionated and tested for anticancer activity using the same silica chromatography separation method. Bioassay guided fractionation was done on the egg masses in Chapter 3 to facilitate comparison and build on previous studies that have been undertaken on the *in vitro* anticancer activity of egg mass extracts from *D. orbita* (Benkendorff et al., 2011). Vitamin E was not used in these *in vitro* assays due to the observed interference with the anti-proliferative effects of tyrindoleinone/tyrindolinone. In Chapter 4, similar to the *in vivo* study by Westley et al. (2010), we used the hypobranchial glands for extraction because insufficient egg masses were available to support the amount of extract required for two weeks of daily

oral gavage in 90 mice. However, the same purification method was used for tyrindolinone and 6-bromoisatin, thus providing comparable purified fractions for *in vitro* and *in vivo* testing. A small amount of Vitamin E (0.02%) was added to the oil for storage of the extracts at room temperature prior to oral gavage. This was deemed necessary to stabilise the extract and tyrindoleninone fraction because large batches of the extract had to be prepared simultaneously and then stored to enable consistency in the sample used over the two week experimental trial. In this case stabilisation of the compounds prior to use was deemed more important that any possible reduction in the anti-proliferative effects, which may or may not occur in an animal model. The *in vivo* model used was directed towards prevention of colon cancer and was directed toward detecting apoptosis in the DNA damaged cells, although cell proliferation was also measured.

3. Purified brominated indole derivatives from *Dicathais orbita* induce apoptosis and cell cycle arrest in colorectal cancer cell lines

This chapter has been published as a research paper in *Marine Drugs* on 11 October 2013.

3.1 Abstract

Dicathais orbita is a large Australian marine gastropod known to produce bioactive compounds with anticancer properties. In this research, we used bioassay guided fractionation from the egg mass extract of *D. orbita* using flash column chromatography and identified fractions containing tyrindoleninone and 6-bromoisatin as the most active against colon cancer cells HT29 and Caco-2. Liquid chromatography coupled with mass spectrometry (LCMS) and ^{1}H NMR were used to characterize the purity and chemical composition of the isolated compounds. An MTT assay was used to determine effects on cell viability. Necrosis and apoptosis induction using caspase/LDH assay and flow cytometry (PI/Annexin-V) and cell cycle analysis were also investigated. Our results show that semi-purified 6-bromoisatin had the highest anti-cancer activity by inhibiting cell viability (IC_{50} = ~100 µM) and increasing caspase 3/7 activity in both of the cell lines at low concentration. The fraction containing 6-bromoisatin induced 77.6% apoptosis and arrested 25.7% of the cells in G2/M phase of cell cycle in HT29 cells. Tyrindoleninone was less potent but significantly decreased the viability of HT29 cells at IC_{50} = 390 µM and induced apoptosis at 195 µM by increasing caspase 3/7 activity in these cells. This research will facilitate the development of these molluscan natural products as novel complementary medicines for colorectal cancer.

3.2 Introduction

Colorectal cancer (CRC) is the third most diagnosed cancer worldwide (Jemal et al., 2011) with an incidence of 1.2 million new cases (9.7% of all cancers excluding non-melanoma skin cancers) and 608,000 deaths in 2008 (Ferlay et al., 2010). Many therapeutic strategies are used to fight CRC. However, chemotherapy with drugs such as *5*-fluorouracil and radiotherapy can expose patients to troublesome side effects (Carnesecchi et al., 2002). Surgical treatment of CRC is associated with a high mortality and the risk of local repetition (Line-Edwige, 2009).

Natural products have served as the most productive source of leads for drug development for centuries (Harvey, 2007). In recent decades, many of the new antibiotics and new antitumor drugs approved by the US Food and Drug Administration (FDA), or comparable entities in other countries, are natural products or derived from natural products (Harvey, 2000, Esmaeelian et al., 2007, Newman and Cragg, 2007). Protective effects against a wide range of cancers, including colon cancer, have been shown by several foods such as nuts, spices, grains, fruits, cereals, vegetables, herbs, as well as medicinal plants and their various bioactive constituents including flavonoids, alkaloids, phenolics, carotenoids, and organosulfur compounds (Rajamanickam and Agarwal, 2008). Natural products are usually considered to exhibit low toxicity, and are cost effective and socially acceptable alternatives to pharmaceutical chemopreventatives (Manson et al., 2005). The marine environment is one of the major sources for novel natural products. The immeasurable chemical and biological diversity of the ocean offers a great source for new as yet undiscovered potential bioactive compounds (Blunt et al., 2006, Benkendorff, 2010, Blunt et al.,

2013). Many marine secondary metabolites have shown bioactivity for application as anticancer agents (Simmons et al., 2005, Sato et al., 2007, Jiang et al., 2011).

The Muricidae (Neogastropoda) are a family of predatory marine gastropods that are historically known for the production of Tyrian purple (6,6′-dibromoindigo), an ancient dye, *de novo* biosynthesized from a choline ester precursor salt of tyrindoxyl sulphate after a series of oxidative, enzymatic and photochemical reactions in the hypobranchial gland and egg masses (Baker, 1974, Benkendorff et al., 2000, Cooksey, 2001, Baker and Duke, 1976, Westley et al., 2006). Tyrindoleninone is the main indole precursor found in the extracts, along with 6-bromoisatin, a natural oxidative by-product of Tyrian purple synthesis (Benkendorff et al., 2000, Westley et al., 2010). 6,6-dibromoindirubin is a structural isomer of Tyrian purple that can form from the combination of tyrindoleninone and 6-bromoisatin (Cooksey, 2001, Cooksey, 2006) and is a minor pigment found in hypobranchial and male reproductive gland extracts of some muricids (Cooksey, 2001, Westley and Benkendorff, 2008). Benkendorff (2013) highlights the fact that all of these brominated indole derivatives in Muricidae molluscs conform to Lipinskis' rule of five for druglikeness and orally active drugs in humans.

Anticancer properties of egg mass extracts and the isolated brominated indoles from the Australian Muricidae *Dicathais orbita,* have been shown by several studies (Benkendorff, 2013). The extracts have been tested against a panel of cancer cell lines *in vitro* (Benkendorff et al., 2011). Tyrindoleninone and 6-bromoisatin purified from *D. orbita* extracts were shown to specifically decrease cell viability of female reproductive cancer cells, rather than freshly isolated human granulosa cells (Edwards et al., 2012). Furthermore, in a study by Vine *et al.* (2007a), some substituted isatin

derivatives including 6-bromoisatin have been synthesized and show *in vitro* anticancer properties on a range of human cancer cells, including leukemia, lymphoma and colorectal (HCT-116) cell lines. Bioassay guided fractionation of secretions from hypobranchial gland of a Mediterranean Muricidae *Hexaplex (Murex*) *trunculus* showed that 6,6-dibromoindirubin is an inhibitor of protein kinases and efficiently inhibits cell proliferation by selectively targeting glycogen synthase kinase-3 (GSK-3) (Meijer et al., 2003, Leclerc et al., 2001). In an *in vivo* study using a rodent model for colon cancer prevention by administrating the DNA damaging agent azoxymethane, pro-apoptotic activity of a crude extract from *D. orbita* containing these brominated indoles, was demonstrated in the distal colon (Westley et al., 2010). However, the compound or compounds responsible for the anticancer *in vivo* and *in vitro* activity have not yet been characterized.

Muricidae molluscs are subject to a small scale world-wide fisheries industry and are of growing interest in aquaculture (Noble et al., 2009, Benkendorff, 2009). Given that these edible molluscs have anticancer properties, there is growing interest in their potential use as a medicinal food for prevention of colon cancer (Benkendorff, 2013, Westley et al., 2013). The aim of this study was to perform bioassay guided fractionation on *D. orbita* extracts and to characterize these fractions *in vitro* using cell viability, apoptosis and cell cycle analysis in two human colon adenocarcinoma cell lines, Caco-2 and HT29.

3.3 Material and methods

3.3.1 Egg mass extraction, purification

All chemicals, HPLC grade solvents and silica gel where obtained from Sigma-Aldrich Pty Ltd (Castle Hill, NSW, Australia) unless otherwise stated. *D. orbita* egg capsules (27 g) were collected from a recirculating aquarium in the School of Biological Sciences, Flinders University, South Australia. The eggs capsules were opened and soaked in 100 mL (per 10 g eggs) chloroform and methanol (1:1, v/v) under agitation at room temperature for 2 h, followed by overnight soaking in fresh solvent. Both extracts were combined and filtered. Then a low volume of milli-Q water (~20–30 mL) was added to facilitate the separation of methanol and chloroform into two phases. The chloroform layer was separated and dried under reduced pressure of 474 mbar on a Buchi rotary evaporator at 40 °C. The dried extracts were re-dissolved in a small volume of dichloromethane (~1 mL), transferred to amber vials, then dried under a stream of nitrogen gas, yielding 300 mg of a light brown/red oily extract which was subsequently stored at −20 °C. Previous research has shown that the dominant compounds in *D. orbita* extracts are colored and can be separated by silica chromatography (Benkendorff et al., 2000). Here flash column chromatography pressurized with nitrogen gas was used to separate the bioactive compounds. The stationary phase consisted of approximately 20 g silica gel (100 mesh) mixed with hexane. The chloroform extract (300 mg) was loaded onto the column and eluted using a stepwise gradient of solvents, starting with 100% hexane (100 mL, Fraction 1). Fraction 2 was eluted using 20% DCM in hexane (50 mL), then Fraction 3 was collected using 25% DCM in hexane (200 mL), followed by Fraction 4 with 100% DCM (200 mL). The polarity of the solvent was then increased to 10% methanol in DCM to collect Fractions 5 (15 mL) and 6 (85 mL). Finally, Fraction 7 was collected

by washing the column with 50 mL 100% methanol. All solvents were evaporated from the fractions under reduced pressure by rotary evaporation at 40 °C.

3.3.2 Chemical analysis

All fractions affecting cell viability in the MTT assay (see below) were further analyzed using liquid chromatography coupled with mass spectrometry (LC/MS). Briefly, fractions were dissolved in acetonitrile and analyzed by HPLC (Waters Alliance) that was coupled to a mass spectrometer (MS, Micromass, Quatro micro™) with a Hydro-RP C18 column (250mm × 4.6 mm × 4 μm) and parallel UV/Vis diode-array detection at 300 and 600 nm. The flow rate was 1 mL/min of formic acid and a gradient of acetonitrile in water, according to the methods established by Westley and Benkendorff (2008). Compounds were identified using electrospray ionization-mass spectrometry (ESI-MS) with a flow rate of 300 μL/min. Mass Lynx 4.0 software was used to analyze the data. Additional analysis on bioactive fractions was facilitated by gas chromatography–mass spectrometry (GC-MS, Agilent Technologies (Mulgrave Australia) 5975C Series GC/MS) with a capillary column (SGE HT-5, 15 m × 0.25 mm i.d.) with a 0.25 μm film thickness. The injection port temperature was set at 260 °C. The initial oven temperature was held at 50 °C for 3 min and then ramped with a rate of 15 °C/min to the final temperature of 300 °C and held for 2 min. The carrier gas was helium with a constant flow rate of 2 mL/min. Electron ionisation (EI) was used with the electron energy of 70 eV. The source temperature was set to 230 °C and the MS quadrupole was 150 °C. To confirm the identity of the bioactive compounds, ^{1}H NMR spectroscopy was also used on purified fractions on a Bruker Avance III 400 MHz spectrometer (Bruker Biosciences, Preston, Australia), operating at 294K, in deuterated acetonitrile. Chemical shifts (δ) are reported as parts per million (ppm) and

referenced to residual solvent peaks. Spin multiplicities are indicated by: s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and dd, doublet of doublets.

3.3.3 Cell culture

Two human colorectal cancer cell lines Caco2 (passage no. 26–34) and HT29 (passage no. 18–26) maintained at 37 °C in a 5% CO_2 humidified atmosphere. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4500 mg/L L-glutamine, 10% FBS, 100 U/mL Penicillin/Streptomycin and 1% Non-essential Amino Acid (100×).

3.3.4 MTT viability assay and cell morphology

All fractions and purified compounds were tested using an MTT viability assay which measured the reduction of MTT tetrazolium salt to formazan (Mosmann, 1983, Young et al., 2005). Caco2 and HT29 cells were grown to 70% confluence, detached from flasks with 1X Trypsin-EDTA, counted using trypan blue dye exclusion method, and plated into 96-well plates (Costar®) (2×10^4 cells in 100 µL media/well). The cells were incubated for 48 h before treatment. All extracts and purified compounds were dissolved in 100% dimethylsulphoxide (DMSO) then diluted in media and added to the cell cultures in triplicate (final DMSO concentration of 1%), with final concentrations ranging from 2 to 0.01 mg/mL. 1% DMSO controls were also included on each plate. All extracts were incubated with the cells for 12 h. The media was removed prior to adding 100 µL of 0.05% MTT (Sigma Aldrich) with fresh media to each well. The cells were incubated for 1 h and then 80 µL of 20% SDS in 0.02 M HCl was added to each well. The absorbance of the samples was determined spectrophotometrically after 1 h by measuring the optical density at 480 and 520 nm

on a FLUOstar Omega microplate reader (BMG Labtech. Mornington Victoria). This assay was repeated on three separate occasions (n = 3). The morphological changes in HT29 cells were also observed by Olympus (Mt Waverly, Australia) CK2 inverted optical microscope (original magnification × 400) 12 h after treatment.

3.3.5 Combined caspase 3/7, membrane integrity and cell viability assays

HT29 and Caco-2 cells (2×10^4 cells in 100 µL media/well) were seeded into sterile white (opaque) 96-well plates (Interpath, Heidelberg West, VIC, Australia) (for determination of apoptosis and necrosis) and clear sterile 96-well plates (Costar®) (for measurement of cell viability). All cells were incubated for 48 h to allow attachment of these adherent cells, then the media was removed and the cells were washed with PBS. The cells were treated with different concentrations of crude extract and purified compounds from 0.5 to 0.01 mg/mL in fresh media. Two positive controls were added to each plate in triplicate wells; staurosporin (5 µM/mL) for apoptosis and lysis solution (5 µL/well, Promega, Madison, WI, USA) for necrosis. All cells were treated for 12 h. To measure necrosis, 70 µL of supernatant from each well of the white opaque plate was transferred to another white opaque 96-well plate. The CytoTox-ONE Homogeneous Membrane Integrity Assay reagent (Promega) was applied based on the manufacturer's instructions, in equal volume to the cell culture medium (70 µL). The plates were then incubated at 22 °C for 10 min and the fluorescence recorded with an excitation wavelength of 535 nm and an emission wavelength of 590 nm on a FLUOstar Omegaplate reader (BMG Labtech, Mornington, Australia). To measure apoptosis, the Caspase-Glo 3/7® assay (Promega) was applied. 30 µL Caspase-Glo® 3/7 Reagent was added to the primary white opaque 96-well containing cells and 30 µL cell culture medium and incubated at 22 °C for 1 h. The plates were read on a

FLUOstar Omega with full light to capture total luminescence. This experiment was repeated on three separate occasions (n = 3).

3.3.6 Flow cytometric detection of apoptosis

To confirm the caspase assay results, the most bioactive compounds were used in flow cytometry. HT29 cells were plated in 24 well plates (Nunc®) in duplicate with 1.5 × 10^5 cells/well in 1 mL media, then incubated for 48 h. Media were removed and 1 mL media and treatments including 0.025 and 0.05 mg/mL semi-purified 6-bromoisatin and 0.05 mg/mL tyrindoleninone (final concentration of 1% DMSO) were added to each well. Staurosporin (5 µM/mL, Sigma) was used as a positive control reagent for triggering apoptosis (data not shown). Cells were treated for 12 h and collected from the wells after the trypsinization by 1× trypsin-EDTA (Sigma Aldrich), then were placed in 15 mL tubes before centrifugation (1500 rpm for 3 min). Media were removed and the cells were washed twice with sterilized phosphate buffered saline (PBS) and suspended in 1 × Binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1 × 10^6 cells/mL. 100 µL of the solution (1 × 10^5 cells) were transferred to a 5 mL culture tube then 5 µL of FITC Annexin V (BD Biosciences, NJ, USA) and 5 µL of propidium iodide (BD Biosciences) at 10 µg/mL final concentration were added to each tube. All cells were incubated for 15 min at RT (25 °C) in the dark and cell distribution was analyzed using FACSan Flow Cytometer (Becton Dickinson, North Ryde Australia) and FlowJo analysis software.

3.3.7 Cell cycle analysis

Flow cytometry was used to assess whether the bioactive compounds arrested the cells at a particular stage of the cell cycle. HT29 cells (5 × 10^4 cells in 1 mL media/well)

were seeded into 12-well plates (Costar®). The cells were incubated for 48 h before treating with different concentrations of semi-purified 6-bromoisatin for 12 h (final DMSO concentration of 1%). The supernatant and cells were then harvested by exposing the cells to 0.25%, Trypsin-EDTA solution for 10 min, then centrifuged and washed in phosphate buffered saline (PBS), fixed in 3 mL ice-cold 100% ethanol and stored overnight at −20 °C. At the time of analysis, the cells were centrifuged, washed once again in PBS and stained with a freshly made solution containing 0.1 mg/mL propidium iodide (PI) (Sigma Aldrich), 0.1% Triton x-100 and 0.2 mg/mL ribonuclease A (Sigma Aldrich) in PBS. All samples were incubated for 30 min at room temperature in the dark. Cell cycle distribution was determined by an analytical DNA flow cytometer (Accuri C6, BD Biosciences) and CFlow Plus software on DNA instrument settings (linear FL2) on low.

3.3.8 Statistical analysis

Statistical analyses were performed using SPSS and values of $p \leq 0.05$ were considered to be statistically significant. One way ANOVA test was performed to compare between different concentrations of treatments and control. Tukey post-hoc test was applied to detect which groups significantly differ.

3.4 Results and discussion

3.4.1 Chemical analysis and bioassay guided fractionation

LC-MS analysis of *D. orbita* egg capsule mass crude extract showed five peaks corresponding to brominated indoles (Figure 3.1). The dominant peak in this extract at t_R 6.39 min and major ions in ESI-MS at *m*/*z* 224, 226 was attributed to the molecular mass of 6-bromoisatin. Another dominant peak at t_R 11.03 min

corresponded to the molecular weight of tyrindoleninone with major ions at *m*/*z* 255, 257. Mass spectrum of the peak at t_R 9.40 min with major ions in ESI-MS at *m*/*z* 302, 304 was indicative of tyrindolinone. The peak at t_R 8.58 min corresponds to tyrindoxyl sulphate, with major ions in ESI-MS at *m*/*z* 336, 338 and a smaller peak at t_R 11.90 min occurred with ions in ESI-MS at *m*/*z* 511, 513, 515 corresponding to the molecular mass of tyriverdin with major fragment ions at *m*/*z* 417, 419, 421 formed by the elimination of dimethyl disulphide.

Bioassay guided fractionation using the 3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide (MTT) cell viability assay revealed a statistically significant mean reduction of 27.6% and 72.4% cell viability in HT29 cells respectively at high concentrations of 1 and 2 mg/mL of crude extract compared with the solvent control (Figure 3.2 a). Caco2 cells showed 86.4% ($p < 0.001$) mean reduction in cell viability when exposed to the highest concentration of crude extract 2 mg/mL (Figure 3.2 b). Significant reductions in cell viability also occurred in some fractions. For example, HT29 cells treated with 0.1 and 0.05 mg/mL of fraction 2, showed 57.3% and 30.2% reduction in formazan production (Figure 3.2 a), while this reduction was more than 90% for Caco2 cells treated with the same concentrations of fraction 2 (Figure 3.2 b)**.** At the highest concentration of 0.5 mg/mL, cell viability was less than 2% in both cell types. Similar activity was observed for fraction 3 (Figure 3.2). The highest concentration of fraction 4 (0.5 mg/mL) caused 23.9% and 24.3% reduction in cell viability of HT29 and Caco2 cells respectively. Fraction 5 at the concentrations of 0.05 and 0.1 mg/mL showed 76.3% and 91.4% reduction of cell viability for Caco2 cells respectively and the greatest reduction in cell viability for HT29 cells. Fraction 5 reduced the viability of both cell lines by over 95% at the

highest concentration of 0.5 mg/mL. A mean reduction of 24.4% in the cell viability of Caco2 cells was also observed in fraction 6 with the higher concentration of 0.5 mg/mL. Significant dose effects were observed in both cancer cell lines, with lower viability rates recorded at the higher treatment concentrations. Bioassay guided fractionation using the MTT assay showed that fractions containing both tyrindoleninone and 6-bromoisatin inhibit the viability of HT29 and Caco2 cells, though tyrindoleninone was more potent towards Caco2 cells than HT29. The effect on viability of fraction 3 (mixture of tyrindoleninone and tyrinolinone) was similar to fraction 2 (tyrindoleninone), indicating the additional methyl thiol group on tyrindolinone does not increase the overall activity.

All fractions from flash chromatography of the crude egg capsule extract that were found to effect cell viability using the MTT assay, were then analyzed by LC-MS. In addition to matching the molecular mass of the isolated compounds with tyrindoleninone and 6-bromoisatin, the identity of these compounds was also confirmed by data gained from ^{1}H NMR. One purified compound was identified in fraction 2 at t_R 11.03 min, which was attributed to the molecular mass of tyrindoleninone (*m*/*z* 255, 257). The purity and identity of tyrindoleninone in fraction 2 was confirmed by GC/MS with one peak at t_R 11.24 min and exact MS match to tyrindoleninone in the mass spectrum library (Figure 3.3 a). ^{1}H NMR also confirmed the identity of tyrindoleninone: ^{1}H NMR (400 MHz, CD_3CN) δ 7.46 (1H, dd, *J* = 0.5, 1.4 Hz), 7.42 (1H, dd, *J* = 0.5, 7.6 Hz), 7.39 (1H, dd, *J* = 7.6, 1.4 Hz), 2.63 (3H, s). Our data for tyrindoleninone was consistent with the ^{1}H NMR results for this compound previously reported by Benkendorff *et al*. (2000) and Baker and Duke (1973). LC-MS of fraction 3 revealed two major peaks at t_R 9.40 and 11.03 min

corresponding to the molecular mass of tyrindolinone (*m*/*z* 302, 304) and tyrindoleninone (*m*/*z* 255, 257) respectively. LC/MS of fraction 5 identified one major compound at t_R 6.42 min which was indicative of 6-bromoisatin (*m*/*z* 224, 226). GC/MS revealed several other minor compounds (at least six peaks) in this fraction but confirmed 6-bromoisatin as the major component (90%) with a dominant peak at t_R 13.01 min (Figure 3.3 b). The other minor compounds in fraction 6 were matched with two short chain aldehydes at t_R 11.71 min and t_R 12.35 min, two sterols at t_R 16.82 min (molecular mass of 366% and 93.7% match with cholesta-4,6-dien-3-ol (3β); $C_{27}H_{44}O$) and at t_R 17.02 min (molecular mass of 364), an unidentified ester at t_R 15.96 min (molecular mass of 302) and finally a new brominated indole with a tiny amount was found at t_R 13.61 min (molecular mass of 267/269). ^{1}HNMR confirmed the identity of the major compound in fraction 5 as 6-bromoisatin: ^{1}H NMR (400 MHz, CD_3CN) δ 8.96 (1H, s), 7.44 (1H, d, J = 8.08 Hz), 7.30 (1H, dd, J = 1.64, 8 Hz), 7.19 (1H, d, J = 1.6 Hz) despite also detecting small peaks associated with minor contaminants. Chemical analysis of the most bioactive fractions showed that a good separation for tyrindoleninone producing pure material and a semi-purification for 6-bromoisatin (90% purity) based on the GC/MS analysis.

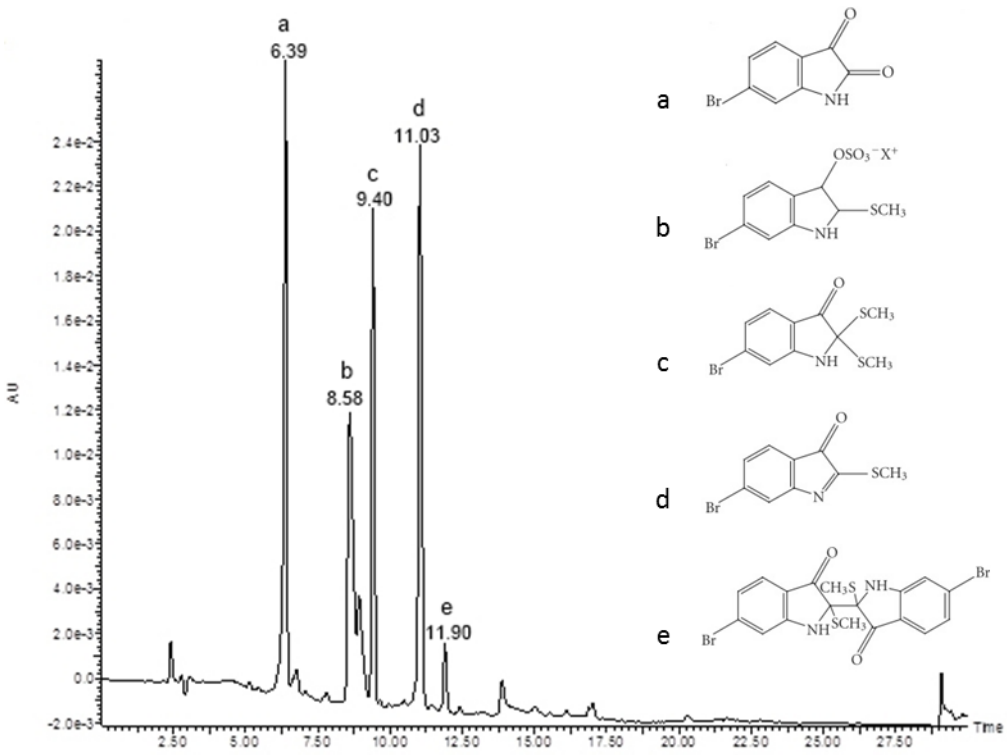

Figure 3.1 Liquid chromatography-mass spectrometry (LC-MS) analysis of extract from *D. orbita* egg capsules. The chromatogram obtained from diode array detection at 300 and 600 nm shows five peaks corresponding to brominated indoles where a: 6-bromoisatin (*m/z* 224, 226); b: tyrindoxylsulphate (*m/z* 336, 338); c: tyrindolinone (*m/z* 302, 304); d: tyrindoleninone (*m/z* 255, 257) and e: tyriverdin (*m/z* 511, 513, 515).

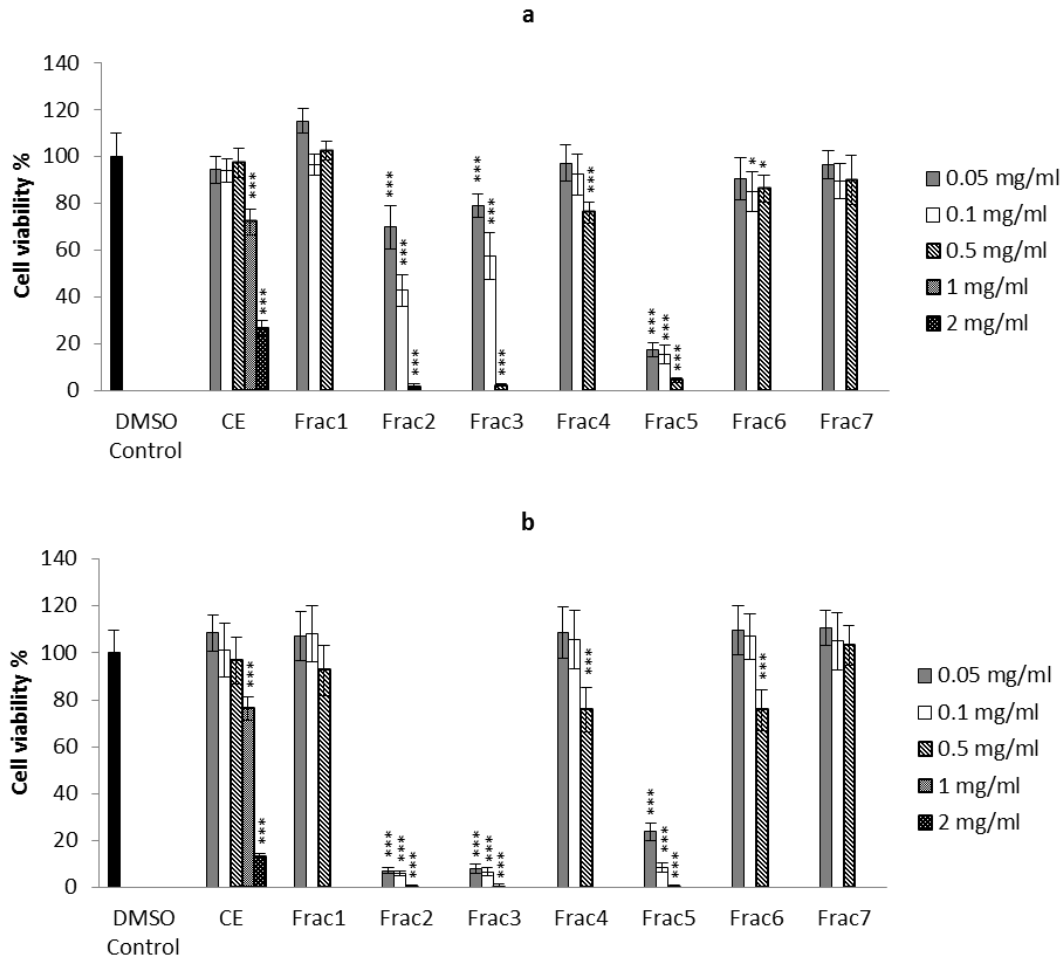

Figure 3.2 MTT viability results of *D. orbita* egg mass crude extract (CE) and all fractions collected from flash column chromatography (Frac 1–7) on HT29 cells (a) and Caco2 cells (b). Fraction 1 is the most lipophilic collected with 100% hexane and fraction 7 is the most polar collected with 100% methanol. Significant difference between each group and the 1% DMSO control are shown as $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

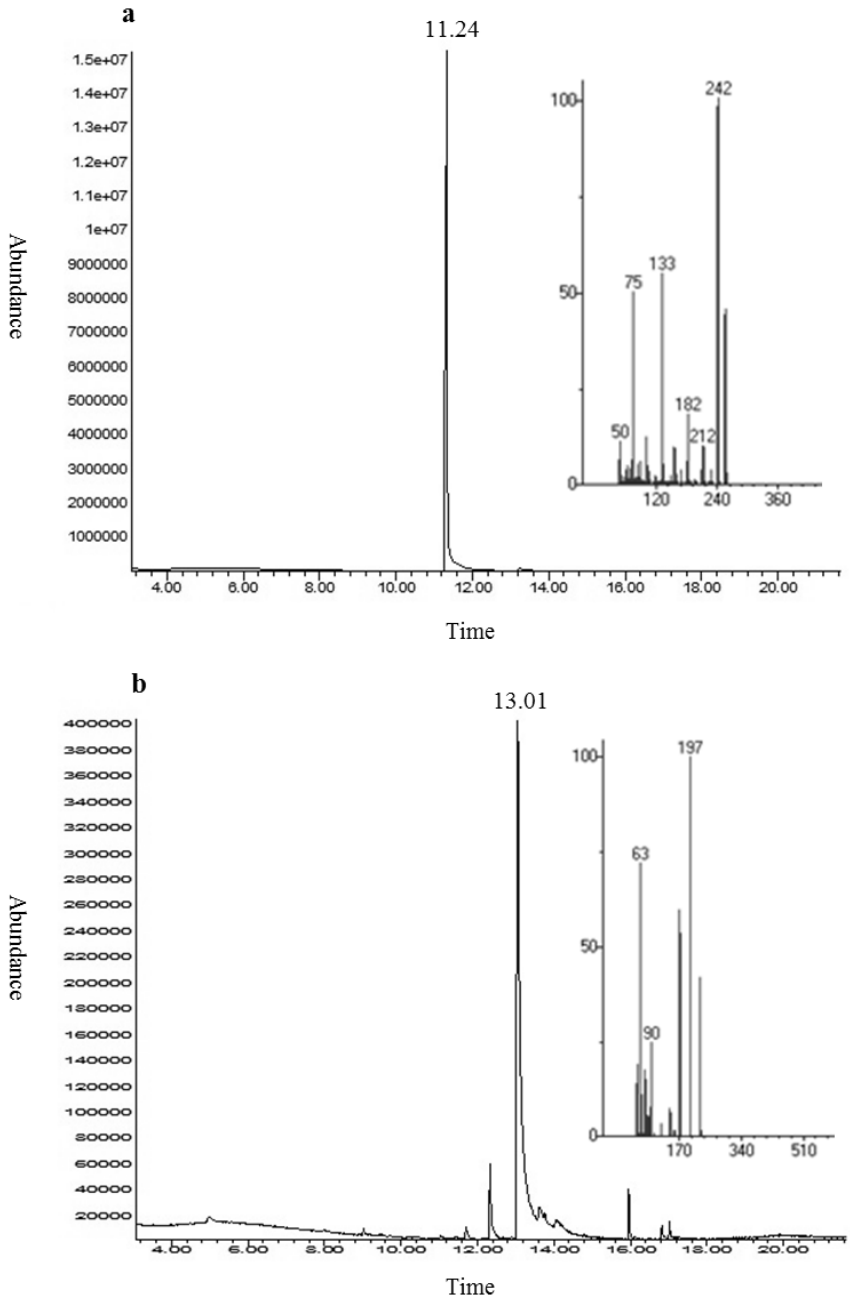

Figure 3.3 Gas chromatography–mass spectrometry (GC-MS) chromatogram of fractions from the egg masses extract of the Australian muricid, *D. orbita*. Fraction 2 (a) at t_R 11.24 min corresponds to tyrindoleninone and fraction 5 (b) with dominant peak at t_R 13.01 min matches the molecular mass of 6-bromoisatin. The mass spectra (ESI-MS) for the major peaks are inset.

3.4.2 Biological activity of the *D. orbita* compounds

3.4.2.1 Apoptosis, necrosis and cell viability

Death by necrosis, which may result in damage to the plasma membrane and releasing of the cytoplasmic contents, including lysosomal enzymes into the extracellular fluid, is often considered as a toxic process in comparision to apoptosis (Jin and El-Deiry, 2005b, Elmore, 2007). The most bioactive fractions from the MTT assay—fraction 2 (tyrindoleninone) and fraction 5 (semi-purified 6-bromoisatin) were examined for their ability to induce either apoptosis or necrosis.

Tyrindoleninone, was found to be more cytotoxic towards Caco2 cells (IC_{50} = 98 μM), than for the HT29 cells (IC50= 390 μM; Figure 3.4 a & d). In a study by Benkendorff *et al.* (2011), greater reduction in cell viability (over 60%) was observed in Caco2 and U937 lymphoma cells treated using a semi-purified egg extract with increased concentration of tyrindoleninone, compared to crude extract, whereas less activity was observed against HT29 cells. This confirms our result that Caco2 cells are more susceptible to tyrindoleninone than HT29 cells. Edwards *et al.* (2012) showed that tyrindoleninone inhibited KGN cell viability (a tumour-derived granulosa cell line), JAr and OVCAR-3 cells with the IC_{50} 39 μM, 39 μM and 156 μM respectively. . In addition, Vine *et al.* (Vine, 2007) demonstrated that tyrindoleninone had less cytotoxic effects on untransformed human mononuclear cells (IC_{50} = 195 μM) than U937 cancer cells (IC_{50} = 4 μM) after 1 h exposure. The current study confirms the different cell line specificity of tyrindoleninone, with a four-fold difference observed here between the two adherent colon cancer cells lines. This difference in drug resistance may be due to the variations in metabolic and signaling pathways and also the difference in

expression and activity of some drug-metabolizing enzymes in different cancer cells (Rochat, 2009).

The other bioactive compound, 6-bromoisatin however, inhibited the viability of both Caco2 and HT29 cells (IC50= 100 μM; Figure 3.4 a & d). Edwards *et al.* (2012) demonstrated that semi-purified 6-bromoisatin significantly reduced cell numbers of three reproductive cancer cell lines KGN, JAr and OVCAR-3, although converse to this study, it was not as potent as tyrindoleninone. The JAr cells were the most susceptible, with cell numbers halved at approximately 223 μM 6-bromoisatin. Vine *et al.* (2007a), on the other hand, showed that a range of isatin derivatives including 7-bromoisatin (IC_{50} = 83 μM) and 6-bromoisatin (IC_{50} = 75 μM) reduced the cell viability of lymphoma cell line U937, which was similar to the efficacy of 6-bromoisatin against Caco2 and HT29 cells in our study (IC_{50} = 100 μM). Vine *et al.* (2007a) also reported different specificity of isatin derivatives against different cancer cell lines. Human leukemic Jurkat cell lines were the most sensitive to isatin treatment (IC_{50} = 5–20.9 μM), the next most sensitive cells were the colon cancer cell line HCT-116 (IC_{50} = 15.9–37.3 μM) and the least sensitive cells were the prostrate PC3 cell line (IC_{50} = 25.9–101 μM). In a review by Vine *et al.* (2009), small electron withdrawing groups, mono, di and tri-halogenation at positions 5, 6 and/or 7 on the isatin molecule were found to enhance cytotoxicity activity. 6-bromoisatin is an example of this kind of halogenated isatin.

Caspase-3 and -7 activity significantly increased only in HT29 cells treated with 195 μM (0.05 mg/mL) tyrindoleninone in 1% DMSO compared to the 1% DMSO control (Figure 3.4 c). An increase in the proportion of Annexin-V positive, PI negative cells (27.6% ± 9.25%) was also observed by flow cytometry in HT29 cells treated with 195

µM (0.05 mg/mL) tyrindoleninone; however, it was not significant (Figure 3.6). Despite a dose-dependent decrease in viability from Caco2 cells treated with tyrindoleninone, no significant increase in caspase-3 and -7 activity was observed (Figure 3.4 d). Tyrindoleninone at high concentrations appears to induce necrosis rather than apoptosis (increase in LDH observed, Figure 3.4 e) towards Caco2 cells, whereas some apoptosis by caspase 3/7 up-regulation was observed in HT29 treated with tyrindoleninone. Apoptotic cells are characterized by particular morphological features (Thompson, 1995, Gamet-Payrastre et al., 2000), such as dense chromatin surrounded by a halo, which were observed in the treated HT29 cells in this study (Figure 3.5 d). Purification of tyrindoleninone from the crude extract consistently increased the cytotoxic potency towards Caco2 cells, but resulted in induction of necrosis rather than apoptosis in these cells, whereas HT29 cells, which were more resilient to the anti-proliferation effects of tyrindoleninone, underwent apoptosis at the concentration of 195 µM. This difference in cell line specificity might be due to the phenotype of the cells, as bioactive compounds may target alternative pathways in different cells (Nguyen and Wells, 2003, Benkendorff et al., 2011). Edwards *et al.* (2012) revealed that purified tyrindoleninone induced 66% apoptosis with 20 µM in KGN compared to 31% apoptosis (391 µM) in freshly isolated human granulosa cells (HGC) using TUNEL assay after 4 h. This study showed that reproductive cancer cell lines were ten times more susceptible than HCG to tyrindoleninone and indicated specificity of this compound toward reproductive cancer cells.

The fraction containing 6-bromoisatin considerably activated caspase-3 and -7 enzymes and induced cell death by apoptosis in both cell lines at approximately 100 µM (0.025 mg/mL) and 200 µM (0.05 mg/mL), much lower concentrations than those

required to cause lactate dehydrogenase (LDH) release and necrosis (~1000 to ~2000 µM; Figures 3.4b and e). For example, the HT29 cells treated with 6-bromoisatin at ~100 µM and 200 µM showed significant increases in caspase-3 and -7 activity, with luminescence values greater than five times the negative (DMSO) control. The light microscopic images from the HT29 cells treated with ~200 µM 6-bromoisatin showed morphological alterations, such as chromatin condensation characteristic of the apoptotic process (Figure 3.5 b). Flow cytometry results (Figure 3.6) also confirmed that HT29 cells treated with ~100 µM (0.025 mg/mL semi-purified) 6-bromoisatin underwent a significant induction of apoptosis (75.3% ± 14.03% Annexin-V positive, PI negative cells) compared with the DMSO control (6.6% ± 3.43% Annexin-V positive, PI negative). Similarly, ~200 µM 6-bromoisatin, induced apoptosis up to 68.1% ± 17.1%, but also with a 9.7% increase in the number of PI positive necrotic cells, as compared to DMSO control. In contrast, the highest concentrations of 6-bromoisatin (~1000 µM and 2000 µM) caused a high release of LDH indicating necrosis in HT29 cells (Figure 3.4 b) without any sign of apoptosis. HT29 cells incubated with approximately 400 µM of 6-bromoisatin underwent a significant induction of apoptosis, while the increase in LDH release did not reach significance at this concentration (Figure 3.4 b). Caco2 cells treated with the three lowest concentrations of semi-purified 6-bromoisatin (~40 µM, 100 µM and 200 µM) showed a significant induction of apoptosis (Figure 3.4 f), but without any significant increase in the release of LDH compared to the DMSO control (Figure 3.4 e). At the highest concentrations of 6-bromoisatin (~1000 µM and 2000 µM) Caco2 cells underwent a significant increase in LDH release (Figure 3.4 e) with no in increase in caspase-3 and -7 activity.

Our results showed that 6-bromoisatin increased the level of caspase 3/7 in both cell lines, while tyrindoleninone only up-regulated the caspase 3/7 in HT29 cells. 6-Bromoisatin also showed more potency than tyrindoleninone producing higher levels of caspase 3/7 in HT29 cells and indicating high induction of apoptosis in these cells. The morphology of condensed chromatin and haloed areas in nearly all cells from the images was also consistent with this type of cell death. Furthermore, Caco2 cells treated with semi-purified 6-bromoisatin also underwent the induction of apoptosis. Therefore, semi-purified 6-bromoisatin in our study had the most consistent anti-cancer efficacy against both colon cancer cell lines at low concentrations. Necrosis, as indicated by LDH release, was only significantly increased with exposure to the highest concentrations of 6-bromoisatin in both cell lines. Our caspase 3/7 and LDH results suggest that 6-bromoisatin induces cell death by apoptosis at low concentrations, while the apoptotic pathway is terminated at higher concentrations and secondary necrosis or necrosis is being triggered (Riss and Moravec, 2004, Pozhilenkova et al., 2008). It has been shown that some structurally similar isatin and indole compounds at low concentrations induce apoptosis through the activation of caspase 3 in a range of cell lines (Vine et al., 2007a, Vine et al., 2007b, Weng et al., 2007). For example, caspase 3/7 was activated by 5,6,7-tribromoisatin at a concentration of 8 μM in the Jurkat cell line after 5 h (Vine et al., 2007a). Edwards *et al.*(2012) showed that caspase 3/7 was up-regulated significantly with approximately 22 μM 6-bromoisatin in KGN cells and apoptosis was also confirmed by Tunnel staining in these cells.

Our results suggest that both tyrindoleninone and semi-purified 6-bromoisatin induce apoptosis through caspase-dependent pathways on HT29 cells. However, more

investigation on initiator caspase 8 and 9 would be required to distinguish between the extrinsic and intrinsic apoptosis pathways (Nicholson, 1999, Elmore, 2007) induced by these brominated indoles. In a review by Vine *et al.*(2009), the mode of action of some halogenated isatins, such as 6-bromoisatin, was proposed to be linked to the reduction in extracellular signal-regulated protein kinase (ERK) activity. Another study by Cane *et al*. (2000) suggests that isatin and indole inhibit cell proliferation and induce apoptosis via inhibiting the signaling of ERK. Inhibition of ERK can suppress cell growth and results in induction of apoptosis in the cells (Steinmetz et al., 2004). Moreover, some other apoptosis pathways, including both caspase-dependent or caspase-independent, can occur via inhibition of ERK, as has been reported by Georgakis *et al*. (2006). ERK may also act through suppression of the anti-apoptotic signaling molecule Akt (Zhuang and Schnellmann, 2006). Therefore, further study on ERK and Akt inhibition, especially with pure 6-bromoisatin, is required to evaluate the exact mode of action of these brominated compounds.

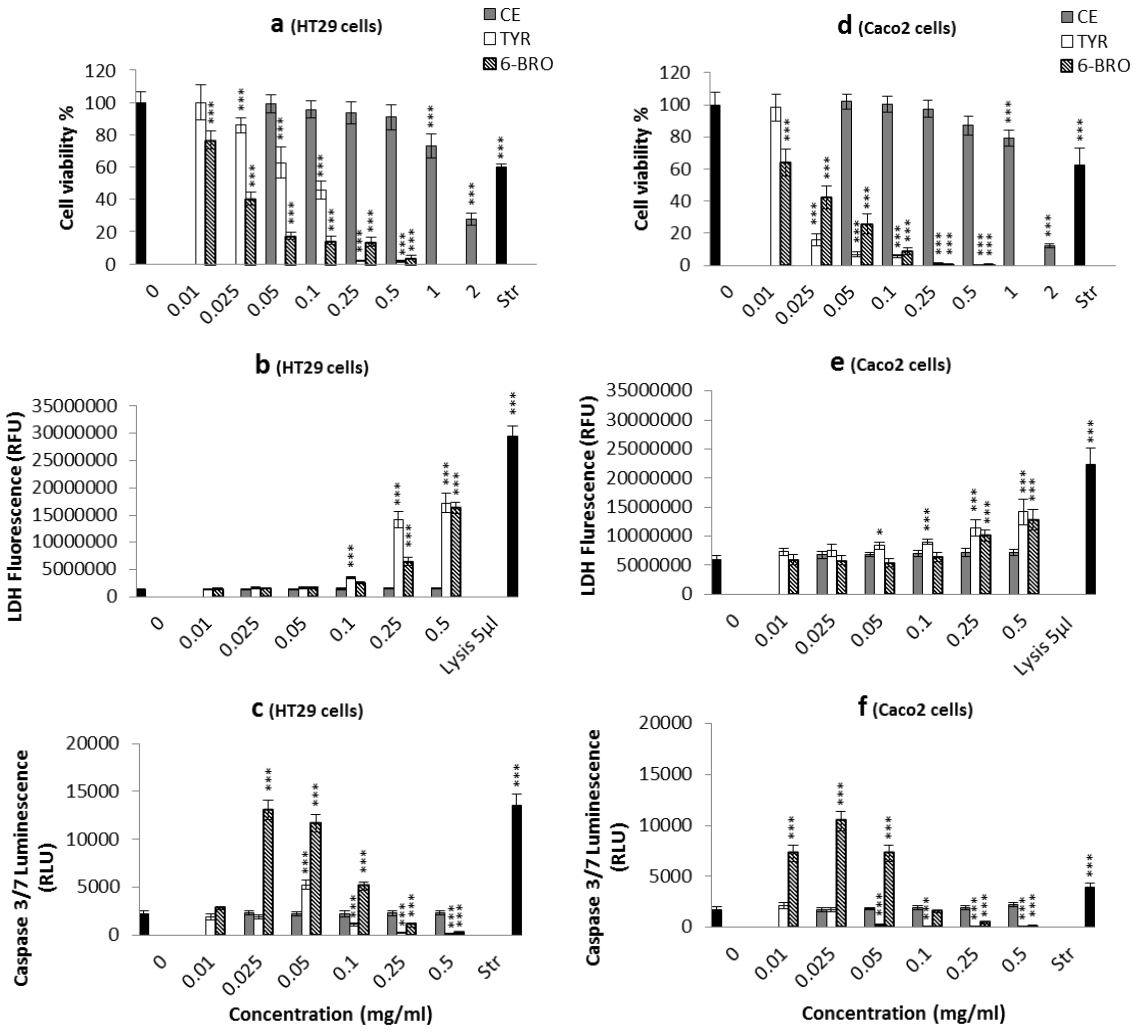

Figure 3.4 Effects of *D. orbita* egg mass crude extract (CE), purified tyrindoleninone (TYR) and semi-purified 6-bromoisatin (6-BRO) in mg/mL on HT29 (left panels) and Caco2 (right panels) cells. Cell viability (a,d), lactate dehydrogenase (LDH) release (b,e) and caspase-3/7 activity (c,f). LDH release was measured by fluorescence at 535EX/590EM and caspase-3/7 activity was measured at full light on a luminescence plate reader. Staurosporin (Str) (5 µM; Sigma) was used as a positive control for the MTT and caspase-3/7 assay; lysis buffer (5 µL/well; Promega) served as the positive control for the LDH assay. A final concentration of 1% DMSO was used in all control and treated cells. The results are the mean for three independent repeat assays each performed in triplicate (n = 3). Significant difference between each group and the DMSO control are shown as $p \leq 0.05$ (*); $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

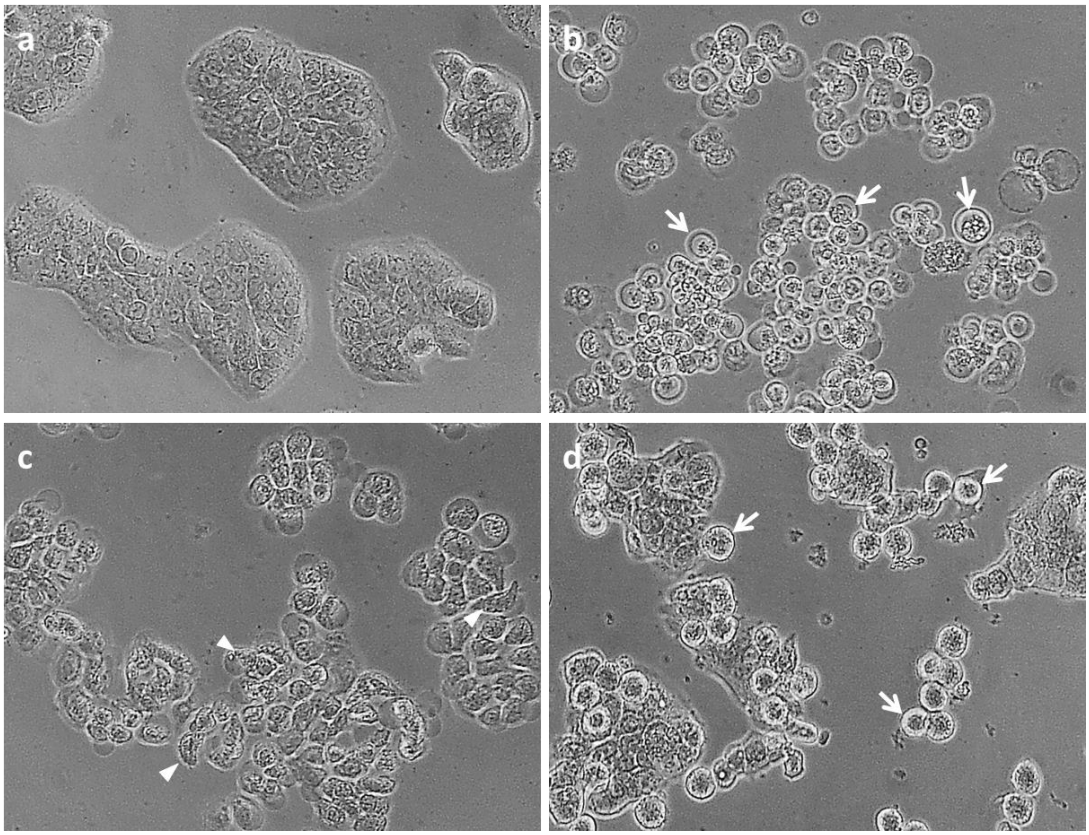

Figure 3.5 HT29 cells at 400× magnification under an Olympus inverted microscope. DMSO control (a); cells treated with 0.05 mg/mL semi-purified 6-bromoisatin (b); cells treated with 0.5 mg/mL semi-purified 6-bromoisatin (c) and cells treated with 0.05 mg/mL tyrindoleninone (d) for 12 h (final concentration of 1% DMSO). Apoptotic cells with chromatin condensation characteristic are shown by arrows and necrotic cells with deformed cell shapes are shown by arrowheads.

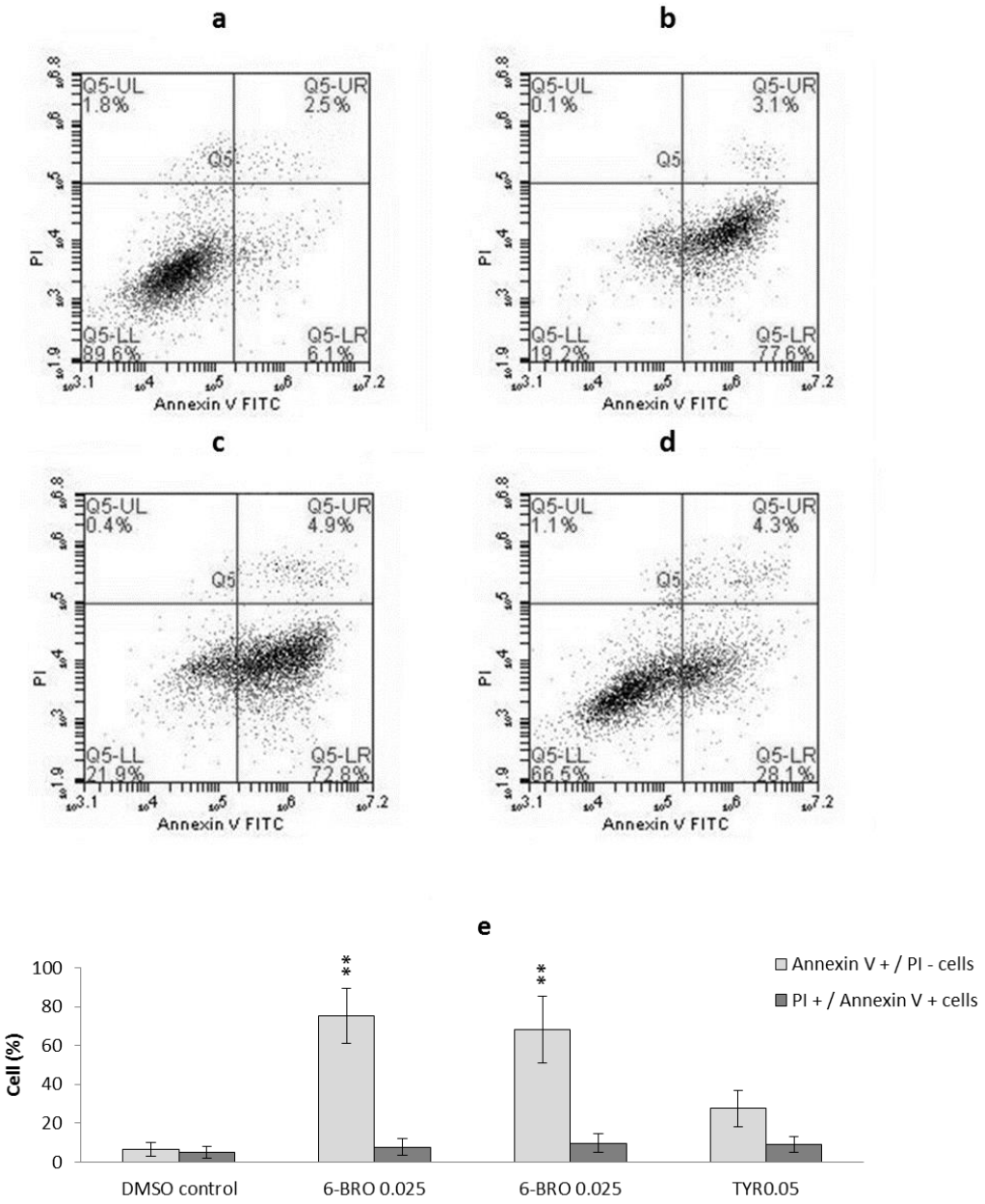

Figure 3.6 Flow cytometric analysis of HT29 cells (1.5×10^5) treated with (a) DMSO only (final concentration 1%); (b) 0.025 mg/mL semi-purified 6-bromoisatin; (c) 0.05 mg/mL semi-purified 6-bromoisatin and (d) 0.05 mg/mL tyrindoleninone purified from *D. orbita* egg masses. Cells were treated for 12 h and stained with Annexin-V-FITC and PI then analyzed by a FACscan flow cytometer and FlowJo analysis software. X-axis shows Annexin-V positive cells and Y-axis shows propidium iodide (PI) positive cells. (e) Histograms of the mean ± SE of three separate experiments for PI and annexin positive cells Significant difference between each group and the DMSO control are shown as $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

3.4.2.2 Cell cycle analysis

Cell cycle analysis revealed three distinct cell populations in HT29 cells, which were indicative of cells in the G0/G1, S and G2/M phases of the cell cycle (Figure 3.7). The DMSO control showed more accumulation of the cells in G0/G1 (64% ± 1.9%) with approximately the same proportion of the cells in S and G2/M (17% *versus* 15.6%). After exposure to ~400 µM (0.1 mg/mL semi-purified) 6-bromoisatin, 26.7% of HT29 cells were in the S phase ($p \leq 0.001$). This switched to significantly more cells in G2/M at the lower and most effective concentrations (100 µM = 25.7% and 200 µM = 23.8%). There were no significant differences in the cell population analysis between the DMSO control negative and the cells treated with 6-bromoisatin at the concentration of 0.01 mg/mL. Our result revealed that the most effective concentration of 6-bromoisatin that induced the highest apoptosis in HT29 cells, also caused the accumulation of cells at G2/M phase of the cell cycle. G2 phase in the cell cycle is where DNA repair might occur in cells, along with preparation for mitosis in M phase (DiPaola, 2002).

Increasing arrest of the cells in G_2/M phase has been shown to be associated with enhanced apoptosis (Manson et al., 2005). CDK1 (cyclin dependent kinase) is one of the protein kinase families that is activated by dephosphorylation and acts as a G2 checkpoint, which controls cell cycle progression from G2 to M phase (DiPaola, 2002). For example, in a study by Singh *et al.* (2004) sulforaphane, a naturally occurring cancer chemopreventive agent, caused an irreversible arrest in the G2/M phase of human prostate cancer cells (PC-3), which was associated with a significant reduction in protein levels of cyclin B1, CDC25B, and CDC25C. In a study by Vine *et al.* (2007b) various *N*-alkylisatins induced G2/M cell cycle arrest. It is known that

the indole based small molecules inhibit the serine/threonine kinases glycogen synthase kinase-3 (GSK3) (Damiens et al., 2001, Leclerc et al., 2001) and CDK5 (Davis et al., 2001, Lane et al., 2001). Another well-known isatin derivative 6,6'dibromoindirubin has also been identified as a specific GSK-3 inhibitor (Meijer et al., 2003). Anti-proliferative activity of indirubin has been shown via ATP-competitive inhibition of both CDK1 and CDK2 (Hoessel et al., 1999, Marko et al., 2001, Jautelat et al., 2005). The modes of action associated with indirubins (Marko et al., 2001) includes the induction of apoptosis through cell cycle arrest at G2/M via the inhibition of GSK3 (Leclerc et al., 2001), as well as induction of the c-Src kinase and nuclear factor-κB signalling pathway and expression (Eisenbrand et al., 2004, Sethi et al., 2006) and activation of the aryl hydrocarbon receptor (Adachi et al., 2001, Spink et al., 2003). Vine (2007) tested the inhibitory effect of six representative *N*-alkyl isatins on a range of tyrosine-specific and serine/threonine-specific protein kinases, but found no inhibition of enzyme activity by these isatins (Vine, 2007). Based on molecular modeling results, neither 6-bromoisatin or tyrindoleninone are predicted to have any kinase receptor binding or enzyme inhibiting activity However, inhibition of tubulin polymerisation in a range of cancer cell lines was shown by an array of imidazole and pyrrole containing 3-substituted isatins, resulting in cell cycle arrest at G2/M and final cell death (Andreani et al., 2005, Chen et al., 2005). Based on morphological examination of treated cells, Vine *et al.* (2007b) suggested that *N*-alkyl isatins may either stabilize or disrupt microtubules in a similar manner. Therefore, the finding that 6-bromoisatin increases the proportion of cells in the G2/M phase is consistent with a range of other studies on isatin derivatives and could be linked to a range of different modes of action that require further investigation.

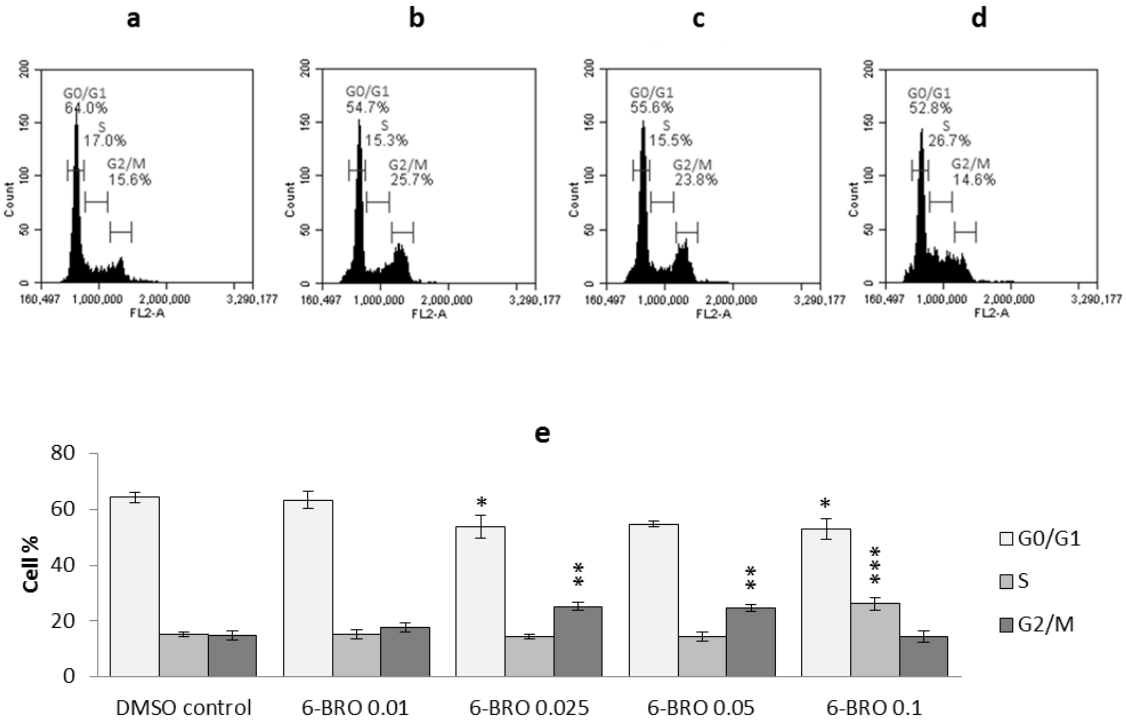

Figure 3.7 Cell cycle analysis using propidium iodide (PI) staining and flow cytometry. HT29 cells (5×10^5 cells in 1 mL media/well) were treated for 12 h with (a) DMSO only (final concentration 1%); (b) 0.025 mg/mL 6-bromoisatin; (c) 0.05 mg/mL 6-bromoisatin; (d) 0.1 mg/mL 6-bromoisatin semi-purified from egg mass of *D. orbita*; (e) Results are the mean ± SE of three separate experiments. Significant difference between each group and the DMSO control are shown as $p \leq 0.05$ (*); $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

Statistical analyses were performed using SPSS and values of $p \leq 0.05$ were considered to be statistically significant. One way ANOVA test was performed to compare between different concentrations of treatments and control. Tukey post-hoc test was applied to detect which groups significantly differ

3.5 Conclusion

Our study demonstrated that both semi-purified 6-bromoisatin and purified tyrindoleninone decreased cell viability in the colon cancer cell lines HT29 and Caco2. In particular, 6-bromoisatin showed more specificity and potency than tyrindoleninone and greater induction of apoptosis toward the colon cancer cells. 6-Bromoisatin also inhibited cell cycle progression of HT29 cells by arresting some cells in the G2/M phase. This data, along with the previously reported *in-vivo* induction of apoptosis in DNA damaged cells of the colon using Muricidae extracts (Westley et al., 2010) suggests that 6-bromoisatin from Muricidae molluscs is promising as an anti-cancer drug against colon cancer.

3.6 Acknowledgments

We are grateful to Daniel Jardine from the Flinders Analytical Laboratory of Flinders University for LC/MS and GC/MS analysis of compounds. We would further like to thank Peta Macardle from the flow cytometry analysis lab, Flinders Medical Centre, Tim Chataway and Nusha Chegeni from proteomics facility, Flinders Medical Centre for their help and advice, and Kathy Schuller in the School of Biological Sciences of Flinders University for housing equipment and facilitating access to her lab.

4. Brominated indoles from a marine mollusc extract prevent early stage colon cancer formation *in vivo*.

4.1 Abstract

The Australian whelk, *Dicathais orbita*, is a novel source of bioactive compounds with anticancer properties. The aim of this research was to purify the bioactive brominated indole derivatives from *D. orbita* and evaluate their *in vivo* safety and efficacy. Specifically these compounds were assessed for their ability to induce apoptosis in DNA damaged cells after azoxymethane (AOM) injection, in a short-term (two week) mouse model for colorectal cancer (CRC). Distal colon epithelial proliferation and apoptosis were evaluated using immunohistochemical and hematoxylin staining. Toxicity was assessed by hematology and serum biochemistry, including the measurement of liver enzymes, along with a histopathological evaluation of liver. A purified fraction containing 6-bromoisatin was found to be more effective than the crude mollusc extract, as evidenced by a significantly higher apoptotic index and reduction of cell proliferation in the distal colon. There was no evidence of liver toxicity associated with 6 bromoisatin, whereas tyrindoleninone showed some evidence for toxicity with elevated aspartate aminotransferase and a reduction in red blood cells, but without increasing the apoptosis index in the distal colon. In conclusion, 6-bromoisatin from Muricidae molluscs holds potential for development as a natural medicine for the prevention of colon cancer.

4.2 Introduction

In the general population worldwide, CRC is the third most commonly diagnosed cancer, with about 1 million cases and >500,000 deaths annually (IARC, 2008). In the United States, CRC is also the second leading cause of cancer death overall in men and women combined (Chan and Giovannucci, 2010). The lifetime risk of CRC is about 5 to 6% (Lynch and de la Chapelle, 2003). Symptoms of CRC usually appear

with more advanced disease and most people with early colon cancer do not have any symptoms of the disease. Therefore the majority of patients often remain undetected until they present with metastasis and micrometastases (Beart Jr et al., 1990, Brown and DuBois, 2005), thus increasing global mortality from CRC, which is approximately 50% of incidence (IARC, 2008).

Most of the CRC incidence (80-95%) is sporadic and related to environmental factors (Lynch and de la Chapelle, 2003). Exposure to exogenous carcinogens such as alkylating agents has been linked with increased risk of sporadic CRC. G→A transitions in K-*ras* gene are a characteristic effect of alkylating agents, such as *N*-nitroso compounds (Bingham et al., 1996). Laboratory results have shown some other potential mutagens, such as heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs), are in meat cooked diets (Cross and Sinha, 2004). Alkylating agents, HCAs and PAHs produce different types of DNA-adducts which can cause damage and mutation in DNA and form aberrant crypt foci (ACF), mucosal cell clusters, polyps, adenomas or colonic polyps, and ultimately adenocarcinomas (Al-Saleh et al., 2008, Cappell, 2007).

Almost forty to fifty percent of patients relapse and ultimately die of metastatic disease after undergoing curative surgery alone (André et al., 2004). The combination of fluorouracil, irinotecan and leucovorin (FOLFIRI) is currently the standard and first-line chemotherapy for metastatic colorectal cancer (Van Cutsem et al., 2011). The risk of treatment failure is reduced by 35% among patients with stage III colon cancer treated with chemotherapy, in comparison to surgery only (Arkenau et al., 2003). Nevertheless, recent therapies are far from curative and many serious side effects have been reported, such as peripheral sensory neuropathy (Cassidy et al., 2008),

myelosuppression, mucositis (Elting et al., 2003, Benson et al., 2004, Peterson et al., 2013), pulmonary embolus, myocardial infarction and death (Rothenberg et al., 2001). Moreover, there are some adverse effects associated with 5-FU due to the lack of selectivity between cancerous and normal cells. This is attributed to the lack of dihydropyrimidine dehydrogenase enzymes, which metabolize 5-FU to 5,6-dihydro 5-fluorouracil. Lack of this enzyme in patients can lead to accumulation of 5-FU which can destroy normal cells (LaFrate and Katzenellenbogen, 2007). Myelosuppression and gastrointestinal toxicity are the most common side effects of this drug, so dosing is limited to increase safety (Saif et al., 2009). Longest durations of hospitalization are for cycles with gastrointestinal mucositis (grade 3 or 4) which can exceed 10 days per cycle during cycles and may cost more than $3500 per cycle (Elting et al., 2003). As a result of the morbidity associated with chemotherapy and poor prognosis of the disease, along with the large economic burden colorectal cancer presents (IARC, 2008), prevention of this disease is an important priority.

Lifestyle alteration is an important way to lower the risk of developing colorectal cancer (Courtney et al., 2004, Cappell, 2007). However, implementing change in global lifestyle is intrinsically difficult. Administration of chemopreventatives agents is limited due to their side effects (Courtney et al., 2004) and can cost up to $400,000/life saved (Ladabaum et al., 2003). Although there is much progress in understanding the process of carcinogenesis, overall mortality from cancer is unlikely to be changed statistically unless there is trend towards increased use of scientifically substantiated natural products as a new chemopreventatives (Reddy et al., 2003). Natural organisms are the source of many potential cancer chemopreventive agents (Cassady et al., 1990). It has been reported that the consumption of food with

anticancer natural products may result in a decrease in the incidence of CRC, by up to 10 times less (Boyle and Levin, 2008).

The Muricid mollusc, *D.orbita*, is known for the production of brominated indole derivatives, including the ancient dye Tyrian purple (Benkendorff, 2013). A minor pigment in Tyrian purple 6,6' dibromoindirubin has been established as a potent inhibitor of protein kinases, particularly of glycogen synthase kinase-3 (GSK-3), which inhibits cell proliferation (Leclerc et al., 2001). Indirubin derivatives have also been shown to inhibit Stat3 signalling, inducing apoptosis in human breast and prostate cancer cells (Meijer et al., 2003, Nam et al., 2005). These natural products also suppress tumour necrosis factor (TNF)-induced NF-κB activation in human leukaemia and lung adenocarcinoma cells (Sethi et al., 2006) and significantly block proliferation in A549 lung carcinoma, SNU-638 stomach carcinoma and HT-1080 fibrosarcoma cell lines (Kim et al., 2007). In addition to these indirubin pigments, the brominated isatin and indole precursors of Tyrian purple from the Muricidae family are known for their anticancer activity (Benkendorff et al., 2011, Benkendorff, 2013). In an *in vitro* study, Edwards et al. (2012) revealed that tyrindoleninone inhibited cell growth (IC50 39 μM) and induced apoptosis in female reproductive cancers cell lines, with 10-100 fold specificity over freshly isolated human granulosa cells. Semi-purified 6-bromoisatin in our previous *in vitro* study induced apoptosis and inhibited the proliferation of both HT29 and Caco2 cells (Chapter 3). Tyrindoleninone was also found to be a cytotoxic compound against these cells (Chapter 3). Furthermore, Muricid extract containing a mixture of tyrindoleninone and 6-bromoisatin was found to be effective for enhancing colonic apoptotic index in a dose-dependent manner in mice (Westeley et al 2010). However, further *in vivo* testing of the crude Muricdae

extract has indicated the potential for idiosyncratic gastrointestinal and liver toxicity (Westley et al., 2013).

The acute apoptotic response to genotoxic carcinogens (AARGC) has been developed as a model for chemopreventative research, with the aim of reducing the incidence of CRC by inducing apoptosis of damaged colon cells (Hu et al., 2002). However, to date this model has been mainly used to test acute apoptotic response of dietary components such as starches and oils (Hong et al., 1999, Le Leu et al., 2002, Le Leu et al., 2003). The aim of this study was to purify the Muricidae anticancer compounds and use these in the AARGC model to identify the specific factor responsible for the induction of apoptosis in damaged colon cells. Additional data was also collected to assess any potential side effects on blood parameters and hepatotoxicity evaluation.

4.3 Materials and Methods

4.3.1 Hypobranchial gland extraction, flash column chromatography and chemical analysis

A total of 1378 *D. orbita* (1146 small ones from 2 to 4 cm and 222 large ones from 4 to 7 cm) were collected from sea based abalone farm at Elliston, South Australia and frozen at -20°C. Hypobranchial glands were dissected from all frozen *D. orbita* and soaked in chloroform and methanol (1:1, v/v, HPLC grade, Sigma Aldrich) under agitation at room temperature. The extract was gained according to the method previously described by Westley et al. (2010). All extracts were combined to obtain a total weight of 7.83g crude extract, which was then stored at -20°C until use. Flash silica chromatography pressurized with nitrogen gas was used to purify tyrindoleninone and 6-bromoisatin (Chapter 2). The extract was passed through a silica column and the fractions were collected; briefly, tyrindoleninone was purified

as a bright orange fraction using dichloromethane and hexane (1:4, v/v) and 6-bromoisatin was subsequently semi-purified using 10% methanol in dichloromethane as the solvent system. Both fractions were dried and analyzed, along with the crude extract, by liquid chromatography (Waters Alliance) coupled to a mass spectrometer (MS, Micromass, Quatro micro™) and stored at -20°C. LC/MS separation was performed on a Hydro-RP C18 column with parallel UV/Vis diode-array detection (300 & 600 nm), a flow rate of 1 mL/min of formic acid and a gradient of acetonitrile in water, according to the methods established by Westley and Benkendorff (2008).

4.3.2 *In vivo* rodent model

In this experiment, an established rodent model was used for early stage prevention of colon cancer (Hu et al., 2002, Le Leu et al., 2002, Hu et al., 2005, Westley et al., 2010). Male mice (wild-type C57BL/6J), 10 weeks old were obtained from the Animal Resource Centre, Perth, Western Australia. Animal ethics and protocol were approved by the Animal Welfare Committee at Flinders University (Approval number: 751/10). Animals were divided randomly into 9 groups (ten mice per group) and housed in 18 cages (five mice per cage). The mice were maintained at the temperature of 22 ± 2°C and humidity of 80 ± 10% with a 12 h light/dark cycle. The mice were given water and food (rodent chow) *ad libitum* and monitored daily for any sign of illness, stool consistency, rectal bleeding and normal behavior such as grooming. All mice were weighed at days 1, 5, 10 and 14 of the experiment. Treatment groups included: 1) 0.5 mg/g crude extract, 2) 0.25 mg/g crude extract, 3) 0.1 mg/g tyrindoleninone, 4) 0.05 mg/g tyrindoleninone, 5) 0.25 mg/g tyrindoleninone, 6) 0.1 mg/g semi-purified 6-bromoisatin and 7) 0.05 mg/g semi-purified 6-bromoisatin. The extracts and purified compounds were administered to mice by daily oral gavage in 100 µl sunflower oil

containing 0.02% Vitamin E for two weeks (Figure 4.1). There were also two control groups gavaged with sunflower oil (containing 0.02% Vitamin E) only. At the end of the two week period each mouse in all of the treatment groups and one of the control groups was injected with a single intraperitoneal (i.p.) injection of AOM (Sigma Aldrich, Australia) at a dosage of 10 mg/kg bodyweight, to induce DNA damage. A second control group received a saline injection instead of the AOM. The mice were euthanized 6 h later by cervical dislocation under ketamine/xylazine anesthesia. The colon was excised immediately post-mortem. Two centimeters of distal colon were fixed in 10% buffered formalin (24 h) and subsequently embedded in paraffin for histological and immunohistological examination.

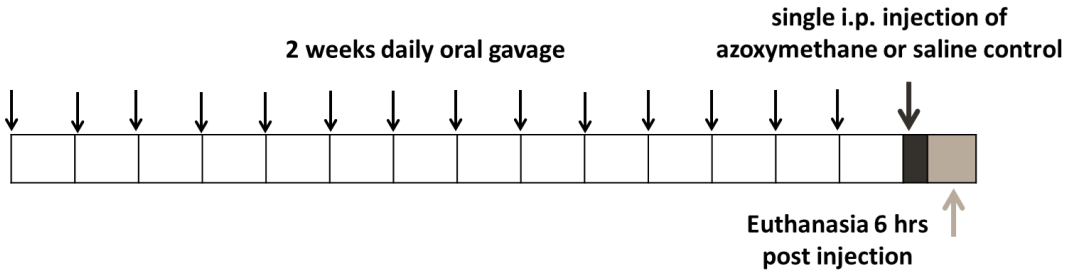

Figure 4.1 Experimental timeline for the two week AOM- induced rodent model for colon cancer prevention.

4.3.3 Measurement of apoptosis using hematoxylin staining

The apoptotic epithelial cells of distal colon sections were determined by hematoxylin staining as described by Hu et al. (2005). Distal colon sections were embedded in paraffin, sectioned at 4 µm (3-4 sections per mouse), stained with haemotoxylin and

examined under a light microscope (Olympus, BH-2) at x400 magnification. Apoptotic cells were identified in 20 randomly chosen complete crypts by characteristic morphological changes of; cell shrinkage, condensed chromatin and sharply delineated cell borders surrounded by an unstained halo (Le Leu et al., 2002, Potten et al., 1992). The percent of apoptotic cells were calculated as the mean number of apoptotic cells/crypt, divided by total number of cells in the crypt and multiplied by 100. The height of each crypt was determined along with the position of apoptotic cells (Le Leu et al., 2005).

4.3.4 Determination of epithelial proliferation (immunohistochemical staining)

Ki-67 is a cell cycle associated antigen and regarded as a useful epithelial cell proliferation marker (Cordes et al., 2009). Distal colon segments were embedded in paraffin then sectioned at 4 μm. Sections were de-waxed in HistoclearTM and hydrogen peroxide (3%) was used for 15 min to quench the endogenous peroxidase activity. Antigen retrieval was achieved by cooking the sections in a pressure cooker at 120°C for 1 hour in 0.1 mol/l citrate buffer (pH= 6.5). Sections were incubated with a primary monoclonal Ki-67 antibody (1:1000; Dako) at 4°C overnight. For detection of the primary antibody, biotinylated secondary rabbit- anti-mouse antibody (1:200; Dako) for 30 min and avidin/biotinylated peroxidase complex (Signet Laboratories USA-HRP kit) for 20 min were used. Slides were visualized by incubating with 3'-diaminobenzamine (DAB) substrate (Signet Laboratories USA-HRP kit) for 3 min, followed by counterstaining for 1 min in haematoxylin and examining under a light microscope (Olympus, BH-2) at x400 magnification. The percent of proliferating cells was calculated in the same way as the apoptosis index described above.

4.3.5 Histopathological evaluation of the liver

Histopathological variables are indicative of toxicity induced by cytotoxic or antimitotic drugs (Greaves, 2007). The liver was therefore removed from each mouse and weighed. Liver weight was standardized by measuring the percent of the liver weight divided by the body weight for each mouse. Livers were then fixed in 10% buffered formalin and embedded in paraffin. Four micrometer sections of liver were stained with hematoxylin and eosin for histopathological examination of potential toxicity (Masson et al., 2010) under light microscopy (Olympus, BH-2). Hepatotoxicity indictors that were assessed included; porphyrin, hepatocellular hyperplasia and hypertrophy, mallory bodies, haemosiderin, sinusoidal dilation, congestion, haemorrhage, lipofuscin, steatosis (fatty change), necrosis and inflammation (Greaves, 2007, Ferrell, 2010).

4.3.6 Blood analysis (hematology and biochemistry)

Before euthanizing the mice, blood samples (0.5-1 mL) were obtained by cardiac puncture into heparinized vacutainer tubes, under anesthesia and transferred to Gribbles Veterinary Pathology laboratory in Adelaide for hematology and biochemistry analysis. The samples were run through the Abbott Cell Dyn 3700 analyzer for hematology assessment and Siemens Advia 1800 chemistry analyzer for biochemistry analysis. The serum levels of liver enzymes including aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were assessed as indicators of hepatotoxicity (Hewawasam et al., 2004).

4.3.7 Statistical analysis

Statistical analyses were performed using SPSS (Smart Viewer 15.0) and P values of ≤ 0.05 were considered to be statistically significant. A one way ANOVA test was performed to compare between treatments and controls across the different concentrations. Tukey HSD post-hoc test was applied to detect which groups significantly differ. Levene test was used to check homogeneity of variances and in all cases $p > 0.05$. The full statistical results for these experiments are provided in Appendix 7.2.

4.4 Results

4.4.1 Chemical analysis

LC-MS analysis of *D. orbita* crude extract revealed at least five peaks attributed to brominated indole compounds (Figure 4.2 a). The dominant peak at t_R 6.37 min occurred with major ions in ESI-MS at *m/z* 224, 226 is indicative of the molecular mass of 6-bromoisatin. Mass spectrum of the peak at t_R 9.35 min with major ions in ESI-MS at *m/z* 302, 304 corresponds to tyrindolinone. The small peak at t_R 10.44 min is attributed to tyrindoxyl sulphate, with major ions in ES-MS at *m/z* 336, 338. The peak at t_R 11.00 min is indicative of the molecular weight of tyrindoleninone with major ions at *m/z* 255, 257 and a peak at t_R 11.71 min with ions in ESI-MS at *m/z* 511, 513, 515 corresponds the molecular mass of tyriverdin with major fragment ions at *m/z* 417, 419, 421 formed by the elimination of dimethyl disulphide. LC-MS analysis of the purified compounds showed a single peak at t_R 11.00 min corresponding to tyrindoleninone (Figure 4.2 b) and a dominant peak at t_R 6.37 min corresponding to 6-bromoisatin with several other minor peaks (Figure 4.2 c).

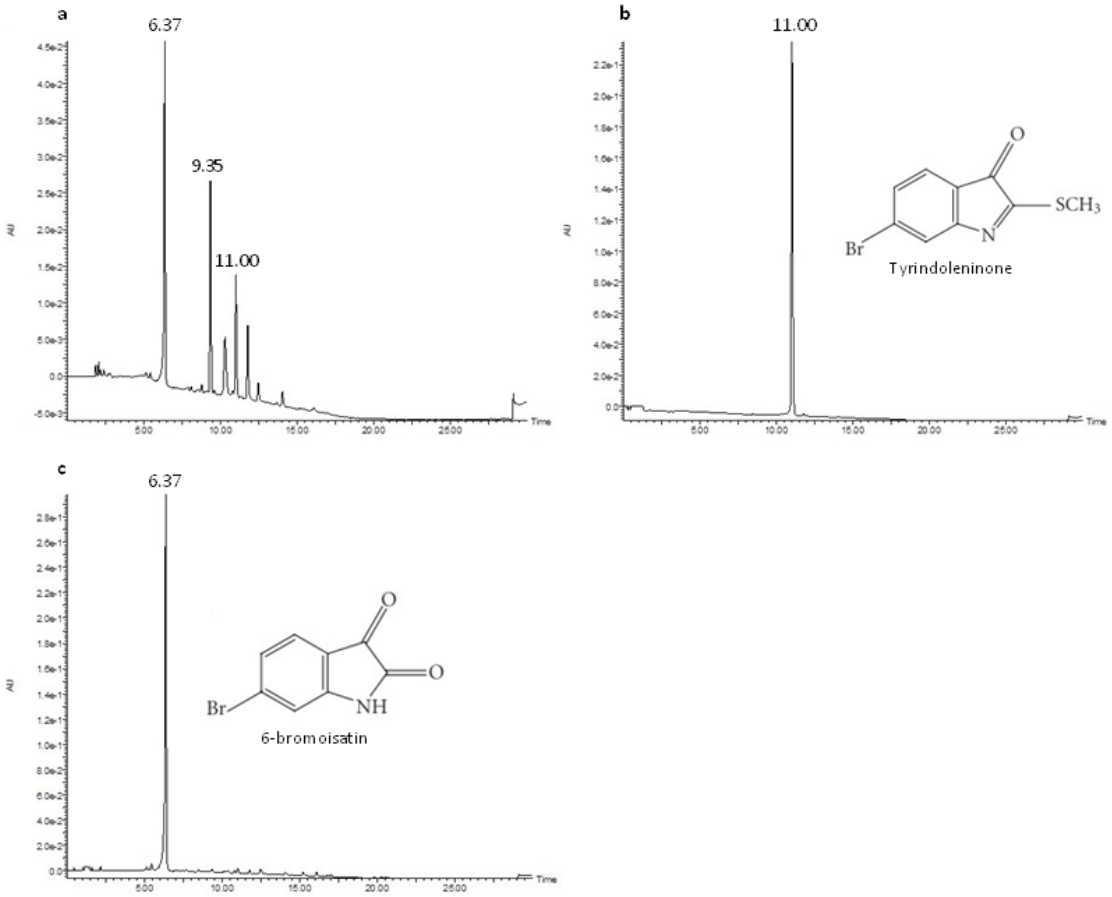

Figure 4.2 Liquid chromatography- mass spectrometry analysis of a) crude extract from hypobranchial gland of *D. orbita*; b) purified tyrindoleninone and c) semi-purified 6-bromoisatin.

4.4.2 *In vivo* model - General observations

There were no signs of illness (i.e. no vomiting, diarrhea, constipation, dysphagia, hematemesis or loss of appetite) in any of the treatment groups or control animals throughout the study. The body weights of all mice increased steadily over the trial duration (Table 4.1); no significant differences in mean total weight gain was revealed either between the saline control and AOM control, or the AOM control group and treatment groups by analysis of variance (Table 4.1). The liver of all mice were excised and weighed at the end of the experiment. The results showed a significant increase in the percent liver to body weight (~25%) for the AOM control compared to saline control ($P \leq 0.01$). No significant change in the liver weight was revealed between AOM control and any treatment group (Table 4.1).

Table 4.1 Comparison of mean (±S.E.) progressive body weight (g) in control and treatment mice on different experimental days; Liver weight (g) and percent of liver weight/body weight were calculated on the day of kill. TYR= tyrindoleninone and 6-BRO= semi-purified 6-bromoisatin, CE= crude extract, (n = 10 mice all groups). Significant difference between the AOM control and treatment or saline control are shown as $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

	Weight (g)						
	Body (day 1)	**Body (day 5)**	**Body (day 10)**	**Body (day 14)**	**Total weight gain**	**Liver**	**Liver / body (%)**
Saline control	24.6±1.1	25.0±0.9	25.2±1.0	25.1±1.1	0.5±0.4	1.1±0.2	4.2±0.6**
AOM control	24.2±1.4	24.5±1.4	25.1±1.6	25.0±1.6	0.7±0.5	1.3±0.1	5.3±0.5
TYR 0.025 mg/g	22.9±1.0	23.0±1.1	23.9±1.4	24.5±1.7	1.6±1.2	1.1±0.1	4.7±0.7
TYR 0.05 mg/g	23.9±1.0	24.1±1.2	24.8±1.3	24.6±1.2	0.7±0.5	1.3±0.1	5.2±0.4
TYR 0.1 mg/g	23.0±1.6	23.3±1.5	23.6±1.5	23.7±1.6	0.6±2.0	1.2±0.2	5.3±0.9
6-BRO 0.05 mg/g	24.9±0.9	25.1±1.2	25.5±1.2	25.5±1.3	0.6±0.9	1.2±0.1	4.8±0.4
6-BRO 0.1 mg/g	25.9±1.1	25.8±1.3	26.2±1.4	26.4±1.4	0.5±0.5	1.4±0.1	5.2±0.2
CE 0.25 mg/g	24.7±1.2	25.3±1.3	25.7±1.6	26.1±1.6	1.4±0.6	1.4±0.1	5.3±0.5
CE 0.5 mg/g	24.0±2.0	24.5±2.1	24.5±2.4	25.1±2.3	1.1±0.4	1.3±0.2	5.2±0.5

4.4.3 Apoptotic index and crypt height

Apoptotic cells were rarely detected in the colon crypts of the saline control, whereas in response to AOM injection, control mice initiated a low background level of apoptosis (Figure 4.3). Apoptosis significantly increased, by 2.3 fold of the AOM control, in the distal colon of animals administered semi-purified 6-bromoisatin, at both concentrations of 0.05 mg/g and 0.1 mg/g ($p \leq 0.001$). A significant increase in the apoptotic index was also revealed between AOM injected control mice and those administered 0.25 mg/g ($p = 0.028$) and 0.5 mg/g ($p \leq 0.001$) of crude extract, with a clear dose effect (Figure 4.3). In contrast, although mice that were treated with tyrindoleninone on average showed a slightly increased apoptosis index at the maximum dose, there was no significant difference when compared to the AOM injected control. Apoptosis in the distal colon of animals occurred mostly in basal crypt cells (Figure 4.4). Mean crypt height in distal colon was similar between all treatment groups and saline control (Figure 4.5). However, two treatment groups showed a significant decrease in mean crypt height; 0.5 mg/g crude extract ($p = 0.05$) and 0.025 mg/g tyrindoleninone ($p = 0.024$), in comparison to the AOM control (Figure 4.5).

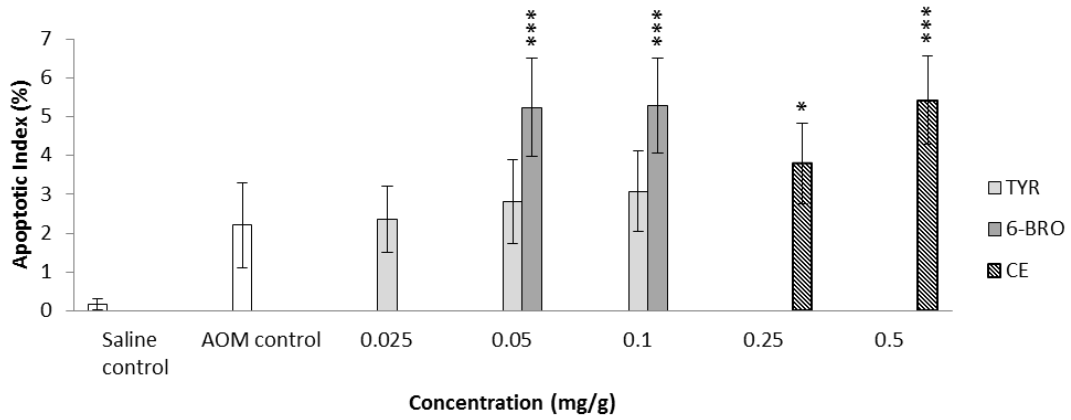

Figure 4.3 Apoptotic response in the epithelial cells of crypts in the distal colon after 14 day oral gavage of different concentrations of tyrindoleninone (TYR), semi-purified 6-bromoisatin (6-BRO) and crude extract (CE). The purified compounds were tested at lower concentrations than the crude extract. All treatments and the AOM control were injected with 10 mg/kg AOM, a genotoxic carcinogen, 6 h prior to kill. Data are expressed as apoptotic index within the distal colon. Data are means ± S.E. for 10 mice per group (10 full crypts/animal). Significant difference between each group and the AOM control are shown as $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

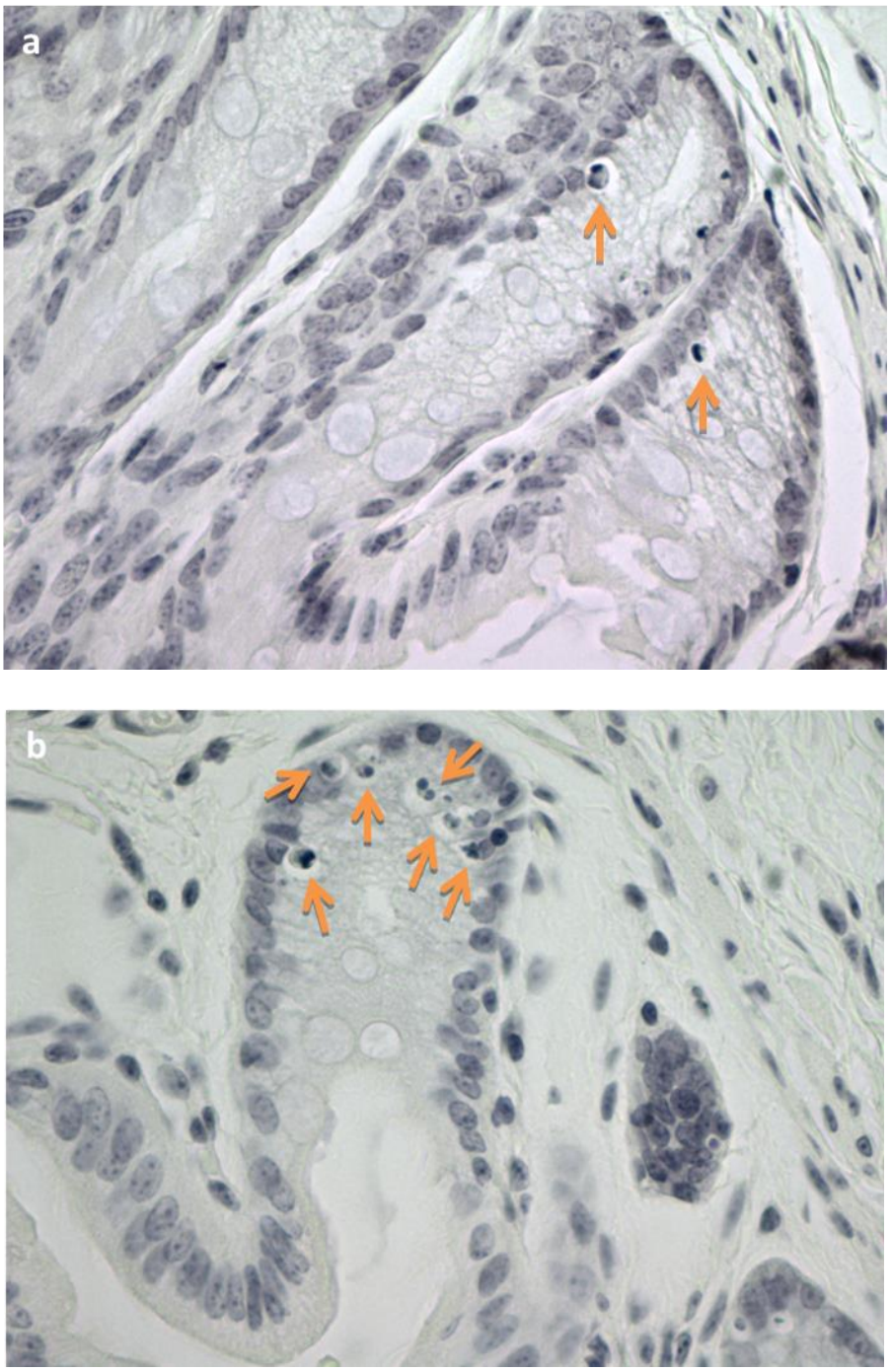

Figure 4.4 Apoptosis in distal colon crypts of a) AOM control and b) mouse administered 0.1 mg/g semi-purified 6-bromoisatin (6-BRO) for 14 days. Apoptotic cells - indicated by arrows - with condensed chromatin occurred mostly in basal crypt cells (Original magnification x400).

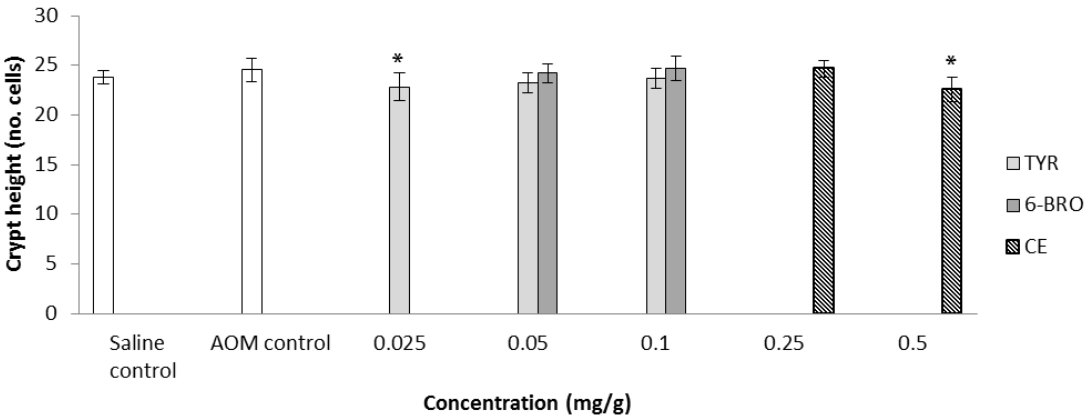

Figure 4.5 Mean (±S.E.) crypt height in the distal colon of control mice and treatment groups including different concentrations of purified tyrindoleninone (TYR), semi-purified 6-bromoisatin (6-BRO) and crude extract (CE). Significant difference between each group and the AOM control are shown as $p \leq 0.05$ (*)

4.4.4 Proliferation index

Mean proliferation index in the distal colon (Figure 4.6) reduced significantly in mice treated with semi-purified 6-bromoisatin at concentrations of 0.05 mg/g and 0.1 mg/g, compared to the AOM control ($p \leq 0.001$), (Figure 4.7). Similarly, tyrindoleninone administration at 0.05 and 0.1 mg/g resulted in a significant reduction of proliferation compared to the AOM control ($p \leq 0.01$), whereas tyrindoleninone at the concentration of 0.025 mg/g had no significant effect on the proliferation index ($p = 0.123$). A significant reduction in proliferative cells was also observed in mice treated with the crude extract at both concentrations of 0.25 and 0.5 mg/g ($p \leq 0.001$).

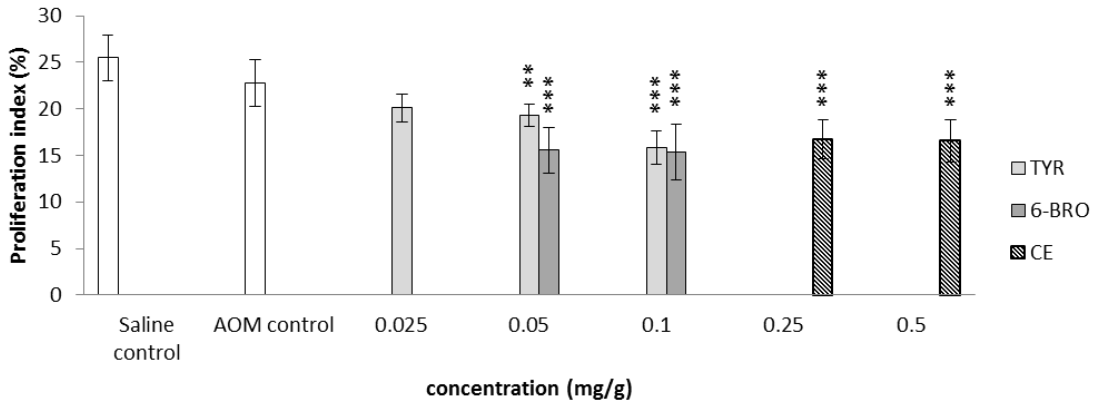

Figure 4.6 Proliferation index in the distal colon of control groups and animals treated with different concentrations of purified tyrindoleninone (TYR), semi-purified 6-bromoisatin (6-BRO) and crude extract (CE) for 14 days. Epithelial cell proliferation was measure using the primary monoclonal Ki-67 antibody. Significant differences between each group and the DMSO control are shown as $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

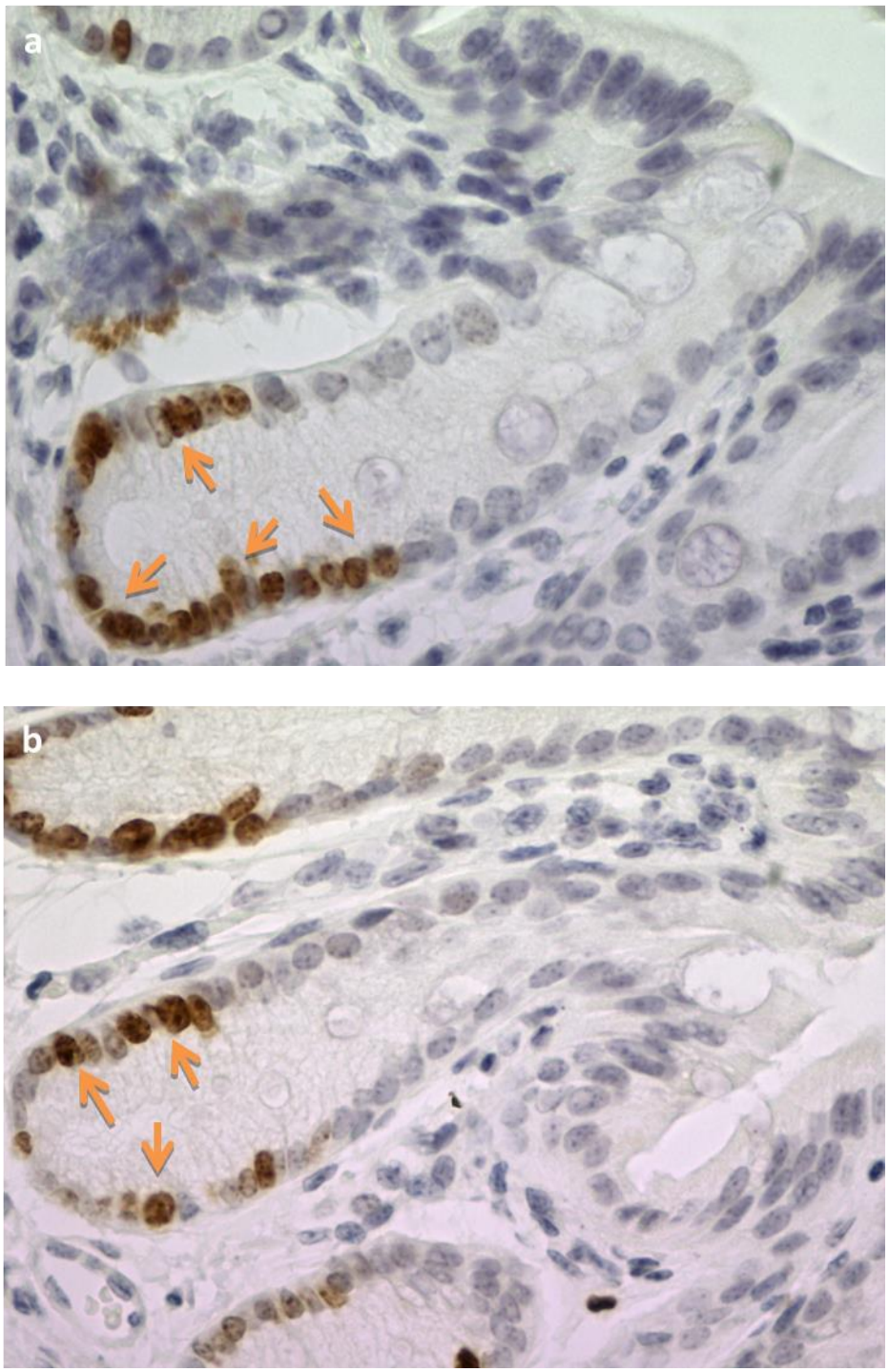

Figure 4.7 Proliferative activity using an antibody specific for the ki-67 antigen in distal colon crypts of a) AOM control and b) mouse administered 0.1 mg/g semi-purified 6-bromoisatin (6-BRO) for 14 days. Proliferating cells are shown by arrows (Original magnification x400).

4.4.5 Histopathology evaluation of the liver

Using light microscopy, the predominant histopathological alteration in the liver was the presence of numerous cytoplasmic vacuoles consistent with microvesicular steatosis (fatty liver), with varying degrees of sinusoidal dilation and congestion (Figure 4.8). This effect was induced by injection with AOM, relative to the saline controls. There were no further changes, with regard to microvesicular steatosis (fatty liver), sinusoidal dilatation, or red blood cell extravasation into the space of Disse, observed in the mice treated with either compound or extract, as compared to AOM injected controls.

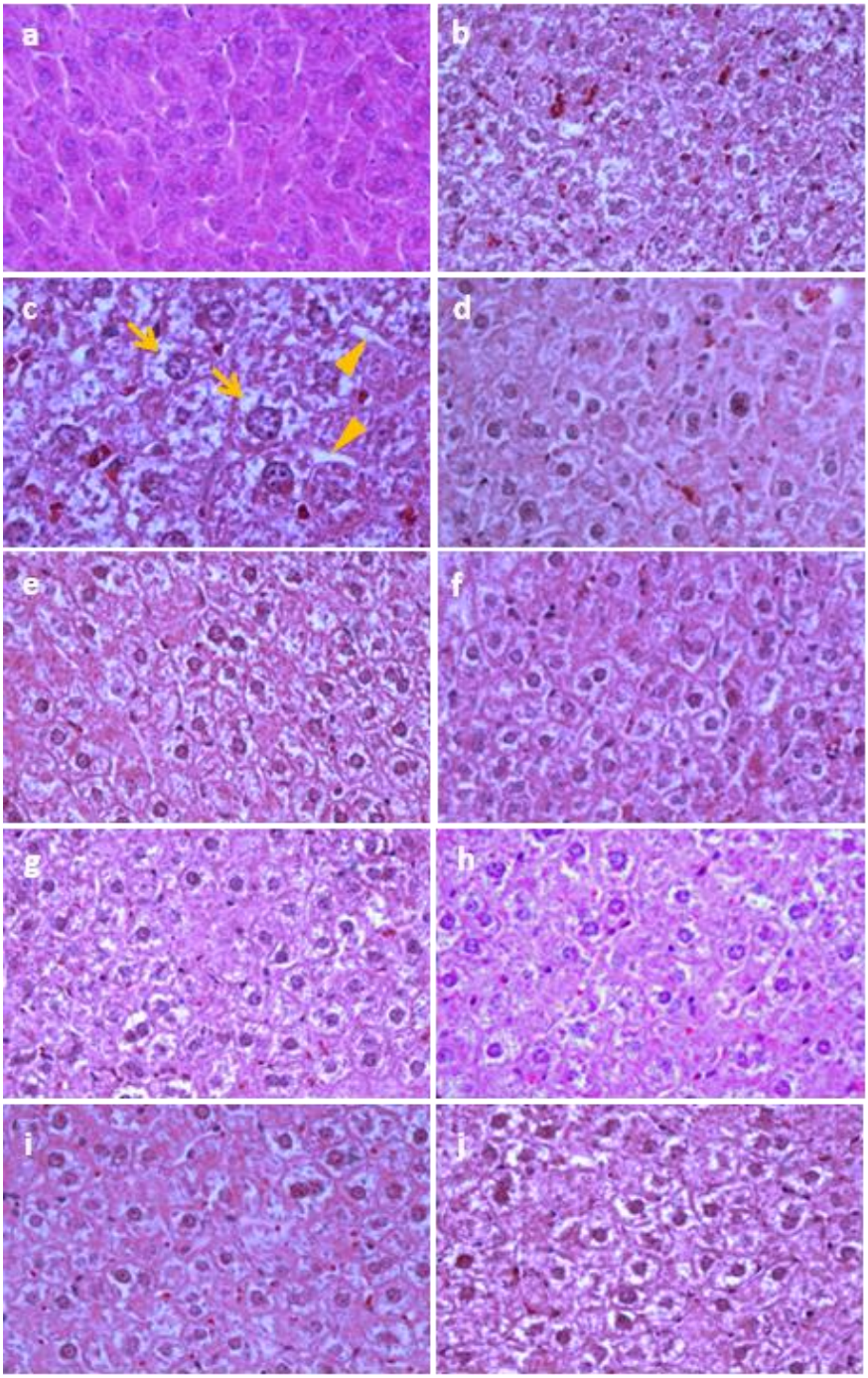

Figure 4.8 Microscopic images of sectioned liver tissue (Original magnification x200 except "c" which is 400x) from mice in control and treatment groups stained by hematoxylin and eosin: a) Saline control; b) AOM control; c) AOM control (400x); d) Tyrindoleninone 0.025 mg/g; e) Tyrindoleninone 0.05 mg/g; f) Tyrindoleninone 0.1 mg/g; g) Semi-purified 6-bromoisatin 0.05 mg/g; h) Semi-purified 6-bromoisatin 0.1 mg/g; i) Crude extract 0.25 mg/g; j) Crude extract 0.5 mg/g. Microvesicular steatosis (shown by arrow) was the predominant histopathological finding with varying degree of sinusoidal dilatation (shown by headarrow) and congestion in all AOM injected mice after 6 h.

4.4.6 Liver enzyme alteration

Liver toxicity was monitored by quantitative analysis of the serum AST, ALT and ALP activities that are known as biochemical markers of liver toxicity. AST levels were higher in AOM controls compared to saline control (Figure 4.9), but these were not significantly different ($p = 0.430$). Similarly, no significant differences were found in the serum AST between AOM controls versus individual treatment groups. However, a combined effect of AOM and tyrindoleninone was observed on the AST levels; mice treated with tyrindoleninone (0.1 mg/g) showed a significant increase in comparison with saline control ($p \leq 0.05$). ALT activity in the serum did not show any significant change in AOM controls, compared to the saline controls ($p = 0.287$), or in treatment groups relative to the AOM control. ALP level was similar between the two control groups ($P = 0.997$) and all treatment groups.

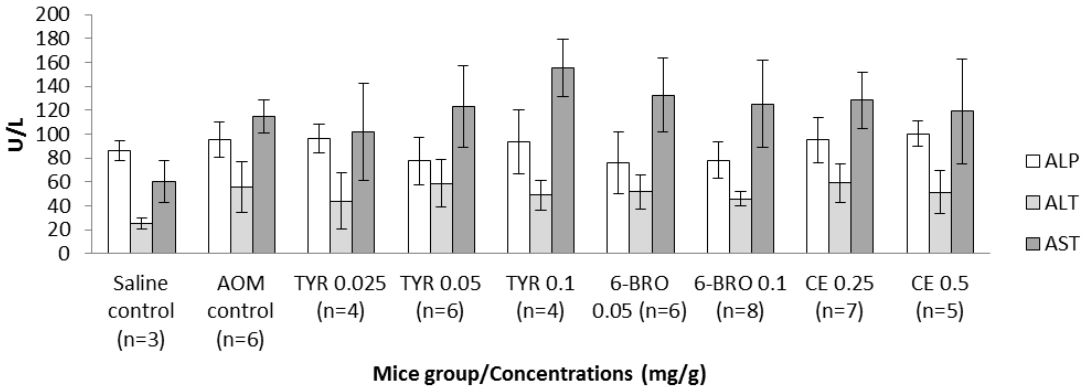

Figure 4.9 Liver enzymes *aspartate aminotransferase* (AST), *alanine aminotransferase* (ALT) *and alkaline phosphatase* (ALP) levels in serum (U/L) of controls and treatment groups including purified tyrindoleninone (TYR), semi-purified 6-bromoisatin (6-BRO) and crude extract (CE). There is no significant difference between AOM control and treatment groups or saline control.

4.4.7 Hematology and other serum biochemical alterations

Hematology results from the mouse blood samples including red blood count, hemoglobin, hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white cell count, neutrophil number, lymphocytes and monocytes, are summarized in Table 4.2. The results revealed a significant reduction of lymphocytes in the AOM control group compared to the saline control ($p \leq 0.01$). By contrast, the number of neutrophils increased significantly in the AOM control ($p \leq 0.05$). In treatment groups, red cell count and hemoglobin concentration showed a significant reduction in the mice treated with 0.1 mg/g tyrindoleninone in comparison with AOM control ($p \leq 0.05$), whereas monocytes were significantly elevated in mice treated with 0.05 mg/g tyrindoleninone. White cell count and the number of neutrophils was reduced significantly in the 6-bromoisatin group at the lower concentration of 0.05 mg/g compared to AOM control ($p \leq 0.05$). However, the number of neutrophils in 6-bromoisatin group (0.05 mg/g) was not significantly different compared with saline control.

The concentration of serum biochemical parameters such as sodium, potassium, sodium/potassium ratio (Na/K), urea, creatinine, calcium, protein, albumin and globulin were also measured (Table 4.3). There was a significant elevation ($p \leq 0.001$) in the urea level of the AOM control (11.81±1.39 mmol/L) in comparison to the saline control (9.01±1.62 mmol/L). Globulin protein level was decreased significantly, but not with a clear dose dependent response, in the mice administered tyrindoleninone (Table 4.3). Conversely, 6-bromoisatin (0.05 mg/g) increased the level of this protein in comparison to the AOM control.

Table 4.2 Hematology results from mouse blood samples at the end of a two week period of oral gavage with oil (controls) or muricid mollusc extract or compounds, followed by injection with azoxymethane (AOM) or saline. (TYR= tyrindoleninone, 6-BRO= semi-purified 6-bromoisatin, CE= crude extract). Significant difference between each group and the AOM control are shown as $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

	Red cell count ($x 10^{12}$/L)	**Hemoglobin (g/L)**	**Hct (L/L)**	**MCV (FL)**	**MCH (Pg)**	**MCHC (g/L)**	**White cell count ($x 10^9$/L)**	**Neutrophils ($x 10^9$/L)**	**Lymphocytes ($x 10^9$/L)**	**Monocytes ($x 10^9$/L)**
Saline control (n=3)	8.9±0.2	135.6±3.5	0.43±0.0	48.3±1.5	15.3±0.5	312.6±3.7	6.4±1.7	0.5±0.3*	5.5±2.0**	0.2±0.1
AOM control (n=6)	9.1±0.2	135.8±1.9	0.42±0.01	46.3±1.3	15.0±0.0	322.1±6.6	5.1±1.1	2.2±1.0	2.6±0.9	0.2±0.1
TYR 0.025 mg/g (n=3)	8.6±0.4	131.7±5.3	0.41±0.01	48.7±0.9	15.2±0.5	316.7±2.5	4.3±0.5	0.9±0.9	3.3±1.0	0.1±0.1
TYR 0.05 mg/g (n=5)	8.8±0.2	133.1±4.5	0.41±0.01	47.3±0.5	15.0±0.0	319.6±3.7	5.3±0.5	1.3±0.5	3.2±0.7	0.6±0.3*
TYR 0.1 mg/g (n=5)	8.2±0.7*	124.8±8.9*	0.40±0.02	48.2±1.9	15.2±1.3	321.5±4.7	5.3±2.1	2.3±1.0	2.7±1.1	0.2±0.3
6-BRO 0.05 mg/g (n=9)	9.4±0.4	140.0±7.6	0.44±0.01	46.6±0.7	14.7±0.4	317.7±6.2	2.8±1.3*	0.8±0.6*	1.8±0.5	0.1±0.1
6-BRO 0.1 mg/g (n=9)	9.0±0.2	136.1±3.2	0.42±0.01	47.0±0.7	15.0±0.0	321.7±2.5	5.1±1.1	2.1±0.4	2.5±0.7	0.4±0.1
CE 0.25 mg/g (n=7)	8.6±0.2	132.0±3.5	0.41±0.01	47.1±1.2	15.1±0.3	321.5±7.4	4.2±1.0	1.7±0.6	2.3±0.3	0.2±0.1
CE 0.5 mg/g (n=5)	8.9±0.1	130.0±4.7	0.42±0.007	47.2±0.8	14.6±0.5	314.0±8.1	5.6±0.4	2.0±0.5	3.3±0.8	0.2±0.1

Hct= Hematocrit, MCV= Mean Corpuscular Volume, MCH= Mean Corpuscular Hemoglobin, MCHC= Mean Corpuscular Hemoglobin Concentration

Table 4.3 Biochemical results of mouse serum samples (CE= crude extract, TYR= tyrindoleninone, 6-BRO= semi-purified 6-bromoisatin).

Significant difference between each group and the AOM control are shown as $p \leq 0.05$ (*) and $p \leq 0.001$ (***).

	Sodium (mmol/L)	**Potassium (mmol/L)**	**NA/K**	**Urea (mmol/L)**	**Creat. (umol/L)**	**Calcium (mmol/L)**	**Protein (g/L)**	**Albumin (g/L)**	**Globulin (g/L)**
Saline control (n=10)	143.0±1.7	6.1±1.4	24.6±6.5	9.0±1.6***	12.3±2.0	2.2±0.0	43.4±1.6	27.3±1.2	16.1±0.8
AOM control (n=7)	144.0±3.9	6.3±2.0	25.3±10.1	11.8±1.3	15.8±3.5	2.1±0.1	46.7±2.3	29.4±1.9	17.2±0.9
TYR 0.025 mg/g (n=10)	139.9±7.0	6.4±3.7	22.3±14.5	10.0±0.6	18.8±10.9	1.9±0.2	47.0±4.2	31.2±4.4	15.8±0.6*
TYR 0.05 mg/g (n=10)	141.9±6.2	6.7±3.7	27.3±9.5	10.8±1.5	14.6±4.8	2.0±0.2	48.2±5.1	31.3±4.6	16.9±0.9
TYR 0.1 mg/g (n=4)	143.0±2.4	6.1±2.7	27.0±11.2	10.2±2.1	15.2±3.5	2.1±0.0	43.7±3.7	28.2±3.3	15.5±0.5*
6-BRO 0.05 mg/g (n=10)	141.1±2.6	5.8±0.7	24.4±2.7	10.0±1.3	12.4±1.0	2.1±0.0	49.3±1.5	30.5±1.0	18.8±0.7*
6-BRO 0.1 mg/g (n=9)	143.5±1.5	5.9±0.4	22.2±7.4	11.0±0.8	13.2±1.0	2.1±0.0	47.4±2.0	29.2±1.3	18.2±0.8
CE 0.25 mg/g (n=10)	142.5±4.4	6.6±3.1	25.2±9.1	10.4±1.0	17.8±8.2	2.0±0.1	46.7±3.3	30.0±3.3	16.6±1.2
CE 0.5 mg/g (n=7)	142.4±4.3	6.5±2.9	24.8±7.9	10.4±0.9	15.2±8.0	2.1±0.1	47.5±1.8	30.2±2.1	17.2±0.9

Na/K= Sodium/Potassium ratio, Creat= Creatinine

4.5 Discussion

This study revealed that both the crude Muricidae extract and semi-purified 6-bromoisatin significantly increased the apoptotic index after administration of a DNA damaging agent, and also inhibited cell proliferation in the distal colon of mice, without any sign of toxicity. LCMS analysis revealed 6-bromoisatin as a major compound in both the crude extract and semi-purified 6-bromoisatin fraction, and the enhanced bioactivity within the semi-purified fraction, indicates that 6-bromoisatin is the main active component. In contrast, purified tyrindoleninone did not increase the apoptotic index after AOM injection, but significantly reduced the cell proliferation index, in a dose-dependent manner, in the distal colon. However, there was also some evidence for toxicity in the liver and mild anaemia in the mice corresponding to the administration of tyrindoleninone.

The inhibition of CRC has been previously linked with AARGC stimulation (Hu et al., 2008, Le Leu et al., 2010). The increased apoptotic index in the lower crypt of distal colonic mucosa, after treatment with muricid mollusc crude extract and semi-purified 6-bromoisatin, agrees with the known enhancement of AOM-induced AARGC in basal crypt cells (Hu et al., 2002) This highlights the CRC chemopreventative effect of the crude extract and semi-purified 6-bromoisatin. In our study, crude extract (0.25 and 0.5 mg/g) had a dose-dependent effect in inducing apoptosis in the distal colon of mice. In a previous study by Westley et al. (2010), oral administration of muricid extract with the concentration of 0.125 mg/g for 4 weeks in an AOM mice model did not have any significant increase in the apoptotic index in the distal colon. The highest concentration of 1mg/g significantly increased apoptotic cells in the distal colon crypts (Westley et al., 2010), but some idiosyncratic toxicity

in the liver was found at this concentration of extract in a separate toxicity study (Westley et al., 2013). In the current study, we were able to simultaneously investigate potential toxicity and efficacy to establish an optimal dose of 0.5mg/g.

The results from semi-purified 6-bromoisatin in this study is consistent with a recent *in vitro* study on colorectal cancer cells (Chapter 3), which revealed the significant induction of apoptosis via activating caspase-3 and -7 enzymes in both human colon adenocarcinoma (HT29) and human epithelial colorectal adenocarcinoma (Caco-2) treated with ~100 µM (0.025 mg/mL) and ~200 µM (0.05 mg/mL) 6-bromoisatin (Chapter 3). In another study by Edwards et al. (2012), approximately 22 µM 6-bromoisatin significantly activated caspase 3/7 and induced apoptosis in a ovarian granulosa tumor (KGN) cell line. Some structurally similar isatin and indole compounds have been also shown to induce apoptosis in a range of cancer cell lines (Vine et al., 2007a, Vine et al., 2007b, Weng et al., 2007). For example, 5,6,7-tribromoisatin at a concentration of 8 µM induced apoptosis through activation of caspase 3/7 in the Jurkat cell line (Vine et al., 2007a). This further supports the potential for developing simple naturally derived brominated isatin derivatives for the prevention or treatment of cancer.

Tyrindoleninone, on the other hand, did not significantly increase the apoptosis index in the distal colon of mice. Our previous *in vitro* study revealed that 195 µM (0.05 mg/mL) tyrindoleninone significantly induced apoptosis by activating caspase -3 and -7 activity in HT29 cell line while this compound caused necrosis in Caco-2 cells and did not induce apoptosis in these cells (Chapter 3). In another *in vitro* study by Edwards et al. (2012), 20 µM tyrindoleninone showed a significant induction of apoptosis on KGN cell line. This indicates an inconsistent apoptotic effect of

tyrindoleninone against different cancer cell lines, and in combination with the lack of *in vivo* activity observed in this study, this compound is a lower priority for future development for colon cancer prevention.

In addition to enhancing AARGC in the mice administered with crude extract, the proliferation index significantly reduced in these mice compared to the AOM control group. This may convey further protection in the crude extract group, by reducing the number of mutated cells that could be otherwise generated during the proliferation of epithelial cells. The previous *in vivo* study by Westley et al. (2010), did not reveal any significant difference in proliferation index in mice distal colon by administrating 1 mg/g crude extract compared with the control group, while this study clearly indicates an anti-proliferative effect at lower doses. *In vitro* studies, support the significant anti-proliferative effect of *D. orbita* egg extracts against the colorectal cancer cell lines HT29 and Caco-2 (Chapter 3); (Benkendorff et al., 2011). Semi- purified 6-bromoisatin (0.05 mg/g) also had a similar anti-proliferative effect *in vivo*, to the crude extract (0.5 mg/g), but at 10 times lower concentration than the crude extract, indicating 6-bromoisatin as the main anti-proliferative compound. In our recent *in vitro* study, 6-bromoisatin significantly inhibited the proliferation of both colorectal cancer cells Caco2 and HT29 with an IC50= ~100 μM (0.025 mg/mL); (Chapter 3). This indicates the consistent anti-proliferative effect of 6-bromoisatin on colorectal cancer cell lines and epithelial cells in distal colon crypts *in vivo*.

The specificity of isatin derivatives, including 6-bromoistain, against cancer cell lines over freshly isolated healthy human cells has been reported by Vine *et al.* (2007a) and Edwards et al. (2012). The other compound tyrindoleninone, while not as potent as 6-bromoisatin, inhibited the proliferation of distal colon epithelial cells at the highest

concentrations (0.05 and 0.1 mg/g). In Chapter 3, tyrindoleninone was found to be cytotoxic towards Caco2 cells at IC50= 98 μM and HT29 cells with IC50= 390 μM. The anti-proliferative effect of tyrindoleninone was also shown in some other cell lines such as KGN, JAr and OVCAR-3 (Edwards et al., 2012). These results all indicate a consistant anti-proliferative effect of tyrindoleninone against colorectal cancer *in vitro* and *in vivo*, which suggests the possibility for synergistic action through a different mode of action to 6-bromoisatin in the extracts.Thus both the crude extract and 6-bromoistatin should be tested in longer term models for colon cancer prevention.

No significant differences in the level of the liver enzymes AST, ALT and ALP were found between AOM control and mice treated with the muricid extract of purified compounds, indicative of low or non-toxicity associated with these extract or compounds. In a study by Bélanger et al. (2006), a significant increase in the serum level of AST and ALT was detected after the injection of AOM (100 mg/kg) in C57BL male mice, followed by the development of acute liver injury, encephalopathy, coma and death in the animals. A slight but nonsignificant increase in AST and ALT levels in the AOM control group was observed compared to saline control. The mice in the study by Belanger et al. (2006) were administrated much 10 times the dose of AOM resulting in severe toxicity in the liver, in comparison to our study which revealed that lower doses (10 mg/kg) of AOM do not produce an immediate effect (within 6 h) on the release of these liver enzymes. Another study by Matkowskyj et al. (1999) showed similar levels of ALT in the serum of C57BL/6J mice following the injection of 0.1 mg/g AOM (56 ± 5 U/l) after 4 h compared with a saline control (64 ± 14 U/l). However, the levels of ALT increased significantly with the presence of centrilobular necrosis in hepatocytes 20 h after AOM administration (5,196 ± 126 U/l),

(Matkowskyj et al., 1999). This indicates the necessity of longer times to induce necrosis in hepatocytes after administration of AOM and also highlights the correlation between hepatic necrosis and increased level of liver enzymes, especially ALT in the mice serum. This finding, along with the results from our study, demonstrate that it is possible to simultaneously assess the effects of chemopreventative agents on blood liver enzymes using the short-term AOM colon cancer model because the background levels of liver enzymes do not show any significant increase in the animals a short time (6 h) after AOM injection, in comparison with saline control.

In our study, although AST levels in AOM controls did not increase significantly compared with the saline control, the combination of AOM and tyrindoleninone (0.1 mg/mL) caused a significant increase in AST, indicating the presence of toxicity in the liver associated with the administration of tyrindoleninone along with a DNA damaging agent. AOM is metabolized via hydroxylation of the methyl group mainly by CYP2E1 to the genotoxic carcinogen methylazoxymethanol in the liver (Weisburger et al., 1998, Chen and Huang, 2009). The tyrindoleninone metabolism pathway has not yet been elucidated. However, of the enzymes needed for debromination and endogenous indole metabolism in the intestinal mucosa, CYP2C19 (Zhang et al., 1999) plays a minor role in indole oxidation (Gillam et al., 2000) and glutathione-S-transferase (GST) (Prueksaritanont et al., 1996) can mediate debromination in phase 2, after oxidation by CYP2E1 (Sherratt et al., 1998). This study suggests that tyrindoleninone metabolism might be associated with CYP2E1, which can affect AOM metabolism and increase the toxic side-effects of AOM in the liver. The other noteable change in biochemical factors was the decreased level of

globulin protein in the mice administered the tyrindoleninone, although this was not a dose dependent alteration. Globulin and albumin proteins are two main serum proteins in the blood that are produced by the liver (Madden and Whiffle, 1940). Damage to the liver by increasing the AST on top of the AOM toxicity corresponding to the highest concentration of tyrindoleninone level was shown in this study. Therefore, decreased production of globulin could provide further evidence of the liver damage associated with the administration of tyrindoleninone.

Liver histopathology, showed further evidence of hepatocyte damage due to AOM, which was consistent with mostly microvesicular steatosis and varying degree of sinusoidal dilation and congestion. These qualitative changes were observed in the AOM control and no further effect was discernible in the treated mice with either compound or extract. Microvesicular steatosis with varying degrees of hemorrhagic congestion and necrosis was shown in 15-week-old male C57BL/6 mice following the injection of 100 mg/kg AOM and sacrificing the animals after reaching the coma stage of encephalopathy (Bémeur et al., 2010a). This study did not show any sign of necrosis or hemorrage in the liver of AOM injected (10 mg/kg B.W) mice; however, the other hepatocyte alterations were consistent with Bémeur et al. (2010a). In another histological study by Matkowskyj et al. (1999), the mice showed microvesicular steatosis after 2 h and sinusoidal dilatation after 4 h of AOM administration (100 mg/kg), while centrilobular necrosis was only apparent after 20 h AOM injection. Macroscopically, the most striking alteration in the liver was the significant increase in the percent liver to body weight in the AOM control compared to saline control. Hepatic enlargement in the liver has been reported in the mice received 100 mg/kg AOM (Bémeur et al., 2010b) and our study confirms that this effect can occur at the

much lower dose of 10 mg/kg AOM. Consequently, although it is possible to assess the effects of chemopreventatives on blood liver enzymes using the short term AOM model, it is not possible to extract further information on the histopathological damage to the liver, due to the rapid over-riding short term effects of AOM.

According to the chart for anaemia severity in male mice established by the World Health Organization, the concentration ranges for mild anaemia is 10.7 to 12.4 g/dL (Groopman and Itri, 1999, Raabe et al., 2011). The mice treated with the highest concentration of tyrindoleninone (0.1 mg/g) had a significantly lower red cell counts and haemoglobin levels (12.4 ± 0.8 g/dL) compared to AOM control, thus indicating mild anaemia in these mice. The AOM control showed a sign of lymphopenia (low lymphocytes) and neutrophilia (high neutrophils) compared to the saline control. Clinical data shows that tumours can impair the function of lymphocytes and cause lymphopenia, leading to immune system dysfunction (de Visser et al., 2006). Immune function is impaired in tumor-bearing mice, manifested by decreased lymphocyte proliferative response and decreased lymphocyte lytic function (Mizoguchi et al., 1992). Despite the fact that AOM does not induce tumours within 6 h of administration, our results suggest that it can still affect the immune system by decreasing the level of lymphocytes. Neutrophils on the other hand, have a protective role in response to infection in the host (Pedrosa et al., 2000); and can increase as part of the general stress response. For example, stress generated by the poisons (e.g. heavy metal arsenic) can increase the serum level of neutrophils in mice (Sarker et al., 2013). In a study by (Haratym-Maj, 2002), poisoning of the mice with the low dose of deltamethrin, significantly elevated neutrophil level in blood compared to the control group. Therefore, occurrence of the neutrophilia in the AOM control is likely to be

related to stress from injecting the DNA damaging AOM in mice. Interestingly, administration of the semi-purified 6-bromoisatin (0.05 mg/g), appeared to return the neutrophils level back to the normal range (similar to saline controls). This indicates that 6-bromoisatin may also have an anti-inflammatory effect that counteracts the AOM side-effect associated with the neutrophilia. However, semi-purified 6-bromoisatin (0.05 mg/g) did not increase the lymphocyte level that was dropped by AOM and in fact the whole white cell count was decreased significantly as compared with both AOM control and saline control.

The most striking alteration among the biochemical factors was significant increase of the serum urea level in AOM control compared to saline control. Urea which is the end product of protein metabolism is produced from ammonia in urea cycle (Krebs, 1973) and excreted through the kidney in urine. Consequently, the rate of renal urea excretion directly affects the maintenance of nitrogen balance in blood. (Knepper and Roch-Ramel, 1987). In a study by Bémeur et al. (2010a), AOM-injected mice showed a significant 5.1 fold increase in ammonia concentration of the serum compared to the saline control mice. The increased concentration of ammonia by AOM in Bémeur et al. (2010a) is consistent with the increased concentration of urea by AOM in our study. Serum concentrations of urea is one of the markers of renal function, which is increased in renal injury (Han et al., 2002). Thus, our study indicates the effect of AOM results in rapid injury (within 6 h) in the kidney, as well as liver. However, mice treated with the crude muricid extract and compounds did not show any increase in urea, indicating non-toxicity of these compounds in the kidney after two weeks administration.

In conclusion, our study determined that both semi-purified 6-bromoisatin and a crude extract from Muricidae molluscs enhanced the apoptotic response to a genotoxic carcinogen in the crypt of the distal colon. 6-bromoisatin was found to be the most bioactive compound, significantly enhancing apoptosis and reducing cell proliferation in the colonic crypts at the lowest dose of 0.05 mg/g. The examination of the lower doses of 6-bromoisatin to establish the lowest effective dose would be beneficial. Our results also showed some potentially toxic effects of purified tyrinoleninone on red blood cells and in the liver (when combined with AOM), but without any efficacy in increasing AARGC. These findings are consistent with previous evidence that tyrindoleninone in the crude extract can be degraded within the stomach Westley et al. (2010). By comparison, 6-bromisatin is relatively stable and thus is a useful target for future prevention of CRC, when combined with the apparent efficacy for inducing apoptosis and preventing the proliferation of DNA damaged cells *in vivo*. However, the longer term studies are required to determine the effect of crude extract and 6-bromoisatin on pre-neoplastic lesions or ACF formation. Further studies to assess any possible side-effects corresponding to the long-term administration of these compounds would also be of interest.

4.6 Acknowledgments

We would like to thank Dr. Daniel Jardine from the Flinders Analytical Laboratory of Flinders University for help with LC/MS analysis of compounds. We are further grateful to Ms Roshini Somashekar and Joanne Wilkins from School of Medicine, Flinders University for their assistance and technical advice.

5. 6-Bromoisatin found in muricid mollusc extracts inhibits colon cancer cell proliferation and induces apoptosis, preventing early stage tumor formation in a colorectal cancer rodent model

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5.1 Abstract

Muricid molluscs are a natural source of brominated isatin with anticancer activity. The aim of this study was to examine the safety and efficacy of synthetic 6-bromoisatin for reducing the risk of early stage colorectal tumor formation. The purity of 6-bromoisatin was confirmed by ^{1}H NMR spectroscopy, then tested for *in vitro* and *in vivo* anticancer activity. A mouse model for colorectal cancer was utilized whereby colonic apoptosis and cell proliferation was measured 6 h after azoxymethane treatment by hematoxylin and immunohistochemical staining. Liver enzymes and other biochemistry parameters were measured in plasma and haematological assessment of the blood was conducted to assess potential toxic side-effects. 6-Bromoisatin inhibited proliferation of HT29 cells at IC_{50} 223 μM (0.05 mg/mL) and induced apoptosis without increasing caspase 3/7 activity. *In vivo* 6-bromoisatin (0.05 mg/g) was found to significantly enhance the apoptotic index ($p \leq 0.001$) and reduced cell proliferation ($p \leq 0.01$) in the distal colon. There were no significant effects on mouse body weight, liver enzymes, biochemical factors or blood cells. However, 6-bromoisatin caused a decrease in the plasma level of potassium, suggesting a diuretic effect. In conclusion this study supports 6-bromoisatin in Muricidae extracts as a promising lead for prevention of colorectal cancer.

5.2 Introduction

Isatin (1*H*-indole-2,3-dione) is a synthetically versatile molecule, and its derivatives possess diverse biological and pharmacological properties, including antibacterial, antifungal, antiviral, anticonvulsant, and anticancer activities (Medvedev et al., 2007, Vine et al., 2009, Pal et al., 2011, Akgul et al., 2013, Benkendorff, 2013). Isatin itself has demonstrated cytotoxic and apoptotic activity. For example, Cane *et al.* (2000) showed that isatin at a concentration of 100 µM reduced cell proliferation of human promyelocytic leukemia (HL60) cancer cells by 80% and induced morphological changes consistent with proapoptotic cells (including DNA fragmentation and chromatin condensation). In another study by Igosheva *et al.* (2005), apoptosis was observed in human neuroblastoma SH-SY5Y cells exposed to 50 µM of isatin. A range of mono-substituted isatins have been studied by Vine *et al.* (2007a) for their *in vitro* cytotoxicity on a lymphoma (U937) cell line. Structure activity relationship studies have shown that substitution with halogens (5-bromo-, 5-iodo-, and 5-fluoroisatin) yielded 5–10 times more activity for killing cancer cells, than the unsubstituted isatin (Vine et al., 2007a). Sunitinib (Sutent®) is a fluorinated isatin derivative that has been approved by FDA as a new anticancer drug to treat advanced renal carcinoma (Motzer et al., 2006) and gastrointestinal stromal tumours (Prenen et al., 2006).

Various substituted isatins have been found in nature including plants (Kapadia et al., 1980), fungi (Gräfe and Radics, 1986) and marine molluscs (Cooksey, 2001, Benkendorff, 2013). Recently 6-bromoisatin (Figure 5.1) from the Australian marine mollusc *Dicathais orbita* has become of particular interest as a major compound of the bioactive extract from this species (Chapter 4). In a study by Edwards et al. (2012), semi-purified 6-bromoisatin from *D. orbita* extracts revealed specific anticancer

activity with >10 fold selective cytotoxicity towards female reproductive cancer cells compared to freshly isolated human granulosa cells. Furthermore, semi-purified 6-bromoisatin was shown to significantly reduce proliferation and induce apoptosis in human colon cancer cell lines HT29 and Caco2 cells (Esmaeelian et al., 2013). In a short-term rodent model for the prevention of colon cancer Westley *et al.* (2010) demonstrated that the crude extract of *D. orbita* increased the apoptotic index of distal colon cells significantly. Due to several contaminants in the naturally purified extract (Esmaeelian et al., 2013, Westley et al., 2010) further work using the pure synthesized compound is required to confirm the activity of 6-bromoisatin against colon cancer cells.

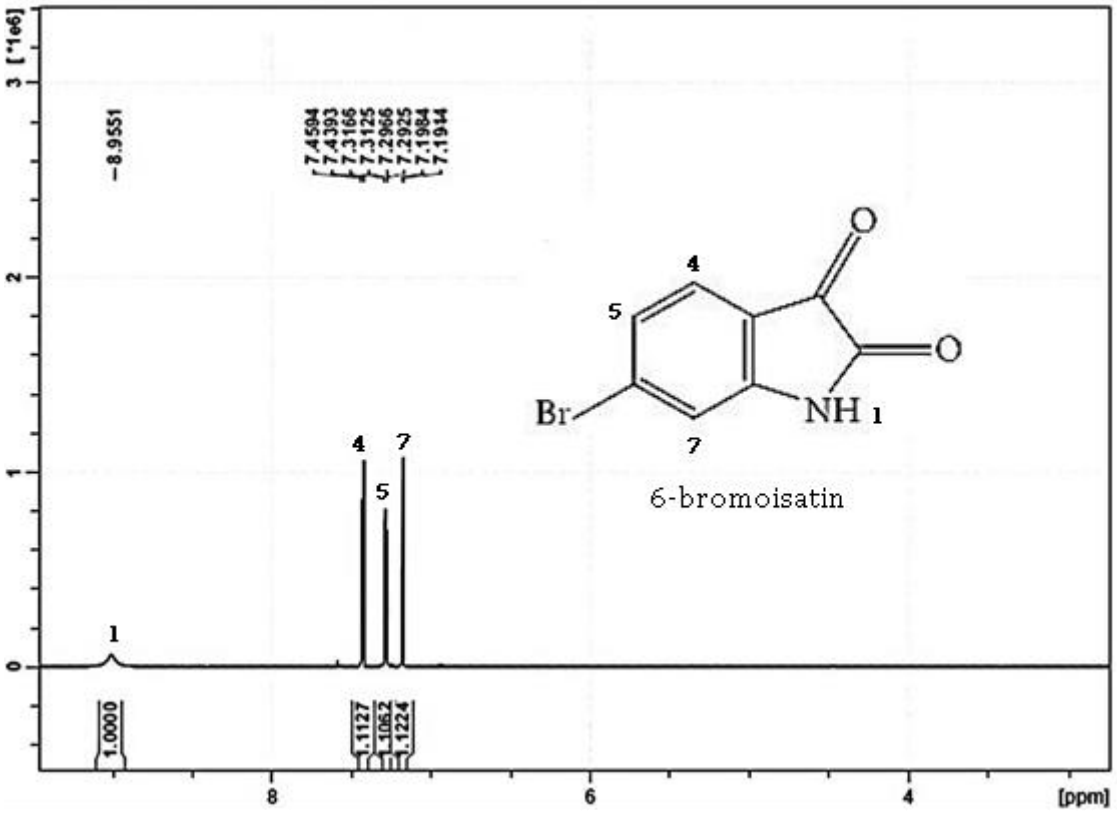

Figure 5.1 ^{1}H NMR spectrum of synthetic 6-bromoisatin on the Bruker Avance III 400 MHz spectrometer in deuterated acetonitrile. Chemical shifts (δ) are as parts per million (ppm) and referenced to residual solvent peaks. The peaks corresponding to the solvent occur below 3 ppm are not shown.

Colorectal cancer (CRC) is the third most common cancer worldwide (McLeod et al., 2009) with the highest incidence rates in Australia, New Zealand, North America and Europe (Jemal et al., 2011). In the United States, CRC is the second highest cause of cancer-related mortality in both males and females (Chan and Giovannucci, 2010). Just 39% of CRCs are diagnosed at early stage and in most cases the cancer spreads to adjacent and distant organs before detection (ACS, 2013). Therefore, prevention of CRC is an important priority (IARC, 2008). Chemoprevention involves the use of functional foods, specific natural products or synthetic chemical agents to suppress or

prevent a wide range of cancers, including colon cancer (Rajamanickam and Agarwal, 2008). The acute apoptotic response to genotoxic carcinogens (AARGC) is a good model for chemopreventative research which has been used in several studies (Hong et al., 1999, Reddy et al., 2000, Hu et al., 2002, Le Leu et al., 2002, Le Leu et al., 2003, Westley et al., 2010). In this model, the carcinogen azoxymethane (AOM) is injected into mice causing DNA damage in epithelial cells in the crypts of the distal colon inducing an acute apoptotic response 6–8 h later. The AARGC model has been mainly used to identify the effect of natural products on inducing apoptosis of the damaged colon cells, with the aim of detecting early stage CRC prevention (Hu et al., 2002).

In a previous *in vivo* study using a two-week preventative treatment with the crude extract from *D. orbita*, we detected an increase in apoptosis in the colon of mice in response to AOM injection. The aim of this study is to test the *in vitro* and *in vivo* effects of pure synthetic 6-bromoisatin, to confirm whether this compound is the key factor in *D. orbita* extracts responsible for the inhibition of colon cancer cells and the induction of apoptosis in damaged colon cells in the AARGC rodent model of colon cancer prevention. We also obtained additional data to assess any potential side effects of synthetic 6-bromoisatin on blood parameters and liver toxicity in the mice.

5.3 Material and Methods

5.3.1 Synthetic 6-bromoisatin and chemical analysis

Synthetic 6-bromoisatin (6-Bromoindole-2,3-dione) was purchased from TCI AMERICA (Portland, OR, USA) (purity of >97.0% GC). To confirm the identity and purity of the compound, ^{1}H NMR spectroscopy (Bruker Avance III 400 MHz spectrometer, Preston, VIC, Australia) was performed in deuterated acetonitrile

(Sigma Aldrich, Castle Hill, NSW, Australia). Chemical shifts (δ) are reported as parts per million (ppm) and referenced to residual solvent peaks. Spin multiplicities are indicated by: s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and dd, doublet of doublets.

5.3.2 *In vitro* experiments using HT29 colorectal cancer cells

All media and chemicals were purchased from Sigma Aldrich (Castle Hill, NSW, Australia) unless otherwise stated. HT29 human colorectal cancer cell line (passage no. 36-42) were cultured (37 °C and 5% CO_2) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4500 mg/L L-glutamine, 10% FBS, 100 U/mL Penicillin/Streptomycin and 1% Non-essential Amino Acid (100×), until the cells reached 70% confluence.

The cells were harvested from flasks by trypsinization (1× Trypsin-EDTA) and were seeded (20,000 cells/well) into clear 96-well plates (Costar®, Mt Martha, VIC, Australia) for measurement of cell viability and white (opaque) 96-well plates (Interpath, Heidelberg West, VIC, Australia) for determination of apoptosis and necrosis. HT29 cells were incubated for 48 h, then the media was removed and the cells were washed with PBS. To treat the cells, synthetic 6-bromoisatin was dissolved in 100% dimethylsulphoxide (DMSO) then diluted in media and added to the cells at a range of concentrations from 0.1 to 0.01 mg/mL, in triplicate (final DMSO concentration of 1%). 1% DMSO controls were also included on each plate. Staurosporine (5 μM) for apoptosis and lysis solution (5 μL/well, Promega, Alexandria, NSW, Australia) for necrosis were added to the white plates in triplicate, as positive controls. All cells were treated for 12 h.

Morphological changes in the cells were observed on an Olympus CK2 inverted optical microscope (×400 magnification) 12 h after treatment. To measure cell viability, the MTT assay was applied, which measures the reduction of MTT tetrazolium salt to formazan, as previously described (Esmaeelian et al., 2013). To measure necrosis and apoptosis, CytoTox-ONE Homogeneous Membrane Integrity Assay reagent (Promega) and Caspase-Glo 3/7® assay (Promega) were applied respectively, according to our previous study (Esmaeelian et al., 2013). These assays were all repeated in triplicate on three separate occasions (n = 3).

5.3.3 *In vivo* model for early stage colon cancer prevention

This experiment was conducted under Flinders animal welfare approval number 751-10. A total of 38 wild-type (C57BL/6J) male mice aged 10 weeks were obtained from the Animal Resource Centre, Perth, Western Australia. Mice were divided randomly into 4 groups (ten mice in each treatment group and 8 mice in the sunflower oil only control group) and housed in 8 cages (four to five mice per cage). The mice were given water and food (rodent chow) *ad libitum* and maintained at the temperature of 22 ± 2 °C and humidity of 80% ± 10% with a 12 h light/dark cycle. Mice were weighed every five days and on the day of kill, and monitored daily for signs of illness, such as weight and hair loss, diarrhea, constipation, rectal bleeding, labored breathing, lethargy, eye and nose discharge.

To detect the early stage prevention of colon cancer, an established AARGC rodent model with injection of the carcinogen AOM was used (Hu et al., 2002, Le Leu et al., 2002, Hu et al., 2005, Westley et al., 2010). Synthetic 6-bromoisatin at 3 different dosages (0.025, 0.05 and 0.1 mg/g body weight) was administered to mice by daily

oral gavage in 100 µL sunflower oil, containing 0.02% Vitamin E, for two weeks. The control group was gavaged with sunflower oil (containing 0.02% Vitamin E) only. After two weeks, all mice were injected with a single intraperitoneal (i.p.) injection of AOM at a dosage of 10 mg/kg bodyweight and euthanized 6 h later by cervical dislocation under anesthesia. Our previous studies have shown that the peak time for the acute apoptotic response to carcinogen occurs between 6 h and 8 h post AOM injection (Hu et al., 2002), hence 6 h post AOM was chosen in the current study. The distal colon of each mouse was excised and fixed in 10% buffered formalin for 24 h and then embedded in paraffin for histological and immunohistological examination.

Distal colon segments were embedded in paraffin and sectioned at 4 µm (3–4 sections per mouse), then stained with hematoxylin, to evaluate apoptosis in epithelial cells of distal colon sections (Hu et al., 2005). The slides were examined under a light microscope (Olympus BH-2, Mt Waverly, VIC, Australia, 400× magnification) to identify the apoptotic cells, by characteristic morphological changes such as cell shrinkage, condensed chromatin and sharply delineated cell borders surrounded by an unstained haloo (Le Leu et al., 2002, Potten et al., 1992). Twenty randomly chosen crypts were used to calculate the percent of apoptotic cells per crypt. The mean crypt column height was also determined.

Ki-67 is a cell cycle associated antigen and regarded as a useful proliferation marker (Cordes et al., 2009). Proliferative activity of distal colonic epithelial cells was measured using an antibody specific for the nuclear proliferating antigen ki-67 (rat-anti-mouse clone TEC-3, Dako, Campbellfield, VIC, Australia) in combination with an immunohistochemistry detection method in paraffin embedded sections, as previously described (Esmaeelian et al., 2013). Sections of 4 µm were examined under

a light microscope (Zeiss, Axio Imager A1, North Ryde, NSW, Australia) at 400× magnification to calculate the proliferation index as a percent of proliferated cells per crypt.

5.3.4 Liver enzymes, blood biochemistry and hematology

Blood samples (0.5–1 mL) were obtained from the mice under anesthesia by cardiac puncture at time of kill and transferred to Gribbles Veterinary Pathology laboratory, Adelaide within heparinized vacutainer tubes then centrifuged to separate into the cell layer for hematology analysis (Abbott Cell Dyn 3700 analyzer, North Ryde, NSW, Australia) and plasma for biochemistry analysis (Siemens Advia 1800 chemistry analyzer, Erlangen, Germany). The plasma levels of the liver enzymes AST, ALT and ALP were assessed as indicators of hepatotoxicity (Hewawasam et al., 2004).

5.3.5 Statistical analysis

Statistical analyses were performed using SPSS and values of $P \leq 0.05$ were considered to be statistically significant. One way ANOVAs with post hoc Tukey HSD multiple comparisons were performed to determine which concentrations of 6-bromoisatin were significantly different to each other and the control.

5.4 Results and Discussion

5.4.1 Chemical analysis

^{1}H NMR results showed four major peaks corresponding to the four hydrogen protons in the 6-bromoisatin molecule: ^{1}H NMR (400 MHz, CD3CN) δ 8.96 (^{1}H, s), 7.44 (^{1}H, d, J = 8.08 Hz), 7.30 (^{1}H dd, J = 1.64, 8 Hz), 7.19 (^{1}H, d, J = 1.6 Hz) and confirming the identity of synthetic 6-bromoisatin and its high purity (Figure 5.1). The ^{1}H NMR

spectra for synthetic 6-bromoisatin matches our previous NMR data for semi-purified 6-bromoisatin in anticancer extracts from the marine mollusc *D. orbita* (Esmaeelian et al., 2013).

5.4.2 *In vitro* apoptosis, necrosis and cell viability:

The effects of 6-bromoisatin on proliferation, apoptosis and necrosis of HT29 cells was examined. A dose dependent effect of 6-bromoisatin on the viability of cells was observed using the 3-(4,5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide (MTT) assay for inhibition of metabolic activity (Figure 5.2). The three highest concentrations of 6-bromoisatin (1 mg/mL, 0.05 mg/mL and 0.025 mg/mL) significantly reduced the cell viability by 64%, 53% and 26% respectively ($p < 0.001$), relative to the DMSO control, but no significant reduction was observed at the lowest dose of 0.01 mg/mL ($p > 0.05$). The IC_{50} for synthetic 6-bromoisatin was calculated at 223 μM (0.05 mg/mL) for HT29 cells (Figure 5.2a). However, our previous *in vitro* study using semi-purified 6-bromoistain, on both HT29 and Caco2 cells, revealed a lower IC_{50} of 100 μM (Esmaeelian et al., 2013), suggesting possible synergistic activity with other factors in the extract. The *in vitro* cytotoxic effects of synthetic 6-bromoisatin in this study could also be due to lower bioavailability of the pure compound to the cells when compared to the natural extract, which contains trace lipids that may help dissolve this lipophylic compound and/or facilitate interactions with cell membrane lipids. Previous studies have reported lower bioavailability of some synthetic compounds, in comparison with the naturally purified compounds (Vinson and Bose, 1983, Lodge, 2005). For example, the bioavailability ratio of natural Vitamin E *versus* synthetic Vitamin E was shown to be close to 2:1 (Lodge, 2005), which is similar to our study.

No increase in the level of lactate dehydrogenase (LDH) (Figure 5.2b) a measure of necrosis or late stage apoptosis was observed in the cells treated with any concentration of 6-bromoisatin, in comparison with DMSO control. Unexpectedly, synthetic 6-bromoisatin did not increase caspase 3/7 activity in HT29 cells *in vitro* at concentrations <0.1 mg/mL. This is in conflict with our previous study on semi-purified 6-bromoisatin, which significantly upregulated caspase 3/7 activity in HT29 cells (Esmaeelian et al., 2013). The positive controls, lysis buffer and staurosporine, resulted in a significant increase in LDH activity (Figure 5.2b, $p < 0.001$) and caspase 3/7 activity (Figure 5.2c, $p < 0.001$) respectively, demonstrating that the assays were working. The cells treated with the highest concentration of 6-bromoisatin (446 μM = 0.1 mg/mL) showed a minor but significant reduction of caspase3/7 activity compared to the DMSO control (Figure 5.2c, $p = 0.011$). Nevertheless, the light microscopic images from the HT29 cells treated with 223 μM and 112 μM 6-bromoisatin showed morphological alterations, such as chromatin condensation, characteristic of the apoptotic process (Figure 5.3b, c). Apoptotic cells were also observed in cultures treated with the highest dose of 6-bromoisatin 446 μM, although in lower numbers than the two other doses (Figure 5.3d). This indicates that synthetic 6-bromoisatin may induce apoptosis in the HT29 cells through a caspase-independent pathway. In the past few years, the existence of caspase-independent programmed cell death pathways have been reported in the literature, which are associated with executioners other than the caspases, such as cathepsins, calpains, serine proteases and also apoptosis inducing factor (AIF) protein (Constantinou et al., 2009).

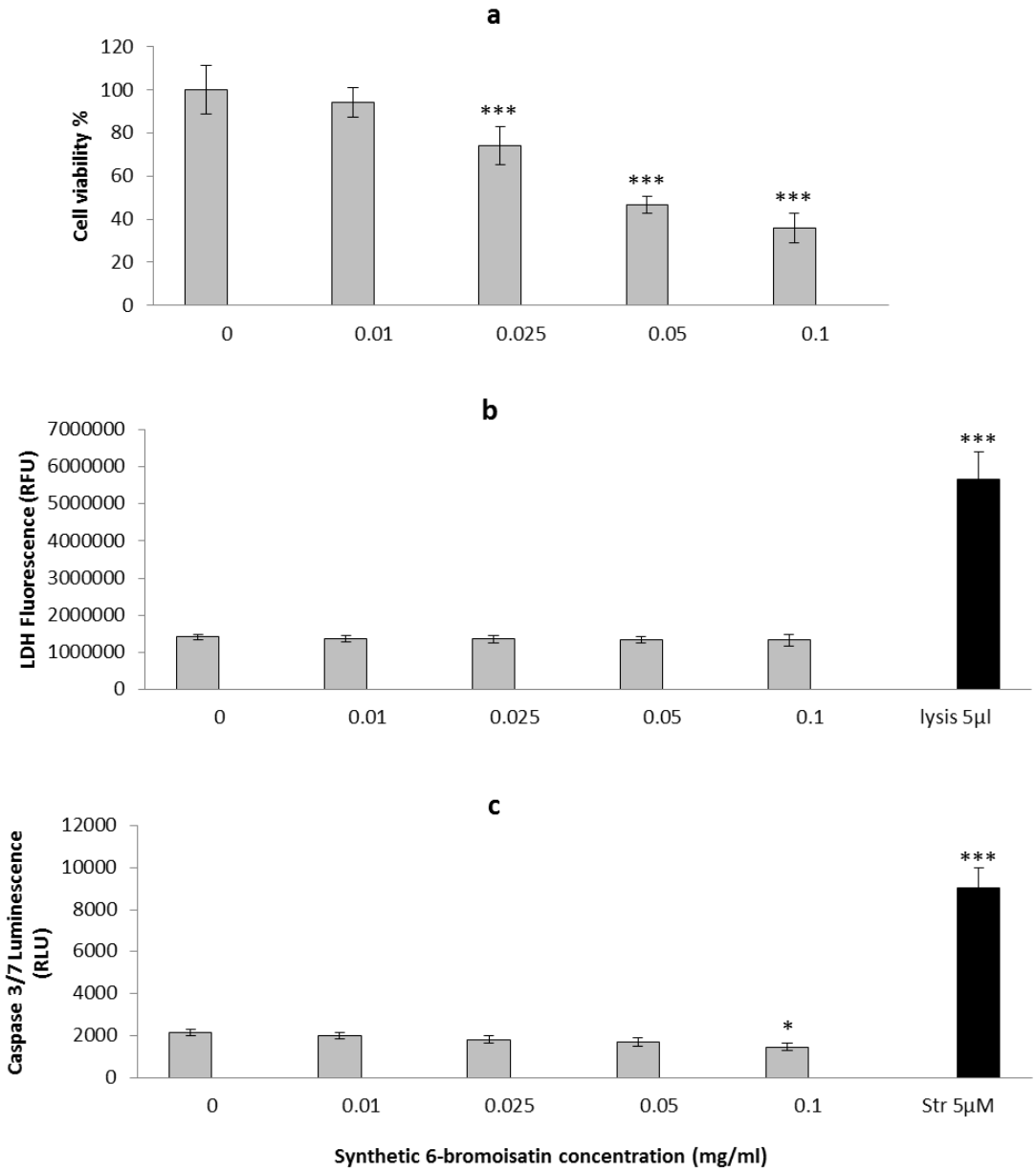

Figure 5.2 Effects of synthetic 6-bromoisatin on HT29 cells: Cell viability (a), LDH release (b) and caspase-3/7 activity (c). LDH release was measured by fluorescence at 535EX/590EM and caspase-3/7 activity was measured at full light on a luminescence plate reader. The positive controls are lysis buffer (5 µL/well) for the LDH assay and staurosporine (Str) (5 µM) for apoptosis. A final concentration of 1% DMSO was used in all control and treated cells. The results are mean for three independent repeat assays (n = 3) each performed in triplicate. Significant differences between each group and the DMSO control are shown as $p \leq 0.05$ (*) and $p \leq 0.001$ (***).

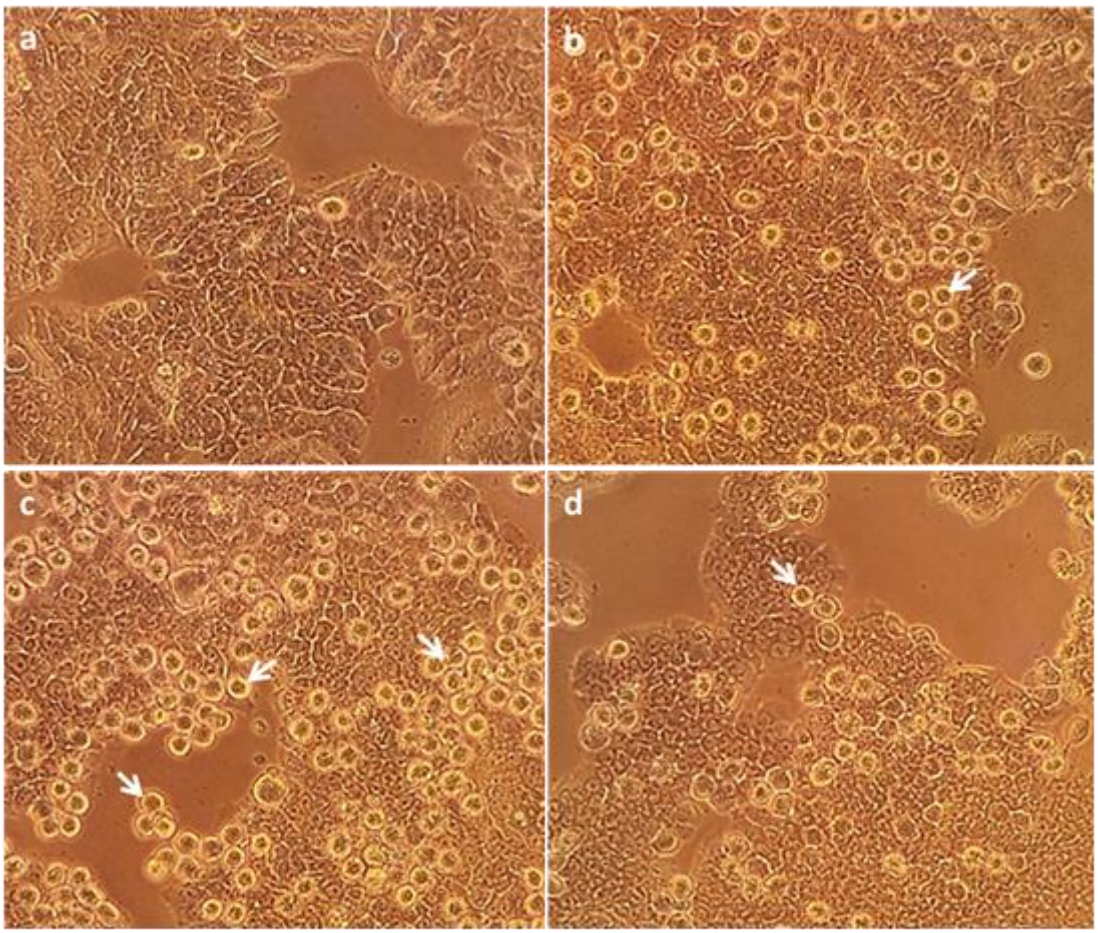

Figure 5.3 HT29 cells at 400× magnification under the Olympus inverted microscope. One percent dimethylsulphoxide (DMSO) control (a); cells treated with 0.025 mg/mL synthetic 6-bromoisatin (b); cells treated with 0.05 mg/mL synthetic 6-bromoisatin (c) and cells treated with 0.1 mg/mL synthetic 6-bromoisatin (d) for 12 h (final concentration of 1% DMSO). Examples of apoptotic cells with chromatin condensation and surrounded by a halo are indicated by the arrows.

In terms of the mode of action, the isatin molecule has been proposed to interact via extracellular signal regulated protein kinases (ERKs) to inhibit cancer cell proliferation and promote apoptosis (Vine et al., 2009). In a study by Cane et al. (2000), isatin at a concentration of 100 µM inhibited the phosphorylation of ERK-2 (but not ERK-1) by 35% compared to the control. ERK is attributed to a survival signaling pathway in several cell types; however, it mediates apoptosis in some cell types and organs (e.g., neuronal and renal epithelial cells) under certain conditions (Zhuang and Schnellmann, 2006). Although the mechanisms for mediating apoptosis by ERK is not fully understood, three mechanisms have been proposed: (1) ERK1/2 may act through the intrinsic apoptotic pathway by up-regulating Bax and P53 followed by mitochondrial cytochrome c release and activation of caspase-3 (Wang et al., 2000, Kim et al., 2005, Zhuang and Schnellmann, 2006); (2) Through the extrinsic pathway by increasing an upstream signal for death receptors, such as TNF-α followed by activation of caspase-8 and caspase-3 (Jo et al., 2005, Zhuang and Schnellmann, 2006) or; (3) Through inhibition of Akt (Protein kinase B) mediated survival signaling (Sinha et al., 2004). As synthetic 6-bromoisatin in this study induced apoptosis in HT29 cells without activating caspase-3 and considering the fact that both intrinsic and extrinsic pathways are associated with upregulation of caspase-3, the third pathway resulting in a decrease in Akt activity is hypothesized as a caspase-independent apoptosis pathway for synthetic 6-bromoisatin. However, further mode of action studies that specifically target Akt gene expression in colon cancer cells are required with 6-bromoisatin to confirm this.

5.4.3 *In vivo* mouse model

5.4.3.1 Mice; general observations

Given synthetic 6-bromoisatin reduced the cell viability of a colon cancer cell line *in vitro*, we tested its effects on the AARGC response in a mouse model of CRC. The mice did not show any signs of illness in the treatment groups or the control group during the study. The body weights of all mice increased steadily over the trial duration, without any significant differences in mean total weight gain between the treatment groups and the control (Table 5.1, $p = 0.999$). No significant change in the liver weight or the percentage liver to body weight ($p = 0.098$) was revealed between treatment groups and the control (Table 5.1).

Table 5.1 Comparison of mean (±S.E.) progressive body weight (g) in controls and mice treated with different concentrations of 6-bromoisatin on different days of the experiment. All treatments and the control were injected with 10 mg/kg AOM 6 h prior to kill. Liver weight (g) and percentage liver weight/body weight were calculated on the day of kill. $n = 10$ mice in treatment groups and $n = 8$ mice in the control.

	Weight (g)						
Concentrations	**Body (day1)**	**Body (day5)**	**Body (day10)**	**Body (day14)**	**Total weight gain**	**Liver**	**Liver / body (%)**
Control	22.0±1.6	22.6±1.6	22.8±1.9	23.5±1.9	1.4±0.7	1.1±0.3	4.8±1.2
0.025 mg/g	22.2±1.3	22.7±1.3	23.0±1.3	23.6±1.4	1.4±0.8	1.0±0.1	4.4±0.6
0.05 mg/g	22.6±1.2	23.4±1.2	23.4±1.5	24.1±1.6	1.4±1.0	1.2±0.1	5.2±0.6
0.1 mg/g	22.3±1.3	22.6±1.3	23.3±1.3	23.8±1.6	1.4±0.8	1.3±0.1	5.3±0.4

5.4.3.2 Apoptotic index, crypt height and cell proliferation

Synthetic 6-bromoisatin was found to significantly increase apoptosis in response to AOM injection (ANOVA $F = 14.660$, $p < 0.001$, $df = 3$), but had no significant effect on colon crypt height (ANOVA $F = 1.013$, $p = 0.403$, $df = 3$); (Figure 5.4). The mice treated for two weeks daily with 0.05 mg/g 6-bromoisatin showed the greatest increase in apoptotic index in the distal colon (Figure 5.4a), with a 2.3 fold increase over the oil control ($p \leq 0.001$). The highest dose of 6-bromoisatin (0.1 mg/g) also significantly induced apoptosis ($p = 0.007$) in the distal colon of the mice compared with the control group. However, this effect was significantly lower, by 40%, than the dose of 0.05 mg/g ($p = 0.031$). In contrast, although the distal colon of the mice administered with the lowest concentration of 6-bromoisatin showed a slightly increased apoptosis index, there was not a significant difference when compared to the AOM injected control (Figure 5.4a, $p = 0.158$). Apoptosis in the distal colon of the mice occurred mostly in basal crypt cells (Figure 5.5).

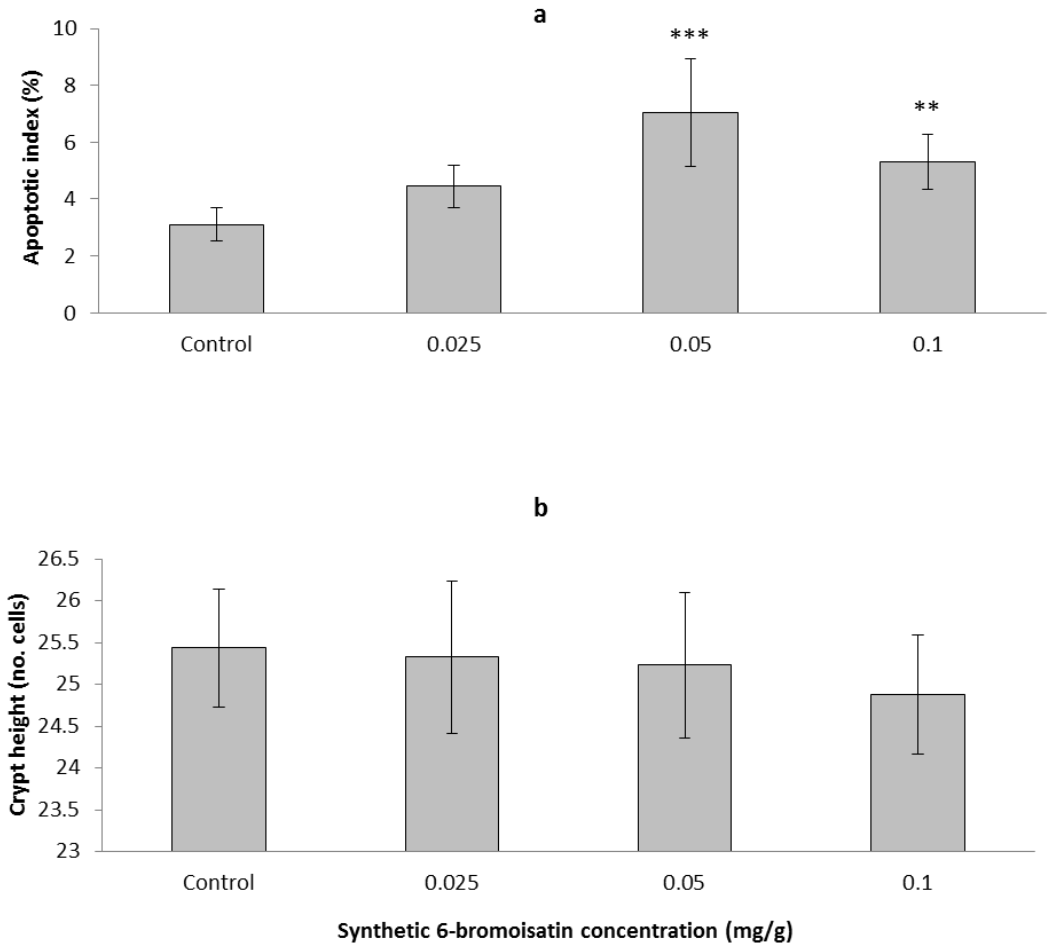

Figure 5.4 Apoptotic response and crypt height in the distal colon of mice after 14 day oral gavage with different concentrations of synthetic 6-bromoisatin, showing: apoptotic index (a) and crypt height (b). All treatments and the oil only control were injected with 10 mg/kg AOM 6 h prior to kill. Data are means ± S.E. for 10 full crypts/animal (n = 10 mice in treatment groups and n = 8 mice in control group). Significant differences between each group and the control are shown as $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

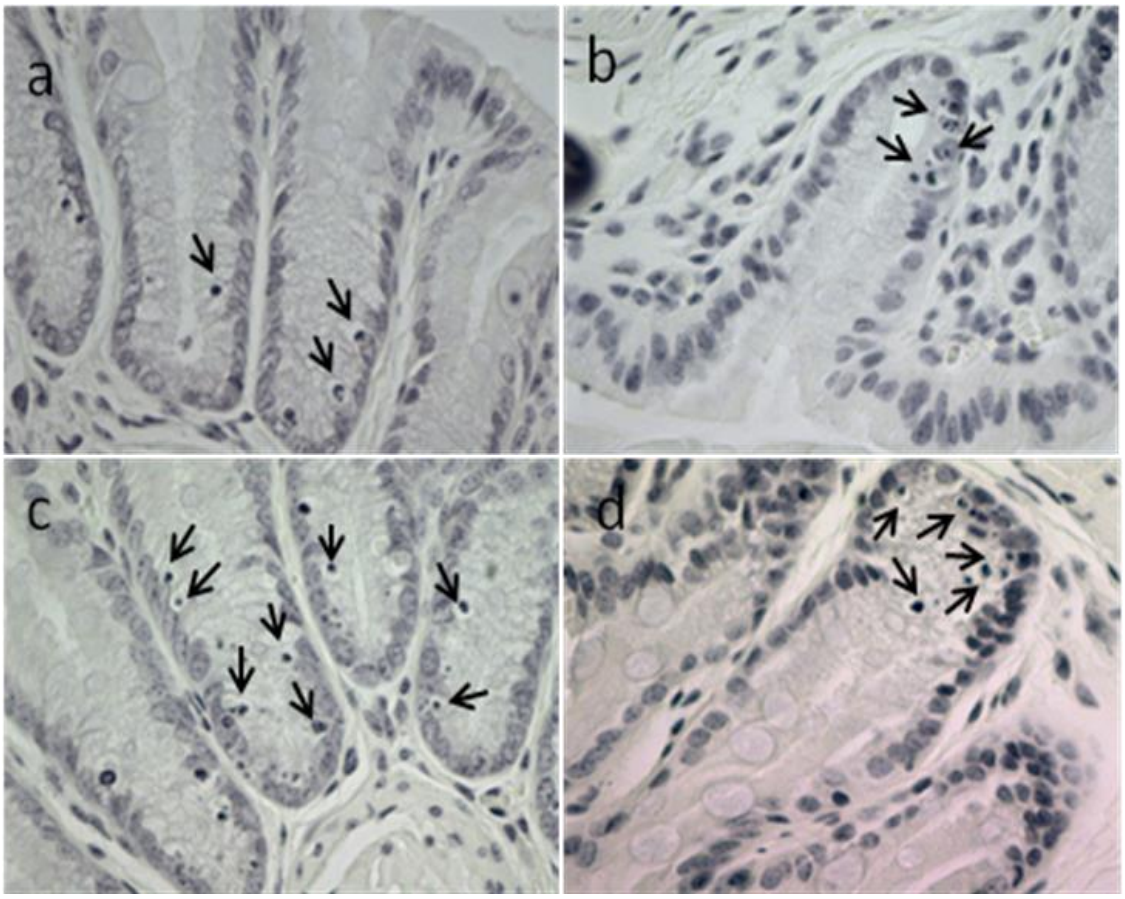

Figure 5.5 Apoptosis in the basal crypt cells of the distal colon of mice 6 h post AOM injection (10 mg/kg). Mice were oral gavaged daily for two weeks prior with oil (a) control; (b) 6-bromoisatin 0.025 mg/g; (c) 6-bromoisatin 0.05 mg/g; (d) 6-bromoisatin 0.1 mg/g. Apoptotic cells with chromatin condensation characteristics are shown by arrows.

Ki-67 immunohistochemistry showed evidence for cell proliferation, also in the basal cells of colon crypts of mice, in response to AOM injection (Figure 5.6). After two weeks daily oral gavage, synthetic 6-bromoisatin was found to significantly reduce this cell proliferation in the distal colon of mice (ANOVA $F = 41.273$, $p < 0.001$, $df = 3$); (Figure 5.7). After AOM injection, the mice treated with the highest concentration of 6-bromoisatin (0.1 mg/g) had the greatest reduction in cell proliferation in the distal colon, by more than 50% compared to control mice gavaged with oil alone ($p \leq 0.001$). Similarly, the dose of 0.05 mg/g significantly reduced the proliferation in the distal colon compared to the oil alone control ($p = 0.006$), and was not significantly different to the higher dose of 0.1 mg/g ($p = 0.652$). In contrast, the lowest dose of 6-bromoisatin (0.025 mg/g) did not show a significant anti-proliferative effect in the distal colon as compared to the oil alone control (Figures 5.6 and 5.7, $p = 0.052$).

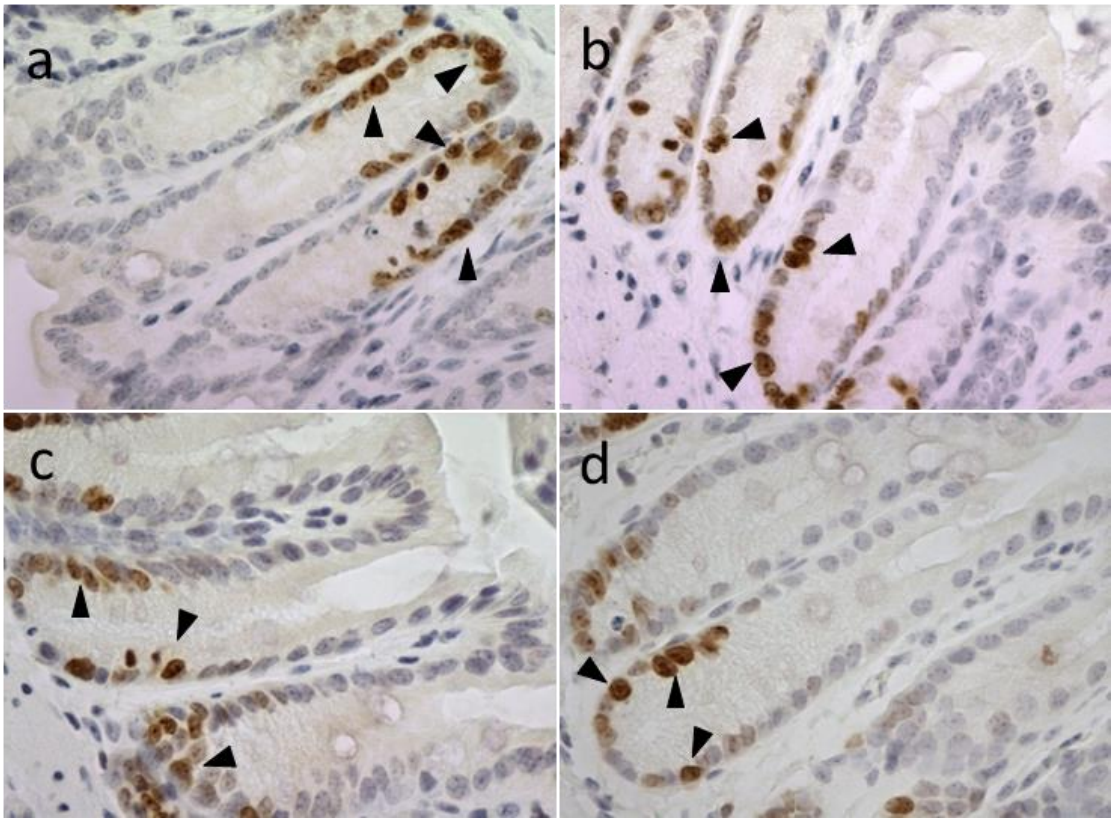

Figure 5.6 Proliferative activity of distal colonic epithelial cells in mice 6 h after AOM injection (10 mg/kg), shown using an antibody specific for the ki-67 antigen. Mice were oral gavaged daily for two weeks prior with oil; (a) control; (b) 6-bromoisatin 0.025 mg/g; (c) 6-bromoisatin 0.05 mg/g; (d) 6-bromoisatin 0.1 mg/g. Proliferating cells are shown by arrowheads

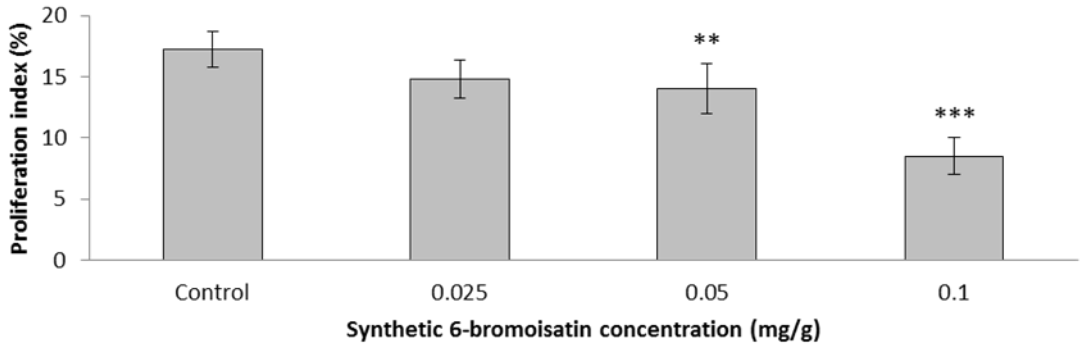

Figure 5.7 Proliferation index in the distal colon of oil control mice and mice treated with different concentrations of 6-bromoisatin by daily oral gavage for 2 weeks, followed by 10 mg/kg AOM injection 6 h prior to kill. Significant differences between each group and the control are shown as $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

A connection between AARGC stimulation and the inhibition of oncogenesis in the distal colon has been previously shown in mice (Hu et al., 2008, Le Leu et al., 2010). In our study, synthetic 6-bromoisatin at the concentration of 0.05 mg/g had the greatest effect in facilitating apoptosis in the distal colon of mice. Interestingly, the apoptotic index at the highest dose of synthetic 6-bromoisatin (0.1 mg/g) was lower than the middle dose of 0.05 mg/g, which is consistent with a previous study using the crude extract from *D. orbita* (Westley et al., 2010). This could be related to the fact that cell proliferation in the colon showed the highest reduction at the highest dose of 0.1 mg/g 6-bromoisatin, indicating that fewer DNA damaged cells may have been present that required removal by the initiation of programmed cell death. In a study by Saini *et al.*, (Saini et al., 1996) the cytotoxic effect of streptozotocin, at low doses, was shown to be associated with the activation of the apoptotic pathway on beta cells, whereas this predominantly changed to necrosis at high doses. Therefore the lower apoptosis index with the highest dose of 6-bromoisatin may indicate that some necrosis or cell cycle

arrest occurred in the crypt cells at this high dose. However, the *in vitro* LDH assays found no evidence of an increase in cell membrane permeability that would suggest necrosis at this concentration

5.4.3.3 Blood biochemistry and hematology

To be useful as a future drug or nutraceutical for the prevention of colon cancer, 6-bromoisatin and/or *D. orbita* extracts must also be safe for oral use. The plasma level of the liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) are indicators of hepatotoxicity (Hewawasam et al., 2004). No significant difference was revealed in the plasma level of these enzymes in the oil control, as compared to treatment groups gavaged for two weeks daily with 6-bromoisatin (Figure 5.8, $p > 0.6$), providing evidence that synthetic 6-bromoisatin is not hepatotoxic at these concentrations. In a study by Westley *et al.* (Westley et al., 2013), mice treated with the crude *D. orbita* extracts containing 6-bromoisatin showed some idiosyncratic toxicity in the liver, whereas pure 6-bromoisatin did not exhibit this hepatotoxicity

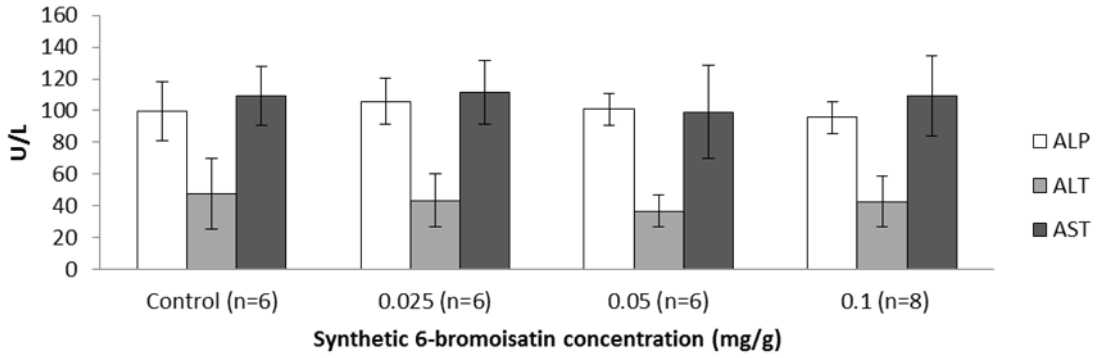

Figure 5.8 Liver enzymes *aspartate aminotransferase* (AST), *alanine aminotransferase* (ALT) and *alkaline phosphatase* (ALP) levels in serum (U/L) of oil control mice and mice treated with different concentrations of 6-bromoisatin by daily oral gavage for two weeks

In the plasma there were no significant differences in sodium, urea, creatinine, calcium, protein, albumin and globulin levels in the mice treated with 6-bromoisatin in comparison to the oil control (Table 5.2; $p > 0.05$). However, a significant dose dependent reduction in potassium plasma levels (Hypokalemia) was observed in the mice administered with both 0.05 mg/g ($p = 0.005$) and 0.1 mg/g 6-bromoisatin ($p = 0.001$), as compared to the oil control (Table 5.2). Consequently, the sodium/potassium ratio (Na/K) increased significantly in these groups compared to the oil control (Table 5.2, $p < 0.005$).

The most common reason for a potassium deficiency is diuretic therapy (loop diuretics, thiazides) that causes urinary potassium excretion (Lindeman, 1976, Weiner and Wingo, 1997), and gastrointestinal potassium wasting from diarrhea (Lindeman, 1976, Gennari, 2002). In this study, no diarrhea or change in stool consistency was observed in either the oil control or 6-bromoisatin treatment groups. Therefore, the

potassium deficiency in the treatment groups might be due to a diuretic effect of 6-bromoisatin leading to an increase in urinary potassium excretion. Diuretic effects of some novel isatin derivatives, especially the derivatives of bromoisatin, were previously shown by Nataraj *et al.* (Nataraj et al., 2010). Consequently, future *in vivo* studies using 6-bromoisatin should carefully monitor this possible diuretic effect

Table 5.2 Plasma biochemistry and blood hematology from mice in the oil only control group and mice treated with different concentrations of 6-bromoisatin, 6 h after injection of 10 mg/kg AOM. Significant differences between each group and the oil control are shown as $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***). Significant differences between 6-bromoistain doses are shown as $p \leq 0.05$ ($^{\#}$) relative to 0.1 mg/mL. Na/K = Sodium/Potassium ratio, Creat = Creatinine, Hct = Hematocrit, MCV = Mean Corpuscular Volume, MCH = Mean Corpuscular Hemoglobin, MCHC = Mean Corpuscular Hemoglobin Concentration.

	AOM control (n=6)	**6-bromoisatin 0.025 mg/g (n=6)**	**6-bromoisatin 0.05 mg/g (n=6)**	**6-bromoisatin 0.1 mg/g (n=8)**
Biochemistry				
Sodium (mmol/L)	146.5±1.2	147.7±0.5	149.7± 1.4	149.7±1.2
Potassium (mmol/L)	5.4±0.3	5.1±0.2#	4.5±0.4*	4.3±0.4**
NA/K	27.0±2.2	29.1±1.2#	33.5±2.7**	34.1±3.6***
Urea (mmol/L)	10.6±1.2	9.7±1.6	9.9±1.4	9.6±1.2
Creat. (umol/L)	14.2±1.2	13.3±0.8	14.7±0.5	14.6±1.2
Calcium (mmol/L)	2.2±0.03	2.2±0.1	2.2±0.04	2.2±0.1
Protein (g/L)	45.8±3.0	46.7±2.8	46.2±1.9	46.1±2.2
Albumin (g/L)	28.2±1.7	28.7±1.5	27.8±1.5	28.2±1.5
Globulin (g/L)	17.7±1.5	18.0±1.4	18.3±0.8	17.9±1.2
Hematology				
Red cell count (x 10^{12}/L)	9.0±0.4	9.3±0.4	9.1±0.4	9.4±0.5
Hemoglobin (g/L)	135.2±2.7	139.7±5.1	135.0±3.5	138.4±5.7
Hct (L/L)	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.02
MCV (FL)	46.0±1.2	46.5±0.8	46.7±1.2	46.4±0.7
MCH (Pg)	15.2±0.4	15.0±0.0	15.0±0.0	15.0±0.0
MCHC (g/L)	323.7±4.5	322.7±1.5	319.0±2.8	319.8±3.0
White cell count (x 10^{9}/L)	4.8±0.9	5.2±1.8	5.8±1.5#	3.2±1.1
Neutrophils (x 10^{9}/L)	1.9±0.3	1.6±0.8	2.2±1.3	0.8±0.7
Lymphocytes (x 10^{9}/L)	2.8±0.9	3.3±1.1	3.4±0.8	2.3±0.9
Monocytes (x 10^{9}/L)	0.2±0.1	0.2±0.3	0.2±0.1	0.1±0.1

The hematological factors including white blood count, red blood count, hemoglobin, hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), band form neutrophils, lymphocytes and monocytes were not significantly altered in treatment groups, in comparison to the oil only control (Table 5.2, $p > 0.05$). However, there was a significant increase in the white blood cell count of mice treated with 0.05 mg/g 6-bromoistain, compared to mice treated with the higher dose of 0.1 mg/g (Table 5.2, $p = 0.037$). This may indicate some mild anti-inflammatory effects at the higher dose of 6-bromoistain. Isatin has been previously found to inhibit NO production, COX-2, TNF and PGE2 in mouse macrophages (Matheus et al., 2007). Furthermore, indirubin derivatives exhibit inflammatory activity in RAW 264.7 cells (Kim and Park, 2012) and in rat brain microglia (Jung et al., 2011).

Overall, this study demonstrates that 6-bromoisatin, a dominant compound found in muricid mollusc extract, has anticancer effects and low toxicity *in vivo*. Pure synthetic 6-bromoisatin effectively reduced the proliferation of colon cells, both *in vitro* against the HT29 colorectal cancer cell line and *in vivo* in mice administered AOM, which causes DNA damage in colon cells. 6-Bromoisatin also enhanced the apoptotic response in DNA damaged colon cells *in vivo*, with 0.05 mg/g found to be the most effective dose, with the only sign of toxicity after two weeks administration being a possible diuretic effect. Although synthetic 6-bromoisatin did not increase caspase 3/7 activity in HT29 cells, light microscopy confirmed the presence of many cells with the morphological appearance of apoptosis, such as a condensed nucleus surrounded by a halo. Consequently it can be concluded that 6-bromoisatin is the main factor in the *D. orbita* anticancer extracts contributing to enhancing the apoptotic response to the genotoxic insult of AOM. Although the effective doses of 6-bromoistain used in this

study were high relative to common chemotherapeutic drugs, cancer prevention strategies are more likely to utilize dietary supplements or nutraceuticals containing higher doses of bioactive secondary metabolites with demonstrated low toxicity. 6-Bromoisatin is the dominant compound in oxidized extracts from the hypobranchial glands of *D. orbita*, an edible marine mollusc (Westley et al., 2013), This compound is stable at low pH in simulated digestive fluid (Westley et al., 2010) and appears to be bioavailable in the distal colon. Therefore, this paper supports the further development of a nutraceutical from Muricidae molluscs with potential application for the prevention of early stage colon cancer, by specifically targeting the 6-bromoisatin fraction.

5.5 Conclusions

In conclusion, this study supports the efficacy of synthetic 6-bromoisatin, at the concentration of 0.05 mg/g, for enhancing the apoptotic response to a genotoxic carcinogen and reducing cell proliferation in the distal colon of mice, without significant toxic effects detected in the liver or blood. The highest dose of 0.1 mg/g 6-bromoisatin showed a saturated dose–response for the induction of apoptosis, but had a stronger effect of inhibiting cell proliferation in the crypt of the distal colon and appears to also reduce the number of circulating white blood cells relative to the lower dose. Synthetic 6-bromoisatin appears to induce apoptosis in HT29 cells by a caspase-independent pathway. Although there was evidence of hypokalemia in the mice, due to the possible diuretic effect of 6-bromoisatin, no further toxicity in the liver or blood cells were observed. Longer term studies in mice are required to assess the effect of 6-bromoisatin on colonic aberrant crypt foci formation and/or tumor formation and also any possible side-effects associated with longer term use of this compound

5.6 Acknowledgments

We appreciate the assistance of Martin Johnston (School of Chemistry and Physical Sciences, Flinders University) with the NMR analysis of 6-bromoistain. Hanna Krysinska and Lisa Pogson (School of Biological Sciences, Flinders University) and Roshini Somashekar, Joanne Wilkins and Jean Winter (Flinders Centre for Innovation in Cancer) provided valuable technical assistance.

6. Final discussion and future directions

6.1 Summary of research

The Australian marine mollusc *D. orbita* produces bioactive brominated compounds that are cytotoxic against colorectal cancer cells. In an *in vitro* trial, purified tyrindoleninone and semi-purified 6-bromoisatin from this mollusc were found to be the most cytotoxic compounds on human colorectal HT29 and Caco2 cancer cells. The effect of semi-purified 6-bromoisatin was consistent by inducing apoptosis in both cell lines, while tyrindoleninone tended to induce more necrosis in both cell types within 12 h (Table 6.1). Semi-purified 6-bromoisatin also arrested HT29 cells in the G2/M phase of cell cycle. Testing these two purified/semi-purified compounds and the pure synthetic 6-bromoisatin *in vivo* confirmed the *in vitro* results, showing that 6-bromoisatin as the most bioactive compound for enhancing the apoptotic response to a carcinogenic agent in the distal colon. The toxicity studies supported the safety of 6-bromoisatin in a short term *in vivo* trial. Similarly in the *in vitro* experiments, tyrindoleninone displayed anti-proliferative effects on the epithelial cells of distal colon; however this compound did not increase the apoptotic index in these cells and produced some potentially toxic side effects. Overall, the *in vitro* and in *vivo* anticancer effects of 6-bromoisatin (natural or synthetic), as well as the molluscan extract containing this compound, highlight the potential for nutraceutical or pharmaceutical development of this compound for the prevention of colorectal cancer.

6.2 The use of natural antioxidants in combinatorial anticancer drug therapies

Natural antioxidants have the potential for improving anticancer activity, by either protecting the bioactive compound from oxidation and or from synergistic interactions. Vitamin A and E are natural antioxidants that are used to prevent oxidation in foods (Sies and Stahl, 1995) and also have cytotoxic activity against some cancer cell lines (Kempná et al., 2004, Sylvester and Shah, 2005, Popadic et al., 2008). However, the usefulness of these compounds needs to be assessed on a case by case basis. For example, the bioactive compound tyrindoleninone is unstable after exposure to oxygen. Vitamin E at 0.1% was effective in preventing the oxidation of tyrindoleninone to 6-bromoistain. However, addition of Vitamin E to the HT29 cells reduced the anti-proliferative effects of tyrindoleninone compared to tyrindoleninone alone. Similar protective effects of Vitamin E, against the cytotoxic effect of bisulfite, have been demonstrated for cervical carcinoma (HeLa) cells (Kuroda, 1975). Consequently, the use of Vitamin E in cell culture has the potential to mask or reduce the effects of anticancer agents. Nevertheless, antioxidants such as Vitamin E could still be used to prevent the oxidative degradation of compounds such as tyrindoleninone during storage. A low level of Vitamin E is typically used in vegetable oils to prevent the oxidation of fatty acids during storage at room temperature. Therefore, this natural antioxidant could be particularly useful for storing bioactive extracts in oil prior to oral gavage in animal models. Despite the effects of 0.1% Vitamin E on the activity of tyrindoleninone *in vitro*, when 0.02% Vitamin E was used *in vivo*, tyrindoleninone caused a significant reduction in cell proliferation in colon cells. The insignificant effect of this compound on apoptosis *in vivo* compared to 6-bromoisatin indicates that tyrindoleninone had not oxidised to 6-bromoisatin in the oil

used to gavage the mice. This suggests that natural antioxidants, such as Vitamins E, can be used at low levels (e.g. 0.02%) during *in vivo* trials to help stabilise the bioactive compounds, without significantly interfering with the activity.

6.3 Cell culture assays for drug screening

In vitro cell-based tests, such as cell culture assays, are a crucial tools for drug discovery at the early preclinical stages (Seiler et al., 2004), which provide a profile of immediate effects of the drug. These assays can also provide some preliminary information about the cell signalling pathways related to the drug (Stockwell et al., 1999) for future in depth investigation. Cell culture tests can save time and costs before undertaking *in vivo* experiments, which are usually more time-consuming and expensive. In this study a suite of complementary *in vitro* assays was used to provide a preliminary assessment of the cytotoxic and apoptotic effects of *D. orbita* extract, tyrindoleninone and 6-bromoisatin (Table 6.1).

Table 6.1 Summary of the different effects of the crude extract and compounds from *D. orbita* from a suite of *in vitro* and *in vivo* experiments. The concentrations (mg/mL for *in vitro* assays and mg/g for *in vivo* models) of different compounds that had significant effects compared to the negative controls are shown, except for the MTT cell proliferation assay which is reported as IC50 of the compounds. Both Caco2 and HT29 cells were treated for 12 h *in vitro* assays. *In vivo* results were obtained after gavaging the compounds for 2 weeks in mice. n/s= not significantly different to the control negative (*in vitro*) or AOM control (*in vivo*) at the maximum concentration tested. n/t= not tested.

Experiment	Cell line or tissue	Crude extract	Tyrindoleninone	Semi-purified 6-bromoisatin	Synthetic 6-bromoisatin
In vitro assays					
MTT cell proliferation	Caco2	2	0.025	0.025	n/t
	HT29	2	0.1	0.025	0.05
LDH necrosis	Caco2	>0.5	≥0.5	≥0.25	n/t
	HT29	>0.5	≥0.1	≥0.25	n/s
Caspase 3/7 apoptosis	Caco2	n/s	n/s	0.01 - 0.05	n/t
	HT29	n/s	0.05	0.025 - 0.1	n/s
Annexin/PI Apoptosis	Caco2	n/t	n/t	n/t	n/t
	HT29	n/t	0.05	0.025 - 0.05	n/t
In vivo models					
Cell proliferation	Colon	0.25 - 0.5	0.05 - 0.1	0.05 - 0.1	0.05 - 0.1
Apoptosis	Colon cells	0.25 - 0.5	n/s	0.05 - 0.1	0.05 - 0.1
Toxicity	Liver enzymes	n/s	n/s	n/s	n/s
	Red cell count	n/s	Decreased using 0.1	n/s	n/s
Homeostatsis	Na/K in blood	n/s	n/s	n/s	Increased using 0.1

6.3.1 Cell viability assays

The US National Cancer Institute NCI60 programme has played a key role in establishing a cell line-based screening platform for anticancer drug discovery and development (Sharma et al., 2010). Practical microplate cytotoxicity assays did not exist when the NCI60 model was perceived, but this platform originally supported the use of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as an amenable assay for use as a growth-inhibition assay (Shoemaker, 2006). The National Cancer Institute originally used the MTT assay, then moved to a modified XTT reagent before trialling other alternatives, such as the sulphorhodimine B reagent (SRB), which includes a fixation step and is thus more suitable for screening large numbers of compounds in high throughput (Shoemaker, 2006). The results of the SRB assay typically correlate with the MTT assay, but with higher IC50s because the SRB assay is based on cell density and does not distinguish between viable and dead cells (Vichai and Kirtikara, 2006). In this study, the MTT assay was used because this is relatively cheap and has been previously optimised for a range of cell lines in our laboratories (Edwards et al., 2012, Nurdin, 2012). Furthermore, the extracts and brominated compounds from *D. orbita* have been previously used in the MTT assay and despite being coloured compounds, these are known not to interfere with the absorbance readings at 490nm for of the end product formazan.

The metabolic enzymatic MTT assay (used in Chapter 2, 3 and 5) is based on measuring the biochemical conversion rate of a substrate to a product in the mitochondria of the cell (Mosmann, 1983). This assay showed with a clear dose effect that the brominated compounds (tyrindoleninone and semi-purified/synthetic 6-bromoisatin) are cytotoxic to colon cancer cells. Tyrindoleninone trends towards

higher toxicity on Caco2 than HT29 cells (Table 6.1). The greater cytotoxic activity of an impure fraction containing tyrindoleninone on some cells such as MCF-7 breast and Caco2 colon (IC50= 0.1 mg/mL) has been also noted compared to HT29 cells (>1 mg/mL) in a study by Benkendorff et al. (2011). However, the cytotoxic effect of tyrindoleninone was even greater in other cell lines such as KGN and JAr cells (IC50= 0.01 mg/mL); (Edwards et al., 2012). A range of factors may be involved in the different activity of tyrindoleninone among these studies. One factor influencing the difference in activity of tyrindoleninone between studies might be the different concentrations of this compound in the purified fractions. For example Benkendorff et al. (2011) has reported the use of a fraction containing 62.7% tyrindoleninone, which was less pure than the tyrindoleninone tested in our study, although the purity of tyrindoleninone on the study by Edwards et al. (2012) appears to be similar. Secondly, the type of assay that is used to determine the cytotoxicity can also contribute to differences in results. For example, Benkendorff et al. (2011) used the MTS assay for screening tyrindoleninone, whereas Edwards et al. (2012) like this study used MTT. It has been reported that the MTS assay is not as sensitive as the MTT assay because it often produces inconsistent results (Pabbruwe et al., 2005). Edwards et al. (2012) also compared the MTT assay and a crystal violet assay and some inconsistencies were observed according to the assay used. For example, the MTT assay was more generally sensitive for detecting the cytotoxic effects of the tyrindoleninone, but the crystal violet assay was more sensitive than MTT assay for detecting the cytotoxic effects of the 6-bromoisatin (Edwards et al., 2012). However, the MTT assay was consistently used with the same purity of tyrindoleninone to test against Caco2 and HT29 cells in this study, thus suggesting a real difference in sensitivity between these cell lines.

Cell type is an important factor that can affect the absorbance, metabolism and excretion of the compounds (Riss and Moravec, 2004, Rochat, 2009). For example, the mechanism for metabolising compounds by choriocarcinoma JAr cells have been shown to involve hepatic excretion (Serrano et al., 2007) in gene expression studies, but this may vary in other cell types, Furthermore, different cancer cell lines have different cell surface receptors and may initiate programmed cell death via different pathways (Sharma et al., 2010). Thus different activity against different cells lines can be used to gain a preliminary insight into the mode of action of anticancer compounds; this is the basis for the use of 60 distinct cancer cell lines in the *in vitro* cytotoxicity screening by the NCI. The cell lines selected for use by the NCI are all well characterised in terms of known mutations that contribute to cancerous attributes (Ikediobi et al., 2006). For example, Ikediobi et al. (2006) showed that HT29 cells have the greatest number of five cancer genes (APC, BRAF, SMAD4, PIK3CA, and TP53) with likely oncogenic mutations compared to the other NCI 60 cell lines. Caco2 cells, however, are not included in NCI60 cell lines, but have a mutation (codon 245) in the beta-catenin (CTNNB1) oncogene (Ilyas et al., 1997). The genomic heterogeneity that is inherent in cancer has been shown by generally low number of clinical response to targeted therapeutics that highlights the necessity of greater representation of cell lines derived from various cancers to cover this genetic diversity (Sharma et al., 2010).

The results of this thesis show a real difference in susceptibility between HT29 and Caco2 cell lines for the cytotoxic effect of tyrindoleninone. The reason for the increased susceptibility of Caco2 cells compared to HT29 cells is unclear; however, it has been proposed that Caco2 cells may absorb more anti-proliferative compounds

than the HT29 cells because Caco-2 cells act as endocrine cells while HT29 cells act as excretory cells in the differentiation process (Parry et al., 2011). In a study by Raymond et al. (1997), oxaliplatin showed a potent cytotoxic activity (as good as, or better than, cisplatin) against human ovarian (IC50 = 10 ± 1.6 μM), breast (IC50 from 7.4 ± 2.7 to 17.9 ± 7.1 μM), and colon (IC50 from 2.1 ± 1.1 to 5.9 ± 1.7 μM) cell lines; however, this cytotoxic effect was surprisingly reduced in human HT29 cells due to a significant inferior DNA binding of oxaliplatin compared to cisplatin in HT29 cells. This indicates that HT29 cells are likely to be more resistant to cytotoxic effects of anticancer drugs in comparison with other cell lines. Our study suggests that it would be well worth expanding these studies to include the NCI 60 cell lines for future screening, and used any further differences between these well characterised cells lines to inform the mode of action.

The other compound 6-bromoisatin, which was semi-purified from *D. orbita* extracts showed a similar anti-proliferative effects on both HT29 and Caco2 cells (Table 6.1; IC50= 100 μM). In a study by Vine *et al.* (2007a), 6-bromoisatin with the IC50= 75 μM reduced the proliferation of lymphoma cell line U937. This shows a similar cytotoxic effect of 6-bromoisatin to U937, HT29 and Caco2 cells. However, Edwards *et al.* (2012) found a lower anti-proliferative effect of semi-purified 6-bromoisatin on KGN cells (MTT assay: IC50= 446 μM; Crystal violet: IC50= 223 μM), JAr cells (MTT assay: IC50= 446 μM; Crystal violet: IC50= 178 μM) and OVCAR-3 cells (MTT assay: IC50= 446 μM; Crystal violet: IC50= 268 μM) after 24 h treatment. The difference in the cytotoxic effect of 6-bromoisatin may again be due to the difference in cell types used and/or different concentrations of 6-bromoisatin in the semi-purified fractions.

Another interesting difference was the greater cytotoxic effect of the semi-purified 6-bromoisatin from the natural extract, compared to synthetic 6-bromoisatin on HT29 cells (Table 6.1). This may due to an unknown bioactive compound in the extract which is active at very low concentrations. Alternatively, there is the possibility of a synergistic effect between 6-bromoisatin and other factors in the extract. Other factors such as sterols and lipids in the extracts, could result in a difference in the bioavailability of the synthetic and natural 6-bromoiatin in cell culture, and certainly synthetic 6-bromoistain did not dissolve as well as the semi-purified compound, with a tendency to precipitate out of the cell culture media at concentrations higher than 0.1 mg/mL (personal observation). Consequently, semi-purified natural extracts have the potential to enhance the bioactivity of some anticancer agents.

6.3.2 Apoptosis tests

To determine the mode of cell death, the MTT cell viability assay can be complimented by apoptosis and necrosis assays, including morphological change of apoptotic cells, caspase 3/7, flow cytometry detection of apoptosis and LDH assay (Chapter 3, 5); (Vermes et al., 1995, Häcker, 2000, Frade et al., 2007, Benachour and Seralini, 2008). Tyrindoleninone at the low concentration of 0.05 mg/mL (195 μM) increased the caspase activity in HT29 cells (Table 6.1). However, the apoptotic effect of tyrindoleninone turned to a necrotic effect when the HT29 cells were treated with higher doses (Table 6.1). Tyrindoleninone failed to increase caspase activity in Caco2 cells after 12 h exposure and with the concentrations of 0.05 mg/mL (and higher) caused necrosis by releasing LDH in these cells (Table 6.1). In another study by Edwards *et al.* (2012), KGN cells treated with 0.05 mg/mL tyrindoleninone underwent apoptosis by activating caspase3/7 after 4 h and 24 h. The KGN cells did not undergo

necrosis after 4 h with 0.05 mg/mL tyrindoleninone, whereas LDH was released in these cells after 24 h (Edwards et al., 2012).

Semi-purified 6-bromoisatin was found to induce apoptosis by the activation of caspase -3 and 7 in both Caco2 and HT29 cells (Table 6.1), while synthetic 6-bromoistain was not effective in increasing apoptosis in these cells (Table 6.1). However, the existence of apoptosis in HT29 cells was confirmed by morphological changes of the cells treated with synthetic 6-bromoisatin under the microscope (Figure 5.3, Chapter 5). After treatment with the semi-purified 6-bromoistain, apoptotic cells were confirmed for HT29 cells treated with ~100 µM by flow cytometry (75.3 ± 14.03 % Annexin-V positive cells, PI negative; Figure 3.6, Chapter 3), in addition to cell morphology changes observed under the light microscope (Figure 3.5, Chapter 3). The apoptotic effect of semi-purified 6-bromoisatin is consistent with a previous study by Edwards *et al.* (2012), where a significant increase in caspase 3/7 activity was detected in KGN cells and 58% of KGN cells were TUNEL positive after 24 h treatment with 22 µM with semi-purified 6-bromoisatin.

The different results of the caspase 3/7 assays for synthetic and semi purified 6-bromoistain suggest a different cell death mechanism may be occurring in the cells exposed to semi-purified and synthetic compound. A caspase-dependant induction of apoptosis occurs for semi-purified 6-bromoisatin, whereas synthetic 6-bromoistain appears to induce apoptosis via a caspase-independent pathway. Due to the higher purity of the synthetic compound, the caspase-independant pathway appears to be the most likely for 6-bromoistain itself. In the semi-purified extract, the other unknown minor compounds may be initiating caspase-dependant apoptosis at very low concentrations. The presence of another unknown active compound is also supported

by the greater anti-proliferative effects of the semi-purified 6-bromoistain fraction (Chapter 2). Therefore, further purification and bioassay guided fractionation of the semi-purified 6-bromoisatin fraction from *D. orbita* is warranted to isolate and characterise the potentially potent unknown anticancer compound/s.

6.4 *In vivo* trials for drug screening

In drug discovery programs, the eventual effect of a drug should be considered in the system that the drug needs to be tested in (Lipinski and Hopkins, 2004). The results of our *in vivo* rodent model, to test whether the Muricidae compounds can enhance the acute apoptotic response to genotoxic carcinogens, found that tyrindoleninone did not increase the apoptotic index in the distal colon of mice, whereas semi-purified 6-bromoisatin increased the apoptotic index by 2.3 fold over the AOM control (Table 6.1). The crude extract (0.5 mg/g) containing 6-bromoisatin also showed a similar effect, but required 10 times higher concentrations than the semi-purified 6-bromoisatin (Figure 4.3, Chapter 4). This is consistent with the 11.4% yield of 6-bromoisatin that could be semi-purified from the crude extract in Chapter 2 and confirms 6-bromoisatin as the main bioactive compound in the crude extract. The crude extract results are consistent with a previous study by Westley et al. (2010) that revealed enhancement of AARGC by 0.1 mg/g muricid extract in a 4 week mice trial.

Furthermore, synthetic 6-bromoisatin also enhanced the apoptotic response to the carcinogenic agent axoymethane (AOM) by 2.3 fold compared to AOM control (Figure 5.4, Chapter 5), which is consistent with the effect of semi-purified 6-bromoisatin (Table 6.1). So regardless of the different mechanism of action that is reported here for apoptotic effects of semi-purified 6-bromoisatin and synthetic 6-

bromoisatin on cell cultures *in vitro*, the *in vivo* study showed that both of natural and synthetic 6-bromoisatin have the same activity, in enhancing the apoptotic index of epithelial cells in distal colon at 0.05 mg/g. When the dose of synthetic 6-bromoisatin went up to 0.1 mg/g, the apoptotic effects were reduced (Figure 5.4, Chapter 5), which could be contributed to the high anti-proliferative effect of this highest dose in epithelial cells, resulting in fewer DNA damaged cells that required removal by the initiation of programmed cell death. Overall the anti-proliferative and anti-apoptotic effects observed at the lower dose of 0.05 mg/g 6-bromoistain could be complimentary in helping prevent the formation of tumors in DNA damaged colon cells.

In terms of the anti-proliferative activity of bioactive compounds in distal colon, tyrindoleninone with the concentration of 0.05 and 0.1 mg/g showed a dose dependent effect (Figure 4.6, Chapter 4). This anti- proliferative effect of the tyrindoleninone without increasing the apoptotic index *in vivo* is consistent with the cytotoxic effect of tyrindoleninone *in vitro*, which also showed a considerable anti-proliferative effect on Caco2 cells, but did not induce apoptosis in these cells (Table 6.1). Semi-purified 6-bromoisatin at both concentrations of 0.05 and 0.1 mg/g also reduced the proliferation of epithelial cells in distal colon by 32% compared to AOM control (Figure 4.6, Chapter 4). However, synthetic 6-bromoisatin showed higher anti-proliferative effects in distal colon. For example, it reduced the proliferation of distal colon by 18.7% at the concentration of 0.05 mg/g, but when the concentration went up to 0.1 mg/g, it inhibited about 50% of the distal colon proliferation (Figure 5.5, Chapter 5). These anti-proliferative effects of synthetic and semi-purified 6-bromoisatin in the *in vivo* experiments are the reverse of the *in vitro* experiments, where semi-purified 6-bromoisatin showed more cytotoxic effects than the synthetic compound. Therefore,

the possible synergic effect of the 6-bromoisatin with other compounds in semi-purified fraction and/or the different bioavailability of the natural semi-purified 6-bromoisatin does not correlate to the anti-proliferative effect of these compounds *in vivo*. In fact, the high purity of the synthetic compound may explain the greater anti-proliferative effect of this compound compared to the semi-purified 6-bromoisatin with the dose of 0.1 mg/g *in vivo*.

6.5 Toxicity studies for drug screening

In addition to the efficacy of drugs, the drug safety must also be tested through animal trials for new drug development and approval (Nicholson et al., 2002). As the liver is the most important organ for drug toxicity, we evaluated any possible toxicity by both histopathological analysis (Greaves, 2007, Ferrell, 2010) and measuring the liver enzymes (Hewawasam et al., 2004) in the serum of the mice. Histopathological analysis did not show any sign of liver damage, over the AOM control, after two weeks daily administration of our compounds to mice (Figure 4.8, Chapter 4). However, the high level of background damage to live cells in the AOM control relative to saline controls implies that this carcinogen model may mask any effects of the test compounds and thus is not really suitable for histological examination of effects of the liver. The results of the serum analyses were however more reliable. Liver enzyme levels did not alter significantly in the treatment groups including crude extract, semi-purified 6-bromoisatin and synthetic 6-bromoisatin (Table 6.1). However, AST levels in the mice treated with tyrindoleninone were elevated significantly compared to the saline control, which could be indicative of some toxicity associated with this compound over the AOM toxicity (Figure 4.9, Chapter 4). Further evidence of liver toxicity was found in the mice treated with tyrindoleninone, which showed a reduced

level of globulin in the serum (Table 4.3, Chapter 4). Globulin is produced by the liver (Madden and Whiffle, 1940), therefore reduction in the level of this protein in the serum could be due to damage of the liver function. By comparison, semi-purified 6-bromoisatin, crude extract dominated with 6-bromoisatin and the synthetic 6-bromoisatin appear to be safe to the liver after two weeks of daily oral ingestion. Westley et al. (2013) reported idiosynchronic liver toxicity associated with administrating of the crude extract dominated by tyrindoleninone in mice. Our study supports the non-toxic effects of 6-bromoisatin and the possible toxicity of tyrindoleninone in the crude extract against the liver.

Some other biochemical factors in the serum were measured to identify any toxicity of our compounds to other organs, such as the kidney (Brzoska et al., 2003), or any possible side effect of the compounds on the balance of electrolytes. The only alteration in biochemical factors, other than those related to the liver, occurred on the potassium levels. Indeed, this element decreased in the mice treated with the synthetic 6-bromoisatin at high concentrations and consequently the sodium/potassium ratio was also altered (Table 5.2, Chapter 5). However, no alteration in the level of potassium was observed in the mice treated with semi-purified 6-bromoisatin (Table 4.3, Chapter 4); (Table 6.1). As the main reason for potassium deficiency is due to diuretic therapy (Lindeman, 1976, Weiner and Wingo, 1997), the reduction in the level of potassium in our study is most likely due to possible diuretic effects of 6-bromoisatin. Indeed in a more recent 12 week trial using this compound and the crude extract, increased levels of urination have been observed (Unpublished data). This effect of 6-bromoisatin is also supported by previous reports of diuretic effect of some novel isatin derivatives, especially the derivatives of bromoisatin by Nataraj et al.

(2010). This difference between the effect of semi-purified 6-bromoisatin and synthetic 6-bromoisatin might again be due to the higher purity of the synthetic compound because no significant difference in Na/K was observed for synthetic 6 bromoistain at the lowest dose of 0.1 mg/mL. Clearly, potassium levels need to be monitored in longer term animal studies to confirm the diuretic effect associated with this compound.

Hematological factors were measured to identify any possible side effect of the compounds on the haematopoietic system (Pellock, 1987) and/or immune system (Veenman and Gavish, 2006). Among these factors, red cell counts and haemoglobin levels were reduced in the mice treated with the tyrindoleninone (Table 4.2, Chapter 4; Table 6.1). The reduced blood cell count is linked to mild anaemia (Groopman and Itri, 1999, Raabe et al., 2011) caused by tyrindoleninone. Another alteration in the hematological factors was an increased number of neutrophils in the control mice that received AOM, compared to the saline control mice, suggesting an inflammatory response to this carcinogen. However, administration of semi-purified 6-bromoisatin (0.05 mg/g) returned the neutrophil level back to the normal range in the saline control, which is indicative of a possible anti-inflammatory effect associated with this compound (Table 4.2, Chapter 4). Elevation of neutrophil level due to AOM has not been reported previously, but this could be due to the stress from injecting the DNA damaging compound in mice. In a study by Haratym-Maj (2002), administration of deltamethrin (a poisonous compound) increased the neutrophils level. This study highlights the importance of simultaneously examining blood samples from *in vivo* experiments for drug discovery to detect the possible side-effects of the compounds on the haematopoietic and immune systems. The toxic effects of AOM within a short

time of six h before killing the animals are also demonstrated in this study (Chapter 4, 5), which indicates the importance of comparing the treatment groups with the relevant controls to ascertain any effects above the AOM. In order to assess the true toxicity of these compounds, it is important to also test their effects on the hematopoietic and immune system in the absence of AOM.

6.6 Pharmaceuticals from isatins

Drug development history has demonstrated that many effective anticancer drugs originate from nature (Chapter 1). Isatin is one of these molecules with a lot of derivatives found in natural organisms, including plants, bacteria and marine molluscs (Kapadia et al., 1980, Gräfe and Radics, 1986, Cooksey, 2001, Benkendorff, 2013). The cytotoxic and apoptotic effects of the isatin molecule have been shown for a range of cancer cell lines studies (Cane et al., 2000, Igosheva et al., 2005, Vine et al., 2007a). However, various other biological and pharmacological effects, such as antibacterial, antifungal, antiviral, anticonvulsant and anticancer properties are also linked with isatin derivatives (Medvedev et al., 2007, Vine et al., 2009, Pal et al., 2011, Akgul et al., 2013, Benkendorff, 2013). A current review by Vine et al. (2013) reports that the cytotoxicity of mono- di- and tri-substituted aromatic isatin derivatives is generally higher when compared to the unsubstituted isatin molecule. In another study by Vine et al. (2007a), a range of mono-substituted isatins were studied for their *in vitro* cytotoxicity and it was shown that substitution with halogens (5-bromo-, 5-iodo-, and 5-fluoroisatin) can increase the cytotoxic effect of these derivatives up to 5-10 times more than unsubstituted isatin. The recent FDA approval of a fluorinated isatin derivative Sunitinib (Sutent®) as a kinase inhibitor and new anticancer drug to treat advanced renal carcinoma (Motzer et al., 2006), gastrointestinal stromal tumours

(Prenen et al., 2006) and more recently for neuroendocrine tumours of the pancreas (Vine et al., 2013), underscore the increasing interest in isatins as new class of antitumor agents. Of importance to Sunitinib activity is the substitution of a halogen atom (fluorine) at C5 which has been linked with the increased biological activity for a range of isatin derivatives (Vine et al., 2013).

The semi-purified 6-bromoisatin in this study showed a clear cytotoxic effect with IC50 of 100 μM on both HT29 and Caco2 cells (Figure 3.4, Chapter 3). This effect was lower in synthetic 6-bromoisatin against HT29 cells with IC50 of 223 μM (Figure 5.2, Chapter 5). The cytotoxic effect of either semi-purified or synthetic 6-bromoisatin in this study seems to be lower than the routine anticancer drugs that are commercially available for treating colorectal cancer. For example, oxaliplatin, a potent inhibitor of DNA synthesis, is one of the chemotherapy drugs for colorectal cancer treatment that showed a good cytotoxic effect on human gastric cell lines MGC803 (IC50= 4.09 - 8.12 μM) and SGC7901 (IC50= 3.49 - 7.11 μM); (Luo et al., 2010). Nevertheless, the low cytotoxic effect of 6-bromoisatin on HT29 and Caco2 does not necessarily limit its anticancer effect because this low cytotoxic effect has been also reported in bevacizumab (Avastin®) with 0.5 mg/mL FDA approved concentration as a targeted therapy for colorectal cancer (Videira et al., 2011) that shows the anticancer effect of this drug in high concentrations. On the other hand, the low cytotoxic effect of 6-bromoisatin on Caco2 and HT29 cells could be due to more resistance of these cells to the cytotoxic effects of anticancer drugs. One evidence to support this idea is the limited cytotoxic effect of staurosporine, a potent inhibitor of protein kinase C, that was used as the positive control on HT29 and Caco2 cells in our study (Chapter 3). Indeed, staurosporine with the concentration of 10 μM could not half the number of

HT29 and Caco2 cells while this compound had very potent cytotoxic effect on different cell lines such as HeLa S3 cells (IC50 =4 × 10^{-12}M); (Tamaoki et al., 1986), MCF-7 and MDA-MB-453 cells (IC50= ~0.5 μM) and MCF-10A cells (IC50 < 0.005μM); (Cyr et al., 2008).

6-bromoistain was non-toxic at high doses indicating the specificity of this compound towards cancer cells rather than normal cells. Therefore, 6-bromoisatin could be used as a treatment at higher doses than other anticancer drugs which have more toxic side effects. The other factor could be the low price of 6-bromoisatin (USD $270.0 for 25g; TCI AMERICA) which is much cheaper than other anticancer drugs such as staurosporine (USD $375.0 for 100μg, Sigma Aldrich). Consequently, more preclinical tests are required to identify whether 6-bromoistain itself has the potential to be used as a single pharmaceutical or chemotherapy drug in the future or not. The anticancer activity of 6-bromoisatin has been previously published and therefore it is not possible to secure a patent on this compound.

6.7 Potential development of 6-bromoisatin as a combinatorial chemotherapy for colorectal cancer

Combinatorial chemotherapy is an option that is increasingly attracting the attention of researchers. More information about the gene and protein changes in cancer cells enable researchers to develop newer drugs that specifically target these changes and work differently from standard chemotherapy drugs. The epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor (VEGF) are two important targets in the treatment of advanced colorectal cancer (Kichenadasse and Karapetis,

2008). Cetuximab, a chimeric monoclonal antibody to EGFR, is one of these targeted agents that is known to enhance the cytotoxicity of chemotherapeutic agents (Luo et al., 2010). In a study by Luo et al (2010), Cetuximab enhanced oxaliplatin-induced cytotoxicity on drug-resistant gastric cancer cells through the inhibition of HIF-1 alpha expression via the MAPK/ERK and PI3K/AKT signalling pathways. In fact, extracellular signal regulated kinase (ERK) mediates important downstream aspects of epidermal growth factor signalling (Li et al., 2008). In chapter 5 it was hypothesised that the mechanism of action of 6-bromoisatin was through the inhibition of ERK/Akt (Chapter 5). Therefore further study on the potential effects of 6-bromoisatin on EGFR through the inhibition of ERK/Akt is justified. The potential use of 6-bromoisatin as a targeted drug is also supported by discovery of the halogenated isatin derivative Sunitinib as a kinase inhibitor and therefore a targeted therapy for renal cell carcinoma (Powles et al., 2011).

Bevacizumab is another targeted drug that acts through VEGF receptors and most often is used in combination with chemotherapy drugs such as oxaliplatin, fluorouracil, and leucovorin to treat advanced colorectal cancer. In a study by Videira et al. (2011), the cytotoxic effect of bevacizumab on bladder cancer cell proliferation was observed at >2.5 mg/mL (Giantonio et al., 2007) showing the low cytotoxic effect of this drug. Overall, the use of simple molecules such as 6-bromoistain together with previously patented chemotherapy drugs could be considered as a novel combination drug therapy. There is also increasing philanthropic funding towards screening combinations of anticancer agents with lapsed and current patents (http://www.standup2cancer.org/dream_teams/view/targeting_pi3k_in_womens_cancers).

6.8 Potential development of 6-bromoisatin as a new product for colorectal cancer prevention or treatment

One main challenge in developing a new anticancer drug is the stability and bioavailability of that drug, which needs to be prolonged enough for its activation in the designated targets *in vivo* (Grabley and Thiericke, 1999). 6-Bromoisatin is a stable oxidation product among the precursors of the ancient dye Tyrian purple, in the muricid extract (Benkendorff et al., 2001). Antioxidant studies show that tyrindolinone and tyrindoleninone oxidised to 6-bromoisatin after 12 h exposure with oxygen (Table 2.2, Chapter 2). In a study by Westley, et al. (2010) 6-bromoisatin was found to be more stable than tyrindoleninone in simulated gastric fluids. Indeed, tyrindoleninone in this environment was readily degraded to 6-bromoisatin or converted to tyriverdin (Westley et al., 2010). Therefore, 6-bromoistain is a more attractive drug target than tyrindoleninone or other similar Muricidae compounds.

Another important issue is the safety of the drug in selectively targeting cancer cells over primary cells (Simmons et al., 2005). Ideally the desired mode of cell death is apoptosis, rather than necrosis (Chapter 3-5), that does not cause trauma to the surrounding tissue (Elmore, 2007). In this study, it was confirmed by different apoptosis assays (morphological change of apoptotic cells under the microscope, caspase 3/7 and flow cytometry detection of apoptosis) that apoptosis was the primary cause of cell death in the colorectal cancer cell lines treated with 6-bromoisatin (Chapter 3, 5).

In terms of the safety of the 6-bromoisatin in the body system, the *in vivo* trial did not show any toxicity associated with a two week administration of 6-bromoisatin (natural

and synthetic) in the liver, kidney and blood (hematology and biochemistry analysis), except a reduction in the serum level of potassium by synthetic 6-bromoisatin, which could simply be counteracted by good hydration. Alternatively, this possible side effect of synthetic 6-bromoisatin could be due to urinating more than usual, which can increase the excretion of potassium (Lindeman, 1976, Weiner and Wingo, 1997). This side effect could be removed by administration of 6-bromoisatin in combination with antidiuretic drugs, such as desmopressin (Lethagen et al., 1998). However, a longer *in vivo* study is required to confirm 6-bromoisatins safety for human use.

Although our short term *in vivo* study (2 weeks) revealed the potential chemopreventive effect of 6-bromoisatin, longer term studies in mice are required to identify the effects of 6-bromoisatin on pre-neoplastic lesions or colonic aberrant crypt foci (ACF) formation (12 weeks *in vivo* study) and/or tumour formation (36 weeks *in vivo* study); (Hu et al., 2008). A study for the effect of synthetic 6-bromoisatin on reducing the numbers of ACF in a 14 week model has recently been conducted; however, the results from this study are yet to be analysed and so they are not included in this thesis. This ongoing experiment will provide key data for establishing the future potential for developing *D. orbita* extracts or 6-bromoisatin as a new chemopreventative agent. The crude extract and/or purified 6-bromoisatin in particular also have potential for nutraceutical development. However, the next step will require effective mechanisms for formulating the extract or fraction into a pill that can be taken orally. In addition, quality assurance methods are required, which could include the LCMS analysis procedure used in this study. Furthermore, a procedure for sustainable supply of the extract or compound must be secured prior to taking a new nutraceutical into phase 1 clinical trials.

6.9 Final conclusion

Our study supports the chemopreventive effect of 6-bromoisatin against colorectal cancer with the potential to develop a new synthetic drug and/or a nutraceutical from the marine mollusc *D. orbita* for the prevention of colorectal cancer.

7. Appendix: Analysis of variance (ANOVA) and Post Hoc Tukey HSD tables

- Post hoc tables are only presented where the main effects are significant

- In the post hoc tables, the red highlights indicate non-significant pairwise comparisons and the yellow highlighting is used for significant pairwise comparison, whereas the cells that are not highlighted are repeats of the same pairwise tests in reverse order and are therefore redundant.

7.1 *In vitro* result analysis from Chapter 2

7.1.1 ANOVA for the effect of semi-purified fraction containing tyrindoleninone and tyrindolinone with and without Vit E on HT29 cell proliferation

Tests of Between-Subjects Effects

Dependent Variable: HT29.Proliferation

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.833[a]	17	.049	53.424	.000
Intercept	2.942	1	2.942	3207.402	.000
MICE	.005	8	.001	.631	.749
GROUP	.828	9	.092	100.352	.000
Error	.066	72	.001		
Total	3.841	90			
Corrected Total	.899	89			

a. R Squared = .927 (Adjusted R Squared = .909)

7.1.2 Tukey HSD for the effect of semi-purified fraction containing tyrindoleninone and tyrindolinone with and without Vit E on HT29 cell proliferation (E= Vit E)

Multiple Comparisons

Dependent Variable: HT29.Proliferation

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Control+E	-.0478*	.01428	.040	-.0944	-.0012
	TYR0.01	.0079	.01428	1.000	-.0387	.0545
	TYR0.01+E	-.0608*	.01428	.002	-.1074	-.0142
	TYR0.05	.0936*	.01428	.000	.0469	.1402
	TYR0.05+E	.0120	.01428	.998	-.0346	.0586
	TYR0.1	.1576*	.01428	.000	.1109	.2042
	TYR0.1+E	.0589*	.01428	.004	.0123	.1055
	TYR0.5	.2379*	.01428	.000	.1913	.2845
	TYR0.5+E	.1829*	.01428	.000	.1363	.2295
Control+E	Control	.0478*	.01428	.040	.0012	.0944
	TYR0.01	.0557*	.01428	.008	.0091	.1023
	TYR0.01+E	-.0130	.01428	.996	-.0596	.0336
	TYR0.05	.1413*	.01428	.000	.0947	.1879
	TYR0.05+E	.0598*	.01428	.003	.0132	.1064
	TYR0.1	.2053*	.01428	.000	.1587	.2519
	TYR0.1+E	.1067*	.01428	.000	.0601	.1533
	TYR0.5	.2857*	.01428	.000	.2391	.3323
	TYR0.5+E	.2307*	.01428	.000	.1841	.2773

Based on observed means.

The error term is Mean Square(Error) = .001.

*. The mean difference is significant at the .05 level.

7.1.3 ANOVA for the effect of semi-purified fraction containing tyrindoleninone and tyrindolinone with and without Vit E on Caco2 cell proliferation

Tests of Between-Subjects Effects

Dependent Variable: Caco2.Proliferation

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.104[a]	17	.006	24.111	.000
Intercept	.232	1	.232	909.704	.000
MICE	.002	8	.000	1.061	.400
GROUP	.102	9	.011	44.600	.000
Error	.018	72	.000		
Total	.355	90			
Corrected Total	.123	89			

a. R Squared = .851 (Adjusted R Squared = .815)

7.1.4 Tukey HSD for the effect of semi-purified fraction containing tyrindoleninone and tyrindolinone with and without Vit E on Caco2 cell proliferation (E= Vit E)

Multiple Comparisons

Dependent Variable: Caco2.Proliferation

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Control+E	-.0329*	.00753	.002	-.0574	-.0083
	TYR0.01	-.0060	.00753	.999	-.0305	.0186
	TYR0.01+E	-.0346*	.00753	.001	-.0592	-.0100
	TYR0.05	.0551*	.00753	.000	.0305	.0797
	TYR0.05+E	-.0195	.00753	.240	-.0441	.0051
	TYR0.1	.0551*	.00753	.000	.0305	.0797
	TYR0.1+E	.0006	.00753	1.000	-.0240	.0252
	TYR0.5	.0565*	.00753	.000	.0319	.0811
	TYR0.5+E	.0033	.00753	1.000	-.0213	.0278
Control+E	Control	.0329*	.00753	.002	.0083	.0574
	TYR0.01	.0269*	.00753	.021	.0023	.0515
	TYR0.01+E	-.0017	.00753	1.000	-.0263	.0228
	TYR0.05	.0880*	.00753	.000	.0634	.1126
	TYR0.05+E	.0134	.00753	.748	-.0112	.0379
	TYR0.1	.0880*	.00753	.000	.0634	.1125
	TYR0.1+E	.0335*	.00753	.001	.0089	.0581
	TYR0.5	.0894*	.00753	.000	.0648	.1140
	TYR0.5+E	.0361*	.00753	.000	.0116	.0607

Based on observed means.

The error term is Mean Square(Error) = .000.

*. The mean difference is significant at the .05 level.

7.2 *In vitro* result analysis from Chapter 3

Table 7.2.1 ANOVA for the effect of bioassay guided fractions from *D. orbita* egg mass extract on HT29 cell proliferation

Tests of Between-Subjects Effects

Dependent Variable: HT29.PROLIFERATION

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	84.869[a]	34	2.496	205.841	.000
Intercept	346.056	1	346.056	28537.207	.000
REPLICATION	.102	8	.013	1.054	.397
GROUP	84.766	26	3.260	268.853	.000
Error	2.522	208	.012		
Total	433.447	243			
Corrected Total	87.391	242			

a. R Squared = .971 (Adjusted R Squared = .966)

Table 7.2.2 Tukey HSD for the effect of bioassay guided fractions from *D. orbita* egg mass extract on HT29 cell proliferation

Multiple Comparisons

Dependent Variable: HT29.PROLIFERATION

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	CE0.05	.0786	.05191	.999	-.1160	.2732
	CE0.1	.0803	.05191	.999	-.1143	.2748
	CE0.5	.0258	.05191	1.000	-.1688	.2204
	CE1	.4582*	.05191	.000	.2636	.6528
	CE2	1.2315*	.05191	.000	1.0370	1.4261
	F1,0.05	-.2792*	.05191	.000	-.4738	-.0846
	F1,0.1	.0413	.05191	1.000	-.1533	.2358
	F1,0.5	-.0596	.05191	1.000	-.2542	.1350
	F2,0.05	.4950*	.05191	.000	.3004	.6895
	F2,0.1	.9578*	.05191	.000	.7633	1.1524
	F2,0.5	1.6572*	.05191	.000	1.4626	1.8518
	F3,0.05	.3391*	.05191	.000	.1445	.5337
	F3,0.1	.7064*	.05191	.000	.5118	.9010
	F3,0.5	1.6470*	.05191	.000	1.4524	1.8416
	F4,0.05	.0297	.05191	1.000	-.1649	.2243
	F4,0.1	.1111	.05191	.924	-.0835	.3057
	F4,0.5	.3883*	.05191	.000	.1937	.5829
	F5,0.05	1.3889*	.05191	.000	1.1943	1.5835
	F5,0.1	1.4261*	.05191	.000	1.2315	1.6207
	F5,0.5	1.6115*	.05191	.000	1.4169	1.8060
	F6,0.05	.1447	.05191	.507	-.0498	.3393
	F6,0.1	.2363*	.05191	.003	.0418	.4309
	F6,0.5	.2141*	.05191	.014	.0195	.4087
	F7,0.05	.0403	.05191	1.000	-.1543	.2349
	F7,0.1	.1612	.05191	.282	-.0334	.3557
	F7,0.5	.1545	.05191	.366	-.0401	.3491

Based on observed means.

The error term is Mean Square(Error) = .012.

*. The mean difference is significant at the 0.05 level.

Table 7.2.3 ANOVA for the effect of bioassay guided fractions from *D. orbita* egg mass extract on Caco2 cell proliferation

Tests of Between-Subjects Effects

Dependent Variable: Caco2.PROLIFERATION

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	19.949[a]	34	.587	232.997	.000
Intercept	41.224	1	41.224	16370.386	.000
REPLICATION	.022	8	.003	1.094	.369
GROUP	19.927	26	.766	304.352	.000
Error	.524	208	.003		
Total	61.697	243			
Corrected Total	20.473	242			

a. R Squared = .974 (Adjusted R Squared = .970)

Table 7.2.4 Tukey HSD for the effect of bioassay guided fractions from *D. orbita* egg mass extract on Caco2 cell proliferation

Multiple Comparisons

Dependent Variable: Caco2.PROLIFERATION

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	CE0.05	-.0378	.02366	.998	-.1265	.0509
	CE0.1	.0091	.02366	1.000	-.0796	.0977
	CE0.5	.0373	.02366	.999	-.0514	.1260
	CE1	.1649*	.02366	.000	.0762	.2536
	CE2	.5618*	.02366	.000	.4731	.6504
	F1,0.05	-.0299	.02366	1.000	-.1186	.0588
	F1,0.1	-.0353	.02366	.999	-.1240	.0533
	F1,0.5	.0627	.02366	.616	-.0260	.1514
	F2,0.05	.5982*	.02366	.000	.5095	.6869
	F2,0.1	.6053*	.02366	.000	.5167	.6940
	F2,0.5	.6385*	.02366	.000	.5498	.7271
	F3,0.05	.5931*	.02366	.000	.5044	.6817
	F3,0.1	.6013*	.02366	.000	.5126	.6900
	F3,0.5	.6373*	.02366	.000	.5487	.7260
	F4,0.05	-.0387	.02366	.997	-.1274	.0500
	F4,0.1	-.0189	.02366	1.000	-.1076	.0698
	F4,0.5	.1681*	.02366	.000	.0795	.2568
	F5,0.05	.4945*	.02366	.000	.4058	.5831
	F5,0.1	.5897*	.02366	.000	.5010	.6784
	F5,0.5	.6392*	.02366	.000	.5506	.7279
	F6,0.05	-.0444	.02366	.983	-.1330	.0443
	F6,0.1	-.0278	.02366	1.000	-.1165	.0609
	F6,0.5	.1684*	.02366	.000	.0797	.2571
	F7,0.05	-.0509	.02366	.920	-.1396	.0377
	F7,0.1	-.0152	.02366	1.000	-.1039	.0734
	F7,0.5	-.0047	.02366	1.000	-.0934	.0839

Based on observed means.

The error term is Mean Square(Error) = .003.

*. The mean difference is significant at the .05 level.

Table 7.2.5 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* egg mass on HT29 cell proliferation

Tests of Between-Subjects Effects

Dependent Variable: HT29.PROLIFERATION

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	52.041[a]	26	2.002	316.856	.000
Intercept	114.055	1	114.055	18055.429	.000
GROUP	51.895	18	2.883	456.397	.000
REPLICATION	.146	8	.018	2.888	.005
Error	.910	144	.006		
Total	167.005	171			
Corrected Total	52.950	170			

a. R Squared = .983 (Adjusted R Squared = .980)

Table 7.2.6 Tukey HSD pairwise comparisons for the effect of the crude extract and purified compounds from *D. orbita* egg mass on HT29 cell proliferation. These comparisons focus on the significant differences between the negative (DMSO) control and the test extracts and compounds.

Multiple Comparisons

Dependent Variable: HT29.PROLIFERATION

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	6BR0.01	.3461*	.03747	.000	.2119	.4804
	6BR0.025	.8824*	.03747	.000	.7482	1.0167
	6BR0.05	1.2217*	.03747	.000	1.0874	1.3559
	6BR0.1	1.2658*	.03747	.000	1.1315	1.4000
	6BR0.25	1.2795*	.03747	.000	1.1452	1.4137
	6BR0.5	1.4218*	.03747	.000	1.2875	1.5560
	CE0.05	.0138	.03747	1.000	-.1205	.1480
	CE0.1	.0639	.03747	.970	-.0704	.1981
	CE0.25	.0966	.03747	.503	-.0377	.2308
	CE0.5	.1338	.03747	.052	-.0005	.2680
	CE1	.4009*	.03747	.000	.2666	.5351
	CE2	1.0696*	.03747	.000	.9353	1.2038
	TYR0.01	.0000	.03747	1.000	-.1342	.1342
	TYR0.025	.2088*	.03747	.000	.0745	.3430
	TYR0.05	.5541*	.03747	.000	.4199	.6884
	TYR0.1	.8043*	.03747	.000	.6701	.9386
	TYR0.25	1.4517*	.03747	.000	1.3174	1.5859
	TYR0.5	1.4579*	.03747	.000	1.3237	1.5921

Based on observed means.

The error term is Mean Square(Error) = .006.

*. The mean difference is significant at the .05 level.

Table 7.2.7 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* egg mass on Caco2 cell proliferation

Tests of Between-Subjects Effects

Dependent Variable: Caco2.PROLIFERATION

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.890[a]	26	.034	461.245	.000
Intercept	1.032	1	1.032	13910.643	.000
GROUP	.890	18	.049	666.121	.000
REPLICATION	.000	8	2.019E-005	.272	.974
Error	.011	144	7.419E-005		
Total	1.932	171			
Corrected Total	.900	170			

a. R Squared = .988 (Adjusted R Squared = .986)

Table 7.2.8 Tukey HSD pairwise comparisons for the effect of the crude extract and purified compounds from *D. orbita* egg mass on Caco2 cell proliferation. These comparisons focus on the significant differences between the negative (DMSO) control and the test extracts and compounds.

Multiple Comparisons

Dependent Variable: Caco2.PROLIFERATION

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	6BR0.01	.0642*	.00406	.000	.0496	.0787
	6BR0.025	.1019*	.00406	.000	.0874	.1165
	6BR0.05	.1301*	.00406	.000	.1155	.1446
	6BR0.1	.1598*	.00406	.000	.1453	.1744
	6BR0.25	.1740*	.00406	.000	.1595	.1886
	6BR0.5	.1741*	.00406	.000	.1596	.1887
	CE0.05	-.0020	.00406	1.000	-.0166	.0125
	CE0.1	.0009	.00406	1.000	-.0136	.0155
	CE0.25	.0059	.00406	.995	-.0087	.0204
	CE0.5	.0238*	.00406	.000	.0093	.0384
	CE1	.0378*	.00406	.000	.0232	.0523
	CE2	.1538*	.00406	.000	.1393	.1684
	TYR0.01	.0044	.00406	1.000	-.0102	.0189
	TYR0.025	.1472*	.00406	.000	.1327	.1618
	TYR0.05	.1633*	.00406	.000	.1487	.1778
	TYR0.1	.1652*	.00406	.000	.1506	.1797
	TYR0.25	.1737*	.00406	.000	.1591	.1882
	TYR0.5	.1742*	.00406	.000	.1597	.1888

Based on observed means.

The error term is Mean Square(Error) = 7.42E-005.

*. The mean difference is significant at the .05 level.

Table 7.2.9 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* egg mass on HT29 cell membrane integrity (LHD release)

Tests of Between-Subjects Effects

Dependent Variable: HT29.LDH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9994412912101726 70.000[a]	26	384400496619297 20.000	684.919	.000
Intercept	550646203467005380.000	1	550646203467005380.000	9811.332	.000
REPLICATION	629583496821633.100	8	78697937102704.140	1.402	.200
GROUP	998811707713351040.000	18	55489539317408392.000	988.704	.000
Error	8081782384584565.000	144	56123488781837.260		
Total	1558169277061760260.000	171			
Corrected Total	1007523073594757250.000	170			

a. R Squared = .992 (Adjusted R Squared = .991)

Table 7.2.10 Tukey HSD pairwise comparisons for the effect of the crude extract and purified compounds from *D. orbita* egg mass on HT29 cell membrane integrity (LHD release)

Multiple Comparisons

Dependent Variable: HT29.LDH

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.neg	6BR0.01	-1118841.7778	3531555.80389	1.000	-13772533.3336	11534849.7780
	6BR0.025	-2394362.4444	3531555.80389	1.000	-15048054.0002	10259329.1113
	6BR0.05	-3964630.6667	3531555.80389	1.000	-16618322.2225	8689060.8891
	6BR0.1	-12184941.3333	3531555.80389	.074	-24838632.8891	468750.2225
	6BR0.25	-51616258.8889*	3531555.80389	.000	-64269950.4447	-38962567.3331
	6BR0.5	-150150553.1111*	3531555.80389	.000	-162804244.6669	-137496861.5553
	C.pos	-282090618.4444*	3531555.80389	.000	-294744310.0002	-269436926.8887
	CE0.025	-451634.0000	3531555.80389	1.000	-13105325.5558	12202057.5558
	CE0.05	-834939.6667	3531555.80389	1.000	-13488631.2225	11818751.8891
	CE0.1	-1499853.6667	3531555.80389	1.000	-14153545.2225	11153837.8891
	CE0.25	-2471678.3333	3531555.80389	1.000	-15125369.8891	10182013.2225
	CE0.5	-2465483.0000	3531555.80389	1.000	-15119174.5558	10188208.5558
	TYR0.01	-808075.1111	3531555.80389	1.000	-13461766.6669	11845616.4447
	TYR0.025	-3389802.2222	3531555.80389	1.000	-16043493.7780	9263889.3336
	TYR0.05	-3992194.8889	3531555.80389	1.000	-16645886.4447	8661496.6669
	TYR0.1	-22643127.5556*	3531555.80389	.000	-35296819.1113	-9989435.9998
	TYR0.25	-128134558.4444*	3531555.80389	.000	-140788250.0002	-115480866.8887
	TYR0.5	-158812633.1111*	3531555.80389	.000	-171466324.6669	-146158941.5553
C.pos	6BR0.01	280971776.6667*	3531555.80389	.000	268318085.1109	293625468.2225
	6BR0.025	279696256.0000*	3531555.80389	.000	267042564.4442	292349947.5558
	6BR0.05	278125987.7778*	3531555.80389	.000	265472296.2220	290779679.3336
	6BR0.1	269905677.1111*	3531555.80389	.000	257251985.5553	282559368.6669
	6BR0.25	230474359.5556*	3531555.80389	.000	217820667.9998	243128051.1113
	6BR0.5	131940065.3333*	3531555.80389	.000	119286373.7775	144593756.8891
	C.neg	282090618.4444*	3531555.80389	.000	269436926.8887	294744310.0002
	CE0.025	281638984.4444*	3531555.80389	.000	268985292.8887	294292676.0002
	CE0.05	281255678.7778*	3531555.80389	.000	268601987.2220	293909370.3336
	CE0.1	280590764.7778*	3531555.80389	.000	267937073.2220	293244456.3336
	CE0.25	279618940.1111*	3531555.80389	.000	266965248.5553	292272631.6669
	CE0.5	279625135.4444*	3531555.80389	.000	266971443.8887	292278827.0002
	TYR0.01	281282543.3333*	3531555.80389	.000	268628851.7775	293936234.8891
	TYR0.025	278700816.2222*	3531555.80389	.000	266047124.6664	291354507.7780
	TYR0.05	278098423.5556*	3531555.80389	.000	265444731.9998	290752115.1113
	TYR0.1	259447490.8889*	3531555.80389	.000	246793799.3331	272101182.4447
	TYR0.25	153956060.0000*	3531555.80389	.000	141302368.4442	166609751.5558
	TYR0.5	123277985.3333*	3531555.80389	.000	110624293.7775	135931676.8891

Based on observed means.

The error term is Mean Square(Error) = 56123488781837.260.

*. The mean difference is significant at the .05 level.

Table 7.2.11 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* egg mass on Caco2 cells membrane integrity (LHD release)

Tests of Between-Subjects Effects

Dependent Variable: Caco2.LDH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2697701342437431.000[a]	26	103757743939901.190	80.206	.000
Intercept	13353089618232400.000	1	13353089618232400.000	10322.160	.000
REPLICATION	14784388644021.754	8	1848048580502.719	1.429	.189
GROUP	2682916953793406.500	18	149050941877411.470	115.219	.000
Error	186283189887419.280	144	1293633263107.078		
Total	16237074150557208.000	171			
Corrected Total	2883984532324850.500	170			

a. R Squared = .935 (Adjusted R Squared = .924)

Table 7.2.12 Tukey HSD pairwise comparisons for the effect of the crude extract and purified compounds from *D. orbita* egg mass on Caco2 cells membrane integrity (LHD release)

Multiple Comparisons

Dependent Variable: Caco2.LDH

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.neg	6BR0.01	-63866.1111	536166.07359	1.000	-1984968.4509	1857236.2287
	6BR0.025	106488.7778	536166.07359	1.000	-1814613.5620	2027591.1175
	6BR0.05	483959.1111	536166.07359	1.000	-1437143.2287	2405061.4509
	6BR0.1	-496733.6667	536166.07359	1.000	-2417836.0064	1424368.6731
	6BR0.25	-4168848.7778*	536166.07359	.000	-6089951.1175	-2247746.4380
	6BR0.5	-6851152.8889*	536166.07359	.000	-8772255.2287	-4930050.5491
	C.pos	-16402942.3333*	536166.07359	.000	-18324044.6731	-14481839.9936
	CE0.025	-872709.3333	536166.07359	.981	-2793811.6731	1048393.0064
	CE0.05	-908784.8889	536166.07359	.972	-2829887.2287	1012317.4509
	CE0.1	-1085032.4444	536166.07359	.871	-3006134.7842	836069.8953
	CE0.25	-1309454.1111	536166.07359	.604	-3230556.4509	611648.2287
	CE0.5	-1272827.6667	536166.07359	.655	-3193930.0064	648274.6731
	TYR0.01	-1389005.7778	536166.07359	.493	-3310108.1175	532096.5620
	TYR0.025	-1697064.0000	536166.07359	.157	-3618166.3398	224038.3398
	TYR0.05	-2441309.1111*	536166.07359	.002	-4362411.4509	-520206.7713
	TYR0.1	-3110506.0000*	536166.07359	.000	-5031608.3398	-1189403.6602
	TYR0.25	-5514240.1111*	536166.07359	.000	-7435342.4509	-3593137.7713
	TYR0.5	-8235412.8889*	536166.07359	.000	-10156515.2287	-6314310.5491

Based on observed means.

The error term is Mean Square(Error) = 1293633263107.079.

*. The mean difference is significant at the .05 level.

Table 7.2.13 ANOVA for the apoptotic effect of the crude extract and purified compounds from *D. orbita* egg mass on HT29 cells (Caspase activity)

Tests of Between-Subjects Effects

Dependent Variable: HT29.Caspase

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	288996776894.070[a]	26	11115260649.772	486.314	.000
Intercept	247871258738.621	1	247871258738.621	10844.833	.000
REPLICATION	199316287.801	8	24914535.975	1.090	.373
GROUP	288797460606.269	18	16044303367.015	701.968	.000
Error	3291287369.310	144	22856162.287		
Total	540159323002.000	171			
Corrected Total	292288064263.380	170			

a. R Squared = .989 (Adjusted R Squared = .987)

Table 7.2.14 Tukey HSD pairwise comparisons for the apoptotic effect of the crude extract and purified compounds from *D. orbita* egg mass on HT29 cells (Caspase activity)

Multiple Comparisons

Dependent Variable: HT29.Caspase

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.neg	6BR0.01	-5632.8889	2253.69634	.561	-13707.9640	2442.1862
	6BR0.025	-107990.3333*	2253.69634	.000	-116065.4084	-99915.2582
	6BR0.05	-93810.6667*	2253.69634	.000	-101885.7418	-85735.5916
	6BR0.1	-28581.4444*	2253.69634	.000	-36656.5195	-20506.3694
	6BR0.25	12162.5556*	2253.69634	.000	4087.4805	20237.6306
	6BR0.5	20040.3333*	2253.69634	.000	11965.2582	28115.4084
	C.pos	-112776.2222*	2253.69634	.000	-120851.2973	-104701.1471
	CE0.025	-839.4444	2253.69634	1.000	-8914.5195	7235.6306
	CE0.05	492.0000	2253.69634	1.000	-7583.0751	8567.0751
	CE0.1	191.0000	2253.69634	1.000	-7884.0751	8266.0751
	CE0.25	-313.7778	2253.69634	1.000	-8388.8529	7761.2973
	CE0.5	-978.0000	2253.69634	1.000	-9053.0751	7097.0751
	TYR0.01	3463.2222	2253.69634	.990	-4611.8529	11538.2973
	TYR0.025	3998.4444	2253.69634	.956	-4076.6306	12073.5195
	TYR0.05	-29570.4444*	2253.69634	.000	-37645.5195	-21495.3694
	TYR0.1	11375.5556*	2253.69634	.000	3300.4805	19450.6306
	TYR0.25	19914.0000*	2253.69634	.000	11838.9249	27989.0751
	TYR0.5	20990.5556*	2253.69634	.000	12915.4805	29065.6306

Based on observed means.

The error term is Mean Square(Error) = 22856162.287.

*. The mean difference is significant at the .05 level.

Table 7.2.15 ANOVA for the apoptotic effect of the crude extract and purified compounds from *D. orbita* egg mass on Caco2 cells (Caspase activity)

Tests of Between-Subjects Effects

Dependent Variable: Caco2.Caspase

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1325187341.825[a]	26	50968743.916	336.378	.000
Intercept	1031971967.251	1	1031971967.251	6810.688	.000
REPLICATION	307844.854	8	38480.607	.254	.979
GROUP	1324879496.971	18	73604416.498	485.766	.000
Error	21819228.924	144	151522.423		
Total	2378978538.000	171			
Corrected Total	1347006570.749	170			

a. R Squared = .984 (Adjusted R Squared = .981)

Table 7.2.16 Tukey HSD pairwise comparisons for the apoptotic effect of the crude extract and purified compounds from *D. orbita* egg mass on Caco2 cells (Caspase activity)

Multiple Comparisons

Dependent Variable: Caco2.Caspase

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.neg	6BR0.01	-5566.8889*	183.49836	.000	-6224.3701	-4909.4076
	6BR0.025	-8735.0000*	183.49836	.000	-9392.4812	-8077.5188
	6BR0.05	-5538.1111*	183.49836	.000	-6195.5924	-4880.6299
	6BR0.1	174.1111	183.49836	1.000	-483.3701	831.5924
	6BR0.25	1274.0000*	183.49836	.000	616.5188	1931.4812
	6BR0.5	1522.1111*	183.49836	.000	864.6299	2179.5924
	C.pos	-2215.0000*	183.49836	.000	-2872.4812	-1557.5188
	CE0.025	-33.3333	183.49836	1.000	-690.8146	624.1479
	CE0.05	-139.3333	183.49836	1.000	-796.8146	518.1479
	CE0.1	-214.3333	183.49836	1.000	-871.8146	443.1479
	CE0.25	-200.0000	183.49836	1.000	-857.4812	457.4812
	CE0.5	-549.0000	183.49836	.235	-1206.4812	108.4812
	TYR0.01	-475.5556	183.49836	.492	-1133.0368	181.9257
	TYR0.025	-47.8889	183.49836	1.000	-705.3701	609.5924
	TYR0.05	1483.2222*	183.49836	.000	825.7410	2140.7035
	TYR0.1	1652.6667*	183.49836	.000	995.1854	2310.1479
	TYR0.25	1662.0000*	183.49836	.000	1004.5188	2319.4812
	TYR0.5	1665.7778*	183.49836	.000	1008.2965	2323.2590

Based on observed means.

The error term is Mean Square(Error) = 151522.423.

*. The mean difference is significant at the .05 level.

Table 7.2.17 ANOVA for the apoptotic effect of the purified compounds from *D. orbita* egg mass extract on HT29 cells (Flow cytometry analysis; Annexine V staining)

Tests of Between-Subjects Effects

Dependent Variable: Annexine

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9861.668[a]	5	1972.334	11.856	.005
Intercept	23709.630	1	23709.630	142.517	.000
REPLICATION	177.545	2	88.773	.534	.612
GROUP	9684.123	3	3228.041	19.404	.002
Error	998.182	6	166.364		
Total	34569.480	12			
Corrected Total	10859.850	11			

a. R Squared = .908 (Adjusted R Squared = .831)

Table 7.2.18 Tukey HSD for the apoptotic effect of the purified compounds from *D. orbita* egg mass extract on HT29 cells (Flow cytometry analysis; Annexine V staining)

Multiple Comparisons

Dependent Variable: Annexine

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6BR0.025	6BR0.05	7.1667	10.53134	.901	-29.2898	43.6231
	control	68.7000*	10.53134	.003	32.2435	105.1565
	TYR0.05	47.6667*	10.53134	.016	11.2102	84.1231
6BR0.05	6BR0.025	-7.1667	10.53134	.901	-43.6231	29.2898
	control	61.5333*	10.53134	.004	25.0769	97.9898
	TYR0.05	40.5000*	10.53134	.032	4.0435	76.9565
control	6BR0.025	-68.7000*	10.53134	.003	-105.1565	-32.2435
	6BR0.05	-61.5333*	10.53134	.004	-97.9898	-25.0769
	TYR0.05	-21.0333	10.53134	.286	-57.4898	15.4231
TYR0.05	6BR0.025	-47.6667*	10.53134	.016	-84.1231	-11.2102
	6BR0.05	-40.5000*	10.53134	.032	-76.9565	-4.0435
	control	21.0333	10.53134	.286	-15.4231	57.4898

Based on observed means.

The error term is Mean Square(Error) = 166.364.

*. The mean difference is significant at the 0.05 level.

Table 7.2.19 ANOVA for the necrotic effect of the purified compounds from *D. orbita* egg mass extract on HT29 cells (Flow cytometry analysis; PI staining)

Tests of Between-Subjects Effects

Dependent Variable: PI

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	143.574[a]	5	28.715	6.128	.024
Intercept	756.841	1	756.841	161.517	.000
GROUP	35.562	3	11.854	2.530	.154
REPLICATION	108.012	2	54.006	11.525	.009
Error	28.115	6	4.686		
Total	928.530	12			
Corrected Total	171.689	11			

a. R Squared = .836 (Adjusted R Squared = .700)

Table 7.2.20 ANOVA for the effect of the semi-purified 6-bromoisatin from *D. orbita* egg mass extract on HT29 cells (Flow cytometry cell cycle analysis; G0-G1 phase)

Tests of Between-Subjects Effects

Dependent Variable: G1

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	369.068[a]	6	61.511	5.222	.018
Intercept	50112.600	1	50112.600	4254.402	.000
REPLICATION	.588	2	.294	.025	.975
GROUP	368.480	4	92.120	7.821	.007
Error	94.232	8	11.779		
Total	50575.900	15			
Corrected Total	463.300	14			

a. R Squared = .797 (Adjusted R Squared = .644)

Table 7.2.21 Tukey HSD for the effect of semi-purified 6-bromoisatin from *D. orbita* egg mass extract on HT29 cells (Flow cytometry cell cycle analysis; G0-G1 phase)

Multiple Comparisons

Dependent Variable: G1
Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6BR0.01	6BR0.025	9.6667	2.80226	.050	-.0144	19.3478
	6BR0.05	8.6000	2.80226	.085	-1.0811	18.2811
	6BR0.1	10.4667*	2.80226	.034	.7856	20.1478
	control	-.9000	2.80226	.997	-10.5811	8.7811
6BR0.025	6BR0.01	-9.6667	2.80226	.050	-19.3478	.0144
	6BR0.05	-1.0667	2.80226	.995	-10.7478	8.6144
	6BR0.1	.8000	2.80226	.998	-8.8811	10.4811
	control	-10.5667*	2.80226	.033	-20.2478	-.8856
6BR0.05	6BR0.01	-8.6000	2.80226	.085	-18.2811	1.0811
	6BR0.025	1.0667	2.80226	.995	-8.6144	10.7478
	6BR0.1	1.8667	2.80226	.958	-7.8144	11.5478
	control	-9.5000	2.80226	.055	-19.1811	.1811
6BR0.1	6BR0.01	-10.4667*	2.80226	.034	-20.1478	-.7856
	6BR0.025	-.8000	2.80226	.998	-10.4811	8.8811
	6BR0.05	-1.8667	2.80226	.958	-11.5478	7.8144
	control	-11.3667*	2.80226	.022	-21.0478	-1.6856
control	6BR0.01	.9000	2.80226	.997	-8.7811	10.5811
	6BR0.025	10.5667*	2.80226	.033	.8856	20.2478
	6BR0.05	9.5000	2.80226	.055	-.1811	19.1811
	6BR0.1	11.3667*	2.80226	.022	1.6856	21.0478

Based on observed means.

The error term is Mean Square(Error) = 11.779.
*. The mean difference is significant at the .05 level.

Table 7.2.22 ANOVA for the effect of semi-purified 6-bromoisatin from *D. orbita* egg mass extract on HT29 cells (Flow cytometry cell cycle analysis; S phase)

Tests of Between-Subjects Effects

Dependent Variable: S

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	337.143[a]	6	56.190	17.591	.000
Intercept	4240.323	1	4240.323	1327.452	.000
REPLICATION	4.145	2	2.073	.649	.548
GROUP	332.997	4	83.249	26.062	.000
Error	25.555	8	3.194		
Total	4603.020	15			
Corrected Total	362.697	14			

a. R Squared = .930 (Adjusted R Squared = .877)

Table 7.2.23 Tukey HSD for the effect of semi-purified 6-bromoisatin from *D. orbita* egg mass extract on HT29 cells (Flow cytometry cell cycle analysis; S phase)

Multiple Comparisons

Dependent Variable: S
Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6BR0.01	6BR0.025	.7333	1.45930	.985	-4.3082	5.7748
	6BR0.05	.9333	1.45930	.964	-4.1082	5.9748
	6BR0.1	-11.0000*	1.45930	.000	-16.0415	-5.9585
	control	1.2667	1.45930	.901	-3.7748	6.3082
6BR0.025	6BR0.01	-.7333	1.45930	.985	-5.7748	4.3082
	6BR0.05	.2000	1.45930	1.000	-4.8415	5.2415
	6BR0.1	-11.7333*	1.45930	.000	-16.7748	-6.6918
	control	.5333	1.45930	.995	-4.5082	5.5748
6BR0.05	6BR0.01	-.9333	1.45930	.964	-5.9748	4.1082
	6BR0.025	-.2000	1.45930	1.000	-5.2415	4.8415
	6BR0.1	-11.9333*	1.45930	.000	-16.9748	-6.8918
	control	.3333	1.45930	.999	-4.7082	5.3748
6BR0.1	6BR0.01	11.0000*	1.45930	.000	5.9585	16.0415
	6BR0.025	11.7333*	1.45930	.000	6.6918	16.7748
	6BR0.05	11.9333*	1.45930	.000	6.8918	16.9748
	control	12.2667*	1.45930	.000	7.2252	17.3082
control	6BR0.01	-1.2667	1.45930	.901	-6.3082	3.7748
	6BR0.025	-.5333	1.45930	.995	-5.5748	4.5082
	6BR0.05	-.3333	1.45930	.999	-5.3748	4.7082
	6BR0.1	-12.2667*	1.45930	.000	-17.3082	-7.2252

Based on observed means.

The error term is Mean Square(Error) = 3.194.
*. The mean difference is significant at the .05 level.

Table 7.2.24 ANOVA for the effect of semi-purified 6-bromoisatin from *D. orbita* egg mass extract on HT29 cells (Flow cytometry cell cycle analysis; G2-M phase)

Tests of Between-Subjects Effects

Dependent Variable: G2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	277.964[a]	6	46.327	14.296	.001
Intercept	5884.561	1	5884.561	1815.849	.000
REPLICATION	.901	2	.451	.139	.872
GROUP	277.063	4	69.266	21.374	.000
Error	25.925	8	3.241		
Total	6188.450	15			
Corrected Total	303.889	14			

a. R Squared = .915 (Adjusted R Squared = .851)

Table 7.2.25 Tukey HSD for the effect of semi-purified 6-bromoisatin from *D. orbita* egg mass extract on HT29 cells (Flow cytometry cell cycle analysis; G2-M phase)

Multiple Comparisons

Dependent Variable: G2
Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6BR0.01	6BR0.025	-7.6000*	1.46985	.006	-12.6779	-2.5221
	6BR0.05	-7.0667*	1.46985	.009	-12.1446	-1.9887
	6BR0.1	3.1000	1.46985	.302	-1.9779	8.1779
	control	.3667	1.46985	.999	-4.7113	5.4446
6BR0.025	6BR0.01	7.6000*	1.46985	.006	2.5221	12.6779
	6BR0.05	.5333	1.46985	.996	-4.5446	5.6113
	6BR0.1	10.7000*	1.46985	.001	5.6221	15.7779
	control	7.9667*	1.46985	.004	2.8887	13.0446
6BR0.05	6BR0.01	7.0667*	1.46985	.009	1.9887	12.1446
	6BR0.025	-.5333	1.46985	.996	-5.6113	4.5446
	6BR0.1	10.1667*	1.46985	.001	5.0887	15.2446
	control	7.4333*	1.46985	.006	2.3554	12.5113
6BR0.1	6BR0.01	-3.1000	1.46985	.302	-8.1779	1.9779
	6BR0.025	-10.7000*	1.46985	.001	-15.7779	-5.6221
	6BR0.05	-10.1667*	1.46985	.001	-15.2446	-5.0887
	control	-2.7333	1.46985	.406	-7.8113	2.3446
control	6BR0.01	-.3667	1.46985	.999	-5.4446	4.7113
	6BR0.025	-7.9667*	1.46985	.004	-13.0446	-2.8887
	6BR0.05	-7.4333*	1.46985	.006	-12.5113	-2.3554
	6BR0.1	2.7333	1.46985	.406	-2.3446	7.8113

Based on observed means.

The error term is Mean Square(Error) = 3.241.
*. The mean difference is significant at the .05 level.

7.3 *In vivo* result analysis from Chapter 4

Table 7.3.1 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on mice weight gain

Tests of Between-Subjects Effects

Dependent Variable: WEIGHTgain

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	393.307[a]	17	23.136	1.345	.191
Intercept	1184.459	1	1184.459	68.862	.000
MICE	129.230	9	14.359	.835	.587
GROUP	264.077	8	33.010	1.919	.070
Error	1238.440	72	17.201		
Total	2816.205	90			
Corrected Total	1631.746	89			

a. R Squared = .241 (Adjusted R Squared = .062)

Table 7.3.2 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on liver / body weight (%)

Tests of Between-Subjects Effects

Dependent Variable: liverPERbody

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	13.469[a]	17	.792	2.411	.005
Intercept	2263.676	1	2263.676	6887.062	.000
GROUP	11.450	8	1.431	4.354	.000
MICE	2.020	9	.224	.683	.722
Error	23.665	72	.329		
Total	2300.811	90			
Corrected Total	37.135	89			

a. R Squared = .363 (Adjusted R Squared = .212)

Table 7.3.3 Tukey HSD for the effect of the crude extract and purified compounds from *D. orbita* on liver / body weight (%)

Multiple Comparisons

Dependent Variable: liverPERbody

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.AOM	6BRO0.05	.4981	.25639	.587	-.3219	1.3180
	6BRO0.1	.0601	.25639	1.000	-.7598	.8801
	C.Saline	1.0438*	.25639	.004	.2238	1.8637
	CRUD0.25	-.0204	.25639	1.000	-.8403	.7996
	CRUD0.5	.0142	.25639	1.000	-.8058	.8341
	TYR0.025	.5949	.25639	.344	-.2250	1.4149
	TYR0.05	.0438	.25639	1.000	-.7762	.8637
	TYR0.1	-.0042	.25639	1.000	-.8242	.8157
C.Saline	6BRO0.05	-.5457	.25639	.463	-1.3656	.2743
	6BRO0.1	-.9837*	.25639	.008	-1.8036	-.1637
	C.AOM	-1.0438*	.25639	.004	-1.8637	-.2238
	CRUD0.25	-1.0641*	.25639	.003	-1.8841	-.2442
	CRUD0.5	-1.0296*	.25639	.004	-1.8495	-.2096
	TYR0.025	-.4488	.25639	.713	-1.2688	.3711
	TYR0.05	-1.0000*	.25639	.006	-1.8200	-.1801
	TYR0.1	-1.0480*	.25639	.003	-1.8680	-.2280

Based on observed means.

The error term is Mean Square(Error) = .329.

*. The mean difference is significant at the 0.05 level.

Table 7.3.4 ANOVA for the apoptotic effect of the crude extract and purified compounds from *D. orbita* on distal colon of mice

Tests of Between-Subjects Effects

Dependent Variable: APOPTOSIS

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	253.351[a]	17	14.903	13.634	.000
Intercept	1026.149	1	1026.149	938.756	.000
MICE	8.228	9	.914	.836	.585
GROUP	245.122	8	30.640	28.031	.000
Error	78.703	72	1.093		
Total	1358.202	90			
Corrected Total	332.053	89			

a. R Squared = .763 (Adjusted R Squared = .707)

Table 7.3.5 Tukey HSD for the apoptotic of effect the crude extract and purified compounds from *D. orbita* on distal colon of mice

Multiple Comparisons

Dependent Variable: APOPTOSIS

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.AOM	6BRO0.05	-3.0239*	.46757	.000	-4.5192	-1.5286
	6BRO0.1	-3.0741*	.46757	.000	-4.5694	-1.5788
	C.Saline	2.0329*	.46757	.001	.5376	3.5282
	CRUD0.25	-1.5908*	.46757	.028	-3.0861	-.0955
	CRUD0.5	-3.2093*	.46757	.000	-4.7046	-1.7140
	TYR0.025	-.1382	.46757	1.000	-1.6335	1.3571
	TYR0.05	-.5931	.46757	.937	-2.0884	.9022
	TYR0.1	-.8703	.46757	.642	-2.3656	.6250

Based on observed means.

The error term is Mean Square(Error) = 1.093.

*. The mean difference is significant at the 0.05 level.

Table 7.3.6 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on distal colon crypt height

Tests of Between-Subjects Effects

Dependent Variable: CRYPT.HEIGHT

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	59.027[a]	17	3.472	2.947	.001
Intercept	51056.917	1	51056.917	43338.544	.000
GROUP	49.597	8	6.200	5.262	.000
MICE	9.430	9	1.048	.889	.539
Error	84.823	72	1.178		
Total	51200.766	90			
Corrected Total	143.850	89			

a. R Squared = .410 (Adjusted R Squared = .271)

Table 7.3.7 Tukey HSD for the effect of the crude extract and purified compounds from *D. orbita* on distal colon crypt height

Multiple Comparisons

Dependent Variable: CRYPT.HEIGHT
Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.AOM	6BRO0.05	.3200	.48541	.999	-1.2323	1.8723
	6BRO0.1	-.1750	.48541	1.000	-1.7273	1.3773
	C.Saline	.7426	.48541	.838	-.8097	2.2950
	CRUD0.25	-.1600	.48541	1.000	-1.7123	1.3923
	CRUD0.5	1.9350*	.48541	.005	.3827	3.4873
	TYR0.025	1.6800*	.48541	.024	.1277	3.2323
	TYR0.05	1.2700	.48541	.198	-.2823	2.8223
	TYR0.1	.7950	.48541	.781	-.7573	2.3473

Based on observed means.

The error term is Mean Square(Error) = 1.178.
*. The mean difference is significant at the .05 level.

Table 7.3.8 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on distal colon proliferation in mice (immunohistochemical staining)

Tests of Between-Subjects Effects

Dependent Variable: PROLIFERATION

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1090.491[a]	17	64.147	14.258	.000
Intercept	31261.745	1	31261.745	6948.451	.000
GROUP	1024.458	8	128.057	28.463	.000
MICE	66.033	9	7.337	1.631	.123
Error	323.935	72	4.499		
Total	32676.171	90			
Corrected Total	1414.426	89			

a. R Squared = .771 (Adjusted R Squared = .717)

Table 7.3.9 Tukey HSD for the effect of the crude extract and purified compounds from *D. orbita* on distal colon proliferation in mice (immunohistochemical staining)

Multiple Comparisons

Dependent Variable: PROLIFERATION
Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6BRO0.05	6BRO0.1	.1861	.94859	1.000	-2.8475	3.2198
	C.AOM	-7.2228*	.94859	.000	-10.2565	-4.1892
	C.Saline	-9.9044*	.94859	.000	-12.9380	-6.8707
	CRUD0.25	-1.1548	.94859	.950	-4.1884	1.8788
	CRUD0.5	-.9818	.94859	.981	-4.0154	2.0518
	TYR0.025	-4.5314*	.94859	.000	-7.5650	-1.4978
	TYR0.05	-3.7515*	.94859	.005	-6.7851	-.7179
	TYR0.1	-.2709	.94859	1.000	-3.3045	2.7627
6BRO0.1	6BRO0.05	-.1861	.94859	1.000	-3.2198	2.8475
	C.AOM	-7.4090*	.94859	.000	-10.4426	-4.3754
	C.Saline	-10.0905*	.94859	.000	-13.1241	-7.0569
	CRUD0.25	-1.3409	.94859	.889	-4.3746	1.6927
	CRUD0.5	-1.1679	.94859	.947	-4.2016	1.8657
	TYR0.025	-4.7176*	.94859	.000	-7.7512	-1.6839
	TYR0.05	-3.9377*	.94859	.003	-6.9713	-.9040
	TYR0.1	-.4571	.94859	1.000	-3.4907	2.5766
C.AOM	6BRO0.05	7.2228*	.94859	.000	4.1892	10.2565
	6BRO0.1	7.4090*	.94859	.000	4.3754	10.4426
	C.Saline	-2.6815	.94859	.126	-5.7152	.3521
	CRUD0.25	6.0680*	.94859	.000	3.0344	9.1017
	CRUD0.5	6.2411*	.94859	.000	3.2074	9.2747
	TYR0.025	2.6914	.94859	.123	-.3422	5.7250
	TYR0.05	3.4713*	.94859	.013	.4377	6.5049
	TYR0.1	6.9519*	.94859	.000	3.9183	9.9855
C.Saline	6BRO0.05	9.9044*	.94859	.000	6.8707	12.9380
	6BRO0.1	10.0905*	.94859	.000	7.0569	13.1241
	C.AOM	2.6815	.94859	.126	-.3521	5.7152
	CRUD0.25	8.7496*	.94859	.000	5.7160	11.7832
	CRUD0.5	8.9226*	.94859	.000	5.8890	11.9562
	TYR0.025	5.3730*	.94859	.000	2.3393	8.4066
	TYR0.05	6.1528*	.94859	.000	3.1192	9.1865
	TYR0.1	9.6335*	.94859	.000	6.5998	12.6671
CRUD0.25	6BRO0.05	1.1548	.94859	.950	-1.8788	4.1884
	6BRO0.1	1.3409	.94859	.889	-1.6927	4.3746
	C.AOM	-6.0680*	.94859	.000	-9.1017	-3.0344
	C.Saline	-8.7496*	.94859	.000	-11.7832	-5.7160
	CRUD0.5	.1730	.94859	1.000	-2.8606	3.2066
	TYR0.025	-3.3766*	.94859	.018	-6.4102	-.3430
	TYR0.05	-2.5967	.94859	.153	-5.6304	.4369
	TYR0.1	.8839	.94859	.990	-2.1497	3.9175
CRUD0.5	6BRO0.05	.9818	.94859	.981	-2.0518	4.0154
	6BRO0.1	1.1679	.94859	.947	-1.8657	4.2016
	C.AOM	-6.2411*	.94859	.000	-9.2747	-3.2074
	C.Saline	-8.9226*	.94859	.000	-11.9562	-5.8890
	CRUD0.25	-.1730	.94859	1.000	-3.2066	2.8606
	TYR0.025	-3.5496*	.94859	.010	-6.5832	-.5160
	TYR0.05	-2.7697	.94859	.101	-5.8034	.2639
	TYR0.1	.7109	.94859	.998	-2.3228	3.7445
TYR0.025	6BRO0.05	4.5314*	.94859	.000	1.4978	7.5650
	6BRO0.1	4.7176*	.94859	.000	1.6839	7.7512
	C.AOM	-2.6914	.94859	.123	-5.7250	.3422
	C.Saline	-5.3730*	.94859	.000	-8.4066	-2.3393
	CRUD0.25	3.3766*	.94859	.018	.3430	6.4102
	CRUD0.5	3.5496*	.94859	.010	.5160	6.5832
	TYR0.05	.7799	.94859	.996	-2.2537	3.8135
	TYR0.1	4.2605*	.94859	.001	1.2269	7.2941

TYR0.05	6BRO0.05	3.7515*	.94859	.005	.7179	6.7851
	6BRO0.1	3.9377*	.94859	.003	.9040	6.9713
	C.AOM	-3.4713*	.94859	.013	-6.5049	-.4377
	C.Saline	-6.1528*	.94859	.000	-9.1865	-3.1192
	CRUD0.25	2.5967	.94859	.153	-.4369	5.6304
	CRUD0.5	2.7697	.94859	.101	-.2639	5.8034
	TYR0.025	-.7799	.94859	.996	-3.8135	2.2537
	TYR0.1	3.4806*	.94859	.013	.4470	6.5142
TYR0.1	6BRO0.05	.2709	.94859	1.000	-2.7627	3.3045
	6BRO0.1	.4571	.94859	1.000	-2.5766	3.4907
	C.AOM	-6.9519*	.94859	.000	-9.9855	-3.9183
	C.Saline	-9.6335*	.94859	.000	-12.6671	-6.5998
	CRUD0.25	-.8839	.94859	.990	-3.9175	2.1497
	CRUD0.5	-.7109	.94859	.998	-3.7445	2.3228
	TYR0.025	-4.2605*	.94859	.001	-7.2941	-1.2269
	TYR0.05	-3.4806*	.94859	.013	-6.5142	-.4470

Based on observed means.

The error term is Mean Square(Error) = 4.499.
*. The mean difference is significant at the .05 level.

Table 7.3.10 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on serum alkaline phosphatase (ALP) level

Tests of Between-Subjects Effects

Dependent Variable: ALP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	8271.854[a]	17	486.580	1.600	.125
Intercept	275437.866	1	275437.866	905.651	.000
MICE	4086.095	9	454.011	1.493	.194
GROUP	4190.252	8	523.782	1.722	.133
Error	9428.105	31	304.132		
Total	395398.000	49			
Corrected Total	17699.959	48			

a. R Squared = .467 (Adjusted R Squared = .175)

Table 7.3.11 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on serum alanine aminotransferase (ALT) level

Tests of Between-Subjects Effects

Dependent Variable: ALT

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4764.692[a]	17	280.276	.966	.517
Intercept	88705.733	1	88705.733	305.681	.000
MICE	1540.471	9	171.163	.590	.795
GROUP	3511.108	8	438.888	1.512	.196
Error	8415.521	29	290.190		
Total	132185.000	47			
Corrected Total	13180.213	46			

a. R Squared = .362 (Adjusted R Squared = -.013)

Table 7.3.12 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on serum aspartate aminotransferase (AST) level

Tests of Between-Subjects Effects

Dependent Variable: AST

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	20648.081[a]	17	1214.593	1.001	.482
Intercept	507528.538	1	507528.538	418.468	.000
MICE	1785.348	9	198.372	.164	.996
GROUP	17466.443	8	2183.305	1.800	.115
Error	37597.593	31	1212.826		
Total	774687.000	49			
Corrected Total	58245.673	48			

a. R Squared = .354 (Adjusted R Squared = .001)

Table 7.3.13 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on red cell count

Tests of Between-Subjects Effects

Dependent Variable: Redcell

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5.983[a]	17	.352	2.400	.013
Intercept	3490.270	1	3490.270	23799.359	.000
MICE	.698	9	.078	.528	.844
GROUP	5.286	8	.661	4.505	.001
Error	5.280	36	.147		
Total	4308.026	54			
Corrected Total	11.262	53			

a. R Squared = .531 (Adjusted R Squared = .310)

Table 7.3.14 Tukey HSD for the effect of the crude extract and purified compounds from *D. orbita* on red cell count

Multiple Comparisons

Dependent Variable: Redcell

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.AOM	6BRO0.05	-.30	.202	.847	-.97	.36
	6BRO0.1	.10	.202	1.000	-.56	.77
	C.Saline	.15	.271	1.000	-.75	1.04
	CRUD0.25	.42	.213	.570	-.28	1.12
	CRUD0.5	.20	.232	.993	-.56	.97
	TYR0.025	.51	.247	.504	-.30	1.33
	TYR0.05	.28	.221	.935	-.45	1.01
	TYR0.1	.82*	.232	.029	.05	1.58

Based on observed means.

The error term is Mean Square(Error) = .147.

*. The mean difference is significant at the .05 level.

Table 7.3.15 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on blood hemoglobin level

Tests of Between-Subjects Effects

Dependent Variable: Hemoglobin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1247.729[a]	17	73.396	2.705	.006
Intercept	786009.575	1	786009.575	28968.176	.000
MICE	299.964	9	33.329	1.228	.309
GROUP	986.166	8	123.271	4.543	.001
Error	976.808	36	27.134		
Total	970509.000	54			
Corrected Total	2224.537	53			

a. R Squared = .561 (Adjusted R Squared = .354)

Table 7.3.16 Tukey HSD for the effect of the crude extract and purified compounds from *D. orbita* on blood hemoglobin level

Multiple Comparisons

Dependent Variable: Hemoglobin

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.AOM	6BRO0.05	-4.17	2.745	.840	-13.22	4.89
	6BRO0.1	-.28	2.745	1.000	-9.33	8.77
	C.Saline	.17	3.683	1.000	-11.98	12.31
	CRUD0.25	3.83	2.898	.918	-5.72	13.39
	CRUD0.5	5.83	3.154	.651	-4.57	16.23
	TYR0.025	4.08	3.362	.948	-7.00	15.17
	TYR0.05	2.67	3.007	.992	-7.25	12.58
	TYR0.1	11.03*	3.154	.031	.63	21.43

Based on observed means.

The error term is Mean Square(Error) = 27.134.

*. The mean difference is significant at the .05 level.

Table 7.3.17 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on hematocrit (Hct)

Tests of Between-Subjects Effects

Dependent Variable: Hct

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.009[a]	17	.001	2.112	.029
Intercept	7.844	1	7.844	33020.705	.000
MICE	.001	9	.000	.615	.776
GROUP	.007	8	.001	3.935	.002
Error	.009	36	.000		
Total	9.610	54			
Corrected Total	.017	53			

a. R Squared = .499 (Adjusted R Squared = .263)

Table 7.3.18 Tukey HSD for the effect of the crude extract and purified compounds from *D. orbita* on hematocrit (Hct)

Multiple Comparisons

Dependent Variable: Hct
Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6BRO0.05	6BRO0.1	.02	.007	.166	.00	.04
	C.AOM	.02	.008	.434	-.01	.04
	C.Saline	.00	.010	1.000	-.03	.04
	CRUD0.25	.03*	.008	.008	.01	.06
	CRUD0.5	.02	.009	.287	-.01	.05
	TYR0.025	.02	.009	.243	-.01	.05
	TYR0.05	.02	.008	.097	.00	.05
	TYR0.1	.04*	.009	.002	.01	.07
6BRO0.1	6BRO0.05	-.02	.007	.166	-.04	.00
	C.AOM	.00	.008	1.000	-.03	.02
	C.Saline	-.02	.010	.842	-.05	.02
	CRUD0.25	.01	.008	.878	-.01	.04
	CRUD0.5	.00	.009	1.000	-.03	.03
	TYR0.025	.00	.009	1.000	-.03	.03
	TYR0.05	.00	.008	1.000	-.02	.03
	TYR0.1	.02	.009	.413	-.01	.05
C.AOM	6BRO0.05	-.02	.008	.434	-.04	.01
	6BRO0.1	.00	.008	1.000	-.02	.03
	C.Saline	-.01	.011	.946	-.05	.02
	CRUD0.25	.01	.009	.822	-.01	.04
	CRUD0.5	.00	.009	1.000	-.03	.03
	TYR0.025	.01	.010	1.000	-.03	.04
	TYR0.05	.01	.009	.998	-.02	.04
	TYR0.1	.02	.009	.377	-.01	.05
C.Saline	6BRO0.05	.00	.010	1.000	-.04	.03
	6BRO0.1	.02	.010	.842	-.02	.05
	C.AOM	.01	.011	.946	-.02	.05
	CRUD0.25	.03	.011	.262	-.01	.06
	CRUD0.5	.02	.011	.857	-.02	.05
	TYR0.025	.02	.012	.783	-.02	.06
	TYR0.05	.02	.011	.660	-.02	.06
	TYR0.1	.03	.011	.083	.00	.07
CRUD0.25	6BRO0.05	-.03*	.008	.008	-.06	-.01
	6BRO0.1	-.01	.008	.878	-.04	.01
	C.AOM	-.01	.009	.822	-.04	.01
	C.Saline	-.03	.011	.262	-.06	.01
	CRUD0.5	-.01	.009	.969	-.04	.02
	TYR0.025	-.01	.010	.997	-.04	.02
	TYR0.05	-.01	.009	.997	-.03	.02
	TYR0.1	.01	.009	.992	-.02	.04
CRUD0.5	6BRO0.05	-.02	.009	.287	-.05	.01
	6BRO0.1	.00	.009	1.000	-.03	.03
	C.AOM	.00	.009	1.000	-.03	.03
	C.Saline	-.02	.011	.857	-.05	.02
	CRUD0.25	.01	.009	.969	-.02	.04
	TYR0.025	.00	.010	1.000	-.03	.04
	TYR0.05	.00	.009	1.000	-.03	.03
	TYR0.1	.02	.010	.652	-.01	.05
TYR0.025	6BRO0.05	-.02	.009	.243	-.05	.01
	6BRO0.1	.00	.009	1.000	-.03	.03
	C.AOM	-.01	.010	1.000	-.04	.03
	C.Saline	-.02	.012	.783	-.06	.02
	CRUD0.25	.01	.010	.997	-.02	.04
	CRUD0.5	.00	.010	1.000	-.04	.03
	TYR0.05	.00	.010	1.000	-.03	.03

	TYR0.1	.02	.010	.849	-.02	.05
TYR0.05	6BRO0.05	-.02	.008	.097	-.05	.00
	6BRO0.1	.00	.008	1.000	-.03	.02
	C.AOM	-.01	.009	.998	-.04	.02
	C.Saline	-.02	.011	.660	-.06	.02
	CRUD0.25	.01	.009	.997	-.02	.03
	CRUD0.5	.00	.009	1.000	-.03	.03
	TYR0.025	.00	.010	1.000	-.03	.03
	TYR0.1	.01	.009	.813	-.02	.05
TYR0.1	6BRO0.05	-.04*	.009	.002	-.07	-.01
	6BRO0.1	-.02	.009	.413	-.05	.01
	C.AOM	-.02	.009	.377	-.05	.01
	C.Saline	-.03	.011	.083	-.07	.00
	CRUD0.25	-.01	.009	.992	-.04	.02
	CRUD0.5	-.02	.010	.652	-.05	.01
	TYR0.025	-.02	.010	.849	-.05	.02
	TYR0.05	-.01	.009	.813	-.05	.02

Based on observed means.

The error term is Mean Square(Error) = .000.
*. The mean difference is significant at the .05 level.

Table 7.3.19 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on mean corpuscular volume (MCV)

Tests of Between-Subjects Effects

Dependent Variable: MCV

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	27.724[a]	17	1.631	1.159	.343
Intercept	99737.188	1	99737.188	70894.841	.000
MICE	1.894	9	.210	.150	.998
GROUP	20.423	8	2.553	1.815	.106
Error	50.646	36	1.407		
Total	120684.000	54			
Corrected Total	78.370	53			

a. R Squared = .354 (Adjusted R Squared = .049)

Table 7.3.20 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on mean corpuscular hemoglobin (MCH)

Tests of Between-Subjects Effects

Dependent Variable: MCH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5.436[a]	17	.320	1.344	.222
Intercept	9990.457	1	9990.457	41996.587	.000
MICE	3.265	9	.363	1.525	.177
GROUP	2.022	8	.253	1.062	.410
Error	8.564	36	.238		
Total	12164.000	54			
Corrected Total	14.000	53			

a. R Squared = .388 (Adjusted R Squared = .099)

Table 7.3.21 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on mean corpuscular hemoglobin concentration (MCHC)

Tests of Between-Subjects Effects

Dependent Variable: MCHC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	846.380[a]	17	49.787	1.858	.061
Intercept	4215415.069	1	4215415.069	157315.297	.000
MICE	394.346	9	43.816	1.635	.145
GROUP	440.182	8	55.023	2.053	.069
Error	911.063	34	26.796		
Total	5304181.000	52			
Corrected Total	1757.442	51			

a. R Squared = .482 (Adjusted R Squared = .222)

Table 7.3.22 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on white cell count

Tests of Between-Subjects Effects

Dependent Variable: Whitecell

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	69.742[a]	17	4.102	3.043	.002
Intercept	1054.142	1	1054.142	781.978	.000
MICE	16.419	9	1.824	1.353	.245
GROUP	51.100	8	6.387	4.738	.000
Error	48.530	36	1.348		
Total	1332.850	54			
Corrected Total	118.272	53			

a. R Squared = .590 (Adjusted R Squared = .396)

Table 7.3.23 Tukey HSD for the effect of the crude extract and purified compounds from *D. orbita* on white cell count

Multiple Comparisons

Dependent Variable: Whitecell

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.AOM	6BRO0.05	2.30*	.612	.016	.28	4.32
	6BRO0.1	-.02	.612	1.000	-2.04	2.00
	C.Saline	-1.27	.821	.828	-3.97	1.44
	CRUD0.25	.86	.646	.914	-1.27	2.99
	CRUD0.5	-.49	.703	.999	-2.80	1.83
	TYR0.025	.76	.749	.982	-1.71	3.23
	TYR0.05	-.22	.670	1.000	-2.43	1.99
	TYR0.1	-.19	.703	1.000	-2.50	2.13

Based on observed means.

The error term is Mean Square(Error) = 1.348.

*. The mean difference is significant at the .05 level.

Table 7.3.24 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on neutrophil number

Tests of Between-Subjects Effects

Dependent Variable: Neutrophils

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	24.671[a]	17	1.451	2.939	.003
Intercept	107.634	1	107.634	217.971	.000
MICE	6.212	9	.690	1.398	.226
GROUP	14.851	8	1.856	3.759	.003
Error	17.777	36	.494		
Total	184.230	54			
Corrected Total	42.448	53			

a. R Squared = .581 (Adjusted R Squared = .383)

Table 7.3.25 Tukey HSD for the effect of the crude extract and purified compounds from *D. orbita* on neutrophil number

Multiple Comparisons

Dependent Variable: Neutrophils

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.AOM	6BRO0.05	1.36*	.370	.019	.14	2.58
	6BRO0.1	.12	.370	1.000	-1.10	1.34
	C.Saline	1.65*	.497	.047	.01	3.29
	CRUD0.25	.50	.391	.929	-.79	1.79
	CRUD0.5	.16	.426	1.000	-1.25	1.56
	TYR0.025	1.27	.454	.153	-.23	2.76
	TYR0.05	.85	.406	.492	-.49	2.19
	TYR0.1	-.10	.426	1.000	-1.51	1.30

Based on observed means.

The error term is Mean Square(Error) = .494.

*. The mean difference is significant at the .05 level.

Table 7.3.26 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on lymphocyte number

Tests of Between-Subjects Effects

Dependent Variable: Lymphocytes

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	41.680[a]	17	2.452	2.863	.004
Intercept	399.139	1	399.139	466.147	.000
MICE	4.437	9	.493	.576	.808
GROUP	32.896	8	4.112	4.802	.000
Error	30.825	36	.856		
Total	493.070	54			
Corrected Total	72.505	53			

a. R Squared = .575 (Adjusted R Squared = .374)

Table 7.3.27 Tukey HSD for the effect of the crude extract and purified compounds from *D. orbita* on lymphocyte number

Multiple Comparisons

Dependent Variable: Lymphocytes
Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.AOM	6BRO0.05	.82	.488	.757	-.79	2.42
	6BRO0.1	.06	.488	1.000	-1.55	1.67
	C.Saline	-2.88*	.654	.003	-5.04	-.73
	CRUD0.25	.32	.515	.999	-1.38	2.01
	CRUD0.5	-.70	.560	.938	-2.55	1.14
	TYR0.025	-.71	.597	.954	-2.68	1.26
	TYR0.05	-.67	.534	.940	-2.43	1.09
	TYR0.1	-.10	.560	1.000	-1.95	1.74
C.Saline	6BRO0.05	3.70*	.617	.000	1.67	5.73
	6BRO0.1	2.94*	.617	.001	.91	4.98
	C.AOM	2.88*	.654	.003	.73	5.04
	CRUD0.25	3.20*	.639	.000	1.09	5.31
	CRUD0.5	2.18	.676	.059	-.05	4.41
	TYR0.025	2.18	.707	.083	-.16	4.51
	TYR0.05	2.22*	.654	.040	.06	4.37
	TYR0.1	2.78*	.676	.006	.55	5.01

Based on observed means.

The error term is Mean Square(Error) = .856.
*. The mean difference is significant at the .05 level.

Table 7.3.28 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on monocyte number

Tests of Between-Subjects Effects

Dependent Variable: Monocytes

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.942[a]	17	.114	3.121	.002
Intercept	3.626	1	3.626	99.077	.000
MICE	.397	9	.044	1.206	.322
GROUP	1.410	8	.176	4.815	.000
Error	1.317	36	.037		
Total	8.240	54			
Corrected Total	3.259	53			

a. R Squared = .596 (Adjusted R Squared = .405)

Table 7.3.29 Tukey HSD for the effect of the crude extract and purified compounds from *D. orbita* on monocytes number

Multiple Comparisons

Dependent Variable: Monocytes
Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.AOM	6BRO0.05	.08	.101	.997	-.25	.41
	6BRO0.1	-.21	.101	.493	-.54	.12
	C.Saline	.07	.135	1.000	-.38	.51
	CRUD0.25	.07	.106	.999	-.28	.42
	CRUD0.5	.05	.116	1.000	-.34	.43
	TYR0.025	.19	.123	.823	-.22	.60
	TYR0.05	-.40*	.110	.022	-.76	-.04
	TYR0.1	-.01	.116	1.000	-.40	.37
C.Saline	6BRO0.05	.01	.128	1.000	-.41	.43
	6BRO0.1	-.28	.128	.440	-.70	.14
	C.AOM	-.07	.135	1.000	-.51	.38
	CRUD0.25	.00	.132	1.000	-.44	.44
	CRUD0.5	-.02	.140	1.000	-.48	.44
	TYR0.025	.13	.146	.994	-.36	.61
	TYR0.05	-.47*	.135	.034	-.91	-.02
	TYR0.1	-.08	.140	1.000	-.54	.38

Based on observed means.

The error term is Mean Square(Error) = .037.
*. The mean difference is significant at the .05 level.

Table 7.3.30 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on serum sodium level

Tests of Between-Subjects Effects

Dependent Variable: Sodium

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	79388.622[a]	17	4669.919	2.537	.003
Intercept	1368013.511	1	1368013.511	743.251	.000
MICE	12120.933	9	1346.770	.732	.679
GROUP	67267.689	8	8408.461	4.568	.000
Error	132521.867	72	1840.581		
Total	1579924.000	90			
Corrected Total	211910.489	89			

a. R Squared = .375 (Adjusted R Squared = .227)

Table 7.3.31 Tukey HSD for the effect of the crude extract and purified compounds from *D. orbita* on serum sodium level

Multiple Comparisons

Dependent Variable: Sodium
Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6BRO0.05	6BRO0.1	11.9000	19.18636	.999	-49.4588	73.2588
	C.AOM	40.3000	19.18636	.481	-21.0588	101.6588
	C.Saline	-1.9000	19.18636	1.000	-63.2588	59.4588
	CRUD0.25	-2.0000	19.18636	1.000	-63.3588	59.3588
	CRUD0.5	27.7000	19.18636	.877	-33.6588	89.0588
	TYR0.025	1.2000	19.18636	1.000	-60.1588	62.5588
	TYR0.05	-.8000	19.18636	1.000	-62.1588	60.5588
	TYR0.1	83.9000*	19.18636	.001	22.5412	145.2588
6BRO0.1	6BRO0.05	-11.9000	19.18636	.999	-73.2588	49.4588
	C.AOM	28.4000	19.18636	.861	-32.9588	89.7588
	C.Saline	-13.8000	19.18636	.998	-75.1588	47.5588
	CRUD0.25	-13.9000	19.18636	.998	-75.2588	47.4588
	CRUD0.5	15.8000	19.18636	.996	-45.5588	77.1588
	TYR0.025	-10.7000	19.18636	1.000	-72.0588	50.6588
	TYR0.05	-12.7000	19.18636	.999	-74.0588	48.6588
	TYR0.1	72.0000*	19.18636	.010	10.6412	133.3588
C.AOM	6BRO0.05	-40.3000	19.18636	.481	-101.6588	21.0588
	6BRO0.1	-28.4000	19.18636	.861	-89.7588	32.9588
	C.Saline	-42.2000	19.18636	.417	-103.5588	19.1588
	CRUD0.25	-42.3000	19.18636	.414	-103.6588	19.0588
	CRUD0.5	-12.6000	19.18636	.999	-73.9588	48.7588
	TYR0.025	-39.1000	19.18636	.523	-100.4588	22.2588
	TYR0.05	-41.1000	19.18636	.454	-102.4588	20.2588
	TYR0.1	43.6000	19.18636	.372	-17.7588	104.9588
C.Saline	6BRO0.05	1.9000	19.18636	1.000	-59.4588	63.2588
	6BRO0.1	13.8000	19.18636	.998	-47.5588	75.1588
	C.AOM	42.2000	19.18636	.417	-19.1588	103.5588
	CRUD0.25	-.1000	19.18636	1.000	-61.4588	61.2588
	CRUD0.5	29.6000	19.18636	.831	-31.7588	90.9588
	TYR0.025	3.1000	19.18636	1.000	-58.2588	64.4588
	TYR0.05	1.1000	19.18636	1.000	-60.2588	62.4588
	TYR0.1	85.8000*	19.18636	.001	24.4412	147.1588

Based on observed means.

The error term is Mean Square(Error) = 1840.581.
*. The mean difference is significant at the .05 level.

Table 7.3.32 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on serum potassium level

Tests of Between-Subjects Effects

Dependent Variable: Potassium

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	285.306[a]	17	16.783	1.833	.040
Intercept	2497.453	1	2497.453	272.743	.000
MICE	149.194	9	16.577	1.810	.081
GROUP	136.112	8	17.014	1.858	.080
Error	659.291	72	9.157		
Total	3442.050	90			
Corrected Total	944.597	89			

a. R Squared = .302 (Adjusted R Squared = .137)

Table 7.3.33 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on serum sodium/ potassium

Tests of Between-Subjects Effects

Dependent Variable: NAK

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	608.649[a]	17	35.803	.394	.982
Intercept	43169.029	1	43169.029	475.149	.000
MICE	364.782	9	40.531	.446	.904
GROUP	260.064	8	32.508	.358	.938
Error	5360.362	59	90.854		
Total	52738.690	77			
Corrected Total	5969.011	76			

a. R Squared = .102 (Adjusted R Squared = -.157)

Table 7.3.34 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on serum urea level

Tests of Between-Subjects Effects

Dependent Variable: Urea

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	79.563[a]	17	4.680	3.580	.000
Intercept	7103.917	1	7103.917	5434.245	.000
MICE	38.618	9	4.291	3.282	.003
GROUP	48.347	8	6.043	4.623	.000
Error	73.206	56	1.307		
Total	8150.370	74			
Corrected Total	152.769	73			

a. R Squared = .521 (Adjusted R Squared = .375)

Table 7.3.35 Tukey HSD for the effect of the crude extract and purified compounds from *D. orbita* on serum urea level

Multiple Comparisons

Dependent Variable: Urea
Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.AOM	6BRO0.05	1.7943	.56345	.056	-.0234	3.6120
	6BRO0.1	.8143	.57619	.888	-1.0446	2.6731
	C.Saline	2.8043*	.56345	.000	.9866	4.6220
	CRUD0.25	1.3043	.56345	.351	-.5134	3.1220
	CRUD0.5	1.4393	.59174	.287	-.4697	3.3483
	TYR0.025	1.8143	.63610	.123	-.2378	3.8664
	TYR0.05	.9943	.56345	.704	-.8234	2.8120
	TYR0.1	1.5643	.71663	.431	-.7476	3.8762
C.Saline	6BRO0.05	-1.0100	.51132	.566	-2.6596	.6396
	6BRO0.1	-1.9900*	.52533	.010	-3.6848	-.2952
	C.AOM	-2.8043*	.56345	.000	-4.6220	-.9866
	CRUD0.25	-1.5000	.51132	.102	-3.1496	.1496
	CRUD0.5	-1.3650	.54234	.246	-3.1146	.3846
	TYR0.025	-.9900	.59042	.758	-2.8947	.9147
	TYR0.05	-1.8100*	.51132	.021	-3.4596	-.1604
	TYR0.1	-1.2400	.67642	.660	-3.4222	.9422

Based on observed means.

The error term is Mean Square(Error) = 1.307.
*. The mean difference is significant at the 0.05 level.

Table 7.3.36 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on serum creatinine level

Tests of Between-Subjects Effects

Dependent Variable: Creat

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	649.500[a]	17	38.206	.793	.694
Intercept	14047.146	1	14047.146	291.455	.000
MICE	350.342	9	38.927	.808	.611
GROUP	266.308	8	33.288	.691	.698
Error	2891.795	60	48.197		
Total	19309.000	78			
Corrected Total	3541.295	77			

a. R Squared = .183 (Adjusted R Squared = -.048)

Table 7.3.37 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on serum calcium level

Tests of Between-Subjects Effects

Dependent Variable: Calcium

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	10976.285[a]	17	645.664	1.042	.429
Intercept	1639.171	1	1639.171	2.645	.109
MICE	5512.777	9	612.531	.988	.459
GROUP	5494.871	8	686.859	1.108	.371
Error	37183.418	60	619.724		
Total	50081.108	78			
Corrected Total	48159.703	77			

a. R Squared = .228 (Adjusted R Squared = .009)

Table 7.3.38 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on serum total protein level

Tests of Between-Subjects Effects

Dependent Variable: Protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	298.154[a]	17	17.538	1.678	.073
Intercept	152077.728	1	152077.728	14546.060	.000
MICE	51.206	9	5.690	.544	.836
GROUP	252.910	8	31.614	3.024	.006
Error	627.295	60	10.455		
Total	172195.000	78			
Corrected Total	925.449	77			

a. R Squared = .322 (Adjusted R Squared = .130)

Table 7.3.39 Tukey HSD for the effect of the crude extract and purified compounds from *D. orbita* on serum total protein level

Multiple Comparisons

Dependent Variable: Protein
Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.AOM	6BRO0.05	-2.5857	1.59344	.789	-7.7128	2.5414
	6BRO0.1	-.7302	1.62948	1.000	-5.9732	4.5129
	C.Saline	3.3143	1.59344	.496	-1.8128	8.4414
	CRUD0.25	.2143	1.59344	1.000	-4.9128	5.3414
	CRUD0.5	-1.0357	1.67345	.999	-6.4202	4.3488
	TYR0.025	-.2857	1.59344	1.000	-5.4128	4.8414
	TYR0.05	-1.4857	1.59344	.990	-6.6128	3.6414
	TYR0.1	2.9643	2.02664	.868	-3.5567	9.4853
C.Saline	6BRO0.05	-5.9000*	1.44602	.004	-10.5528	-1.2472
	6BRO0.1	-4.0444	1.48565	.162	-8.8247	.7358
	C.AOM	-3.3143	1.59344	.496	-8.4414	1.8128
	CRUD0.25	-3.1000	1.44602	.455	-7.7528	1.5528
	CRUD0.5	-4.3500	1.53374	.126	-9.2850	.5850
	TYR0.025	-3.6000	1.44602	.258	-8.2528	1.0528
	TYR0.05	-4.8000*	1.44602	.038	-9.4528	-.1472
	TYR0.1	-.3500	1.91291	1.000	-6.5050	5.8050

Based on observed means.

The error term is Mean Square(Error) = 10.455.
*. The mean difference is significant at the .05 level.

Table 7.3.40 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on serum albumin level

Tests of Between-Subjects Effects

Dependent Variable: Albumin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	161.288[a]	17	9.488	1.001	.470
Intercept	61741.114	1	61741.114	6512.017	.000
MICE	34.354	9	3.817	.403	.929
GROUP	128.723	8	16.090	1.697	.118
Error	568.866	60	9.481		
Total	70212.000	78			
Corrected Total	730.154	77			

a. R Squared = .221 (Adjusted R Squared = .000)

Table 7.3.41 ANOVA for the effect of the crude extract and purified compounds from *D. orbita* on serum globuline level

Tests of Between-Subjects Effects

Dependent Variable: Globulin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	92.544[a]	17	5.444	7.033	.000
Intercept	20020.510	1	20020.510	25864.593	.000
MICE	12.441	9	1.382	1.786	.090
GROUP	80.791	8	10.099	13.047	.000
Error	46.443	60	.774		
Total	22715.000	78			
Corrected Total	138.987	77			

a. R Squared = .666 (Adjusted R Squared = .571)

Table 7.3.42 Tukey HSD for the effect of the crude extract and purified compounds from *D. orbita* on serum globuline level

Multiple Comparisons

Dependent Variable: Globulin

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.AOM	6BRO0.05	-1.5143*	.43357	.024	-2.9094	-.1192
	6BRO0.1	-.9365	.44338	.475	-2.3631	.4901
	C.Saline	1.1857	.43357	.158	-.2094	2.5808
	CRUD0.25	.6857	.43357	.811	-.7094	2.0808
	CRUD0.5	.0357	.45534	1.000	-1.4294	1.5008
	TYR0.025	1.4857*	.43357	.028	.0906	2.8808
	TYR0.05	.3857	.43357	.993	-1.0094	1.7808
	TYR0.1	1.7857*	.55144	.047	.0114	3.5601

Based on observed means.

The error term is Mean Square(Error) = .774.

*. The mean difference is significant at the .05 level.

7.4 *In vitro* and *in vivo* result analysis from Chapter 5

Table 7.4.1 ANOVA for the effect of synthetic 6-bromoisatin on HT29 cell proliferation

Tests of Between-Subjects Effects

Dependent Variable: HT29.Proliferation

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.683[a]	12	.057	37.196	.000
Intercept	5.201	1	5.201	3398.249	.000
REPLICATION	.009	8	.001	.771	.631
GROUP	.674	4	.168	110.046	.000
Error	.049	32	.002		
Total	5.933	45			
Corrected Total	.732	44			

a. R Squared = .933 (Adjusted R Squared = .908)

Table 7.4.2 Tukey HSD for the effect of synthetic 6-bromoisatin on HT29 cell proliferation

Multiple Comparisons

Dependent Variable: HT29.Proliferation

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6-BR0.01	6-BR0.05	.2291*	.01844	.000	.1758	.2824
	6-BR0.1	.2804*	.01844	.000	.2271	.3337
	6BR0.025	.0965*	.01844	.000	.0432	.1498
	C.neg	-.0296	.01844	.505	-.0829	.0237
6-BR0.05	6-BR0.01	-.2291*	.01844	.000	-.2824	-.1758
	6-BR0.1	.0513	.01844	.064	-.0020	.1046
	6BR0.025	-.1326*	.01844	.000	-.1859	-.0793
	C.neg	-.2587*	.01844	.000	-.3120	-.2054
6-BR0.1	6-BR0.01	-.2804*	.01844	.000	-.3337	-.2271
	6-BR0.05	-.0513	.01844	.064	-.1046	.0020
	6BR0.025	-.1839*	.01844	.000	-.2372	-.1306
	C.neg	-.3100*	.01844	.000	-.3633	-.2567
6BR0.025	6-BR0.01	-.0965*	.01844	.000	-.1498	-.0432
	6-BR0.05	.1326*	.01844	.000	.0793	.1859
	6-BR0.1	.1839*	.01844	.000	.1306	.2372
	C.neg	-.1261*	.01844	.000	-.1794	-.0728
C.neg	6-BR0.01	.0296	.01844	.505	-.0237	.0829
	6-BR0.05	.2587*	.01844	.000	.2054	.3120
	6-BR0.1	.3100*	.01844	.000	.2567	.3633
	6BR0.025	.1261*	.01844	.000	.0728	.1794

Based on observed means.

The error term is Mean Square(Error) = .002.

*. The mean difference is significant at the .05 level.

Table 7.4.3 ANOVA for the effect of synthetic 6-bromoisatin on HT29 cells membrane integrity (LHD release)

Tests of Between-Subjects Effects

Dependent Variable: HT29.LDH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	139768284531492.810[a]	13	10751406502422.523	106.381	.000
Intercept	233419412680970.700	1	233419412680970.700	2309.587	.000
REPLICATION	489083900033.696	8	61135487504.212	.605	.768
GROUP	139279200631459.160	5	27855840126291.832	275.622	.000
Error	4042617664796.156	40	101065441619.904		
Total	377230314877260.400	54			
Corrected Total	143810902196288.970	53			

a. R Squared = .972 (Adjusted R Squared = .963)

Table 7.4.4 Tukey of effect of synthetic 6-bromoisatin on HT29 cells membrane integrity (LHD release)

Multiple Comparisons

Dependent Variable: HT29.LDH

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.neg	6-BR0.01	47033.9444	149863.22773	1.000	-401390.4099	495458.2988
	6-BR0.05	70784.3111	149863.22773	.997	-377640.0433	519208.6655
	6-BR0.1	88140.8222	149863.22773	.991	-360283.5322	536565.1766
	6BR0.025	60543.0000	149863.22773	.999	-387881.3544	508967.3544
	C.pos	-4255436.9222*	149863.22773	.000	-4703861.2766	-3807012.5678
C.pos	6-BR0.01	4302470.8667*	149863.22773	.000	3854046.5123	4750895.2210
	6-BR0.05	4326221.2333*	149863.22773	.000	3877796.8790	4774645.5877
	6-BR0.1	4343577.7444*	149863.22773	.000	3895153.3901	4792002.0988
	6BR0.025	4315979.9222*	149863.22773	.000	3867555.5678	4764404.2766
	C.neg	4255436.9222*	149863.22773	.000	3807012.5678	4703861.2766

Based on observed means.

The error term is Mean Square(Error) = 101065441619.904.

*. The mean difference is significant at the .05 level.

Table 7.4.5 ANOVA for the apoptotic effect of synthetic 6-bromoisatin on HT29 cells (Caspase activity)

Tests of Between-Subjects Effects

Dependent Variable: HT29.Caspase

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	395329409.130[a]	13	30409954.548	192.589	.000
Intercept	492580123.130	1	492580123.130	3119.550	.000
REPLICATION	2168411.037	8	271051.380	1.717	.124
GROUP	393160998.093	5	78632199.619	497.984	.000
Error	6316040.741	40	157901.019		
Total	894225573.000	54			
Corrected Total	401645449.870	53			

a. R Squared = .984 (Adjusted R Squared = .979)

Table 7.4.6 Tukey HSD for the apoptotic effect synthetic 6-bromoisatin on HT29 cells (Caspase activity)

Multiple Comparisons

Dependent Variable: HT29.Caspase
Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C.neg	6-BR0.01	149.2222	187.32089	.966	-411.2838	709.7283
	6-BR0.05	436.4444	187.32089	.206	-124.0616	996.9505
	6-BR0.1	671.2222*	187.32089	.011	110.7162	1231.7283
	6BR0.025	330.2222	187.32089	.500	-230.2838	890.7283
	C.pos	-6900.5556*	187.32089	.000	-7461.0616	-6340.0495
C.pos	6-BR0.01	7049.7778*	187.32089	.000	6489.2717	7610.2838
	6-BR0.05	7337.0000*	187.32089	.000	6776.4939	7897.5061
	6-BR0.1	7571.7778*	187.32089	.000	7011.2717	8132.2838
	6BR0.025	7230.7778*	187.32089	.000	6670.2717	7791.2838
	C.neg	6900.5556*	187.32089	.000	6340.0495	7461.0616

Based on observed means.

The error term is Mean Square(Error) = 157901.019.
*. The mean difference is significant at the .05 level.

Table 7.4.7 ANOVA for the effect of synthetic 6-bromoisatin on mice weight gain

Tests of Between-Subjects Effects

Dependent Variable: WEIGHTgain

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	67.044[a]	12	5.587	.345	.971
Intercept	1475.364	1	1475.364	91.119	.000
GROUP	.487	3	.162	.010	.999
MICE	66.176	9	7.353	.454	.891
Error	404.792	25	16.192		
Total	2014.054	38			
Corrected Total	471.836	37			

a. R Squared = .142 (Adjusted R Squared = -.270)

Table 7.4.8 ANOVA for the effect of synthetic 6-bromoisatin on liver / body weight (%)

Tests of Between-Subjects Effects

Dependent Variable: liverPERbody

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6.713[a]	12	.559	.803	.644
Intercept	905.105	1	905.105	1299.901	.000
GROUP	4.889	3	1.630	2.341	.098
MICE	1.747	9	.194	.279	.975
Error	17.407	25	.696		
Total	957.183	38			
Corrected Total	24.120	37			

a. R Squared = .278 (Adjusted R Squared = -.068)

Table 7.4.9 ANOVA for the apoptotic effect of synthetic 6-bromoisatin on distal colon of mice

Tests of Between-Subjects Effects

Dependent Variable: Apoptosis

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	80.211[a]	12	6.684	3.966	.002
Intercept	909.038	1	909.038	539.399	.000
GROUP	74.118	3	24.706	14.660	.000
MICE	6.261	9	.696	.413	.916
Error	42.132	25	1.685		
Total	1102.087	38			
Corrected Total	122.343	37			

a. R Squared = .656 (Adjusted R Squared = .490)

Table 7.4.10 Tukey HSD for the apoptotic of synthetic 6-bromoisatin on distal colon of mice

Multiple Comparisons

Dependent Variable: Apoptosis

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6BR0.025	6BR0.05	-2.5899*	.58056	.001	-4.1869	-.9930
	6BR0.1	-.8630	.58056	.460	-2.4600	.7339
	C.AOM	1.3393	.61578	.158	-.3545	3.0331
6BR0.05	6BR0.025	2.5899*	.58056	.001	.9930	4.1869
	6BR0.1	1.7269*	.58056	.031	.1300	3.3238
	C.AOM	3.9292*	.61578	.000	2.2354	5.6230
6BR0.1	6BR0.025	.8630	.58056	.460	-.7339	2.4600
	6BR0.05	-1.7269*	.58056	.031	-3.3238	-.1300
	C.AOM	2.2023*	.61578	.007	.5085	3.8961
C.AOM	6BR0.025	-1.3393	.61578	.158	-3.0331	.3545
	6BR0.05	-3.9292*	.61578	.000	-5.6230	-2.2354
	6BR0.1	-2.2023*	.61578	.007	-3.8961	-.5085

Based on observed means.

The error term is Mean Square(Error) = 1.685.

*. The mean difference is significant at the 0.05 level.

Table 7.4.11 ANOVA for the effect of synthetic 6-bromoisatin on distal colon crypt height

Tests of Between-Subjects Effects

Dependent Variable: CRYPT.HEIGHT

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	11.045[a]	12	.920	1.772	.110
Intercept	23470.122	1	23470.122	45190.470	.000
GROUP	1.579	3	.526	1.013	.403
MICE	9.373	9	1.041	2.005	.082
Error	12.984	25	.519		
Total	24165.630	38			
Corrected Total	24.029	37			

a. R Squared = .460 (Adjusted R Squared = .200)

Table 7.4.12 ANOVA for the effect of synthetic 6-bromoisatin on distal colon proliferation in mice (immunohistochemical staining)

Tests of Between-Subjects Effects

Dependent Variable: Proliferation

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	346.308[a]	12	28.859	12.123	.000
Intercept	5289.428	1	5289.428	2221.884	.000
GROUP	294.763	3	98.254	41.273	.000
MICE	37.502	9	4.167	1.750	.142
Error	47.612	20	2.381		
Total	6160.998	33			
Corrected Total	393.920	32			

a. R Squared = .879 (Adjusted R Squared = .807)

Table 7.4.13 Tukey HSD for the effect of synthetic 6-bromoisatin on distal colon proliferation in mice (immunohistochemical staining)

Multiple Comparisons

Dependent Variable: Proliferation

Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6BR0.025	6BR0.05	.8294	.70892	.652	-1.1549	2.8136
	6BR0.1	6.3174*	.72734	.000	4.2816	8.3531
	C.AOM	-2.3887	.86060	.052	-4.7975	.0200
6BR0.05	6BR0.025	-.8294	.70892	.652	-2.8136	1.1549
	6BR0.1	5.4880*	.70892	.000	3.5038	7.4722
	C.AOM	-3.2181*	.84509	.006	-5.5835	-.8527
6BR0.1	6BR0.025	-6.3174*	.72734	.000	-8.3531	-4.2816
	6BR0.05	-5.4880*	.70892	.000	-7.4722	-3.5038
	C.AOM	-8.7061*	.86060	.000	-11.1149	-6.2973
C.AOM	6BR0.025	2.3887	.86060	.052	-.0200	4.7975
	6BR0.05	3.2181*	.84509	.006	.8527	5.5835
	6BR0.1	8.7061*	.86060	.000	6.2973	11.1149

Based on observed means.

The error term is Mean Square(Error) = 2.381.
*. The mean difference is significant at the .05 level.

Table 7.4.14 ANOVA for the effect of synthetic 6-bromoisatin on serum alkaline phosphatase (ALP) level

Tests of Between-Subjects Effects

Dependent Variable: ALP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1991.836[a]	12	165.986	.931	.546
Intercept	205024.063	1	205024.063	1150.057	.000
GROUP	317.785	3	105.928	.594	.630
MICE	1638.951	9	182.106	1.021	.471
Error	2317.549	13	178.273		
Total	265110.000	26			
Corrected Total	4309.385	25			

a. R Squared = .462 (Adjusted R Squared = -.034)

Table 7.4.15 ANOVA for the effect of synthetic 6-bromoisatin on serum alanine aminotransferase (ALT) level

Tests of Between-Subjects Effects

Dependent Variable: ALT

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2230.921[a]	12	185.910	.563	.836
Intercept	36341.997	1	36341.997	109.993	.000
GROUP	342.434	3	114.145	.345	.793
MICE	1883.934	9	209.326	.634	.751
Error	4295.233	13	330.403		
Total	53744.000	26			
Corrected Total	6526.154	25			

a. R Squared = .342 (Adjusted R Squared = -.266)

Table 7.4.16 ANOVA for the effect of synthetic 6-bromoisatin on serum aspartate aminotransferase (AST) level

Tests of Between-Subjects Effects

Dependent Variable: AST

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7355.474[a]	12	612.956	1.437	.263
Intercept	241397.657	1	241397.657	565.743	.000
GROUP	360.846	3	120.282	.282	.838
MICE	6798.054	9	755.339	1.770	.169
Error	5546.987	13	426.691		
Total	313150.000	26			
Corrected Total	12902.462	25			

a. R Squared = .570 (Adjusted R Squared = .173)

Table 7.4.17 ANOVA for the effect of synthetic 6-bromoisatin on serum sodium level

Tests of Between-Subjects Effects

Dependent Variable: Sodium

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	11833.964[a]	12	986.164	1.457	.249
Intercept	476046.005	1	476046.005	703.167	.000
GROUP	1824.714	3	608.238	.898	.466
MICE	9749.726	9	1083.303	1.600	.208
Error	9478.036	14	677.003		
Total	573435.000	27			
Corrected Total	21312.000	26			

a. R Squared = .555 (Adjusted R Squared = .174)

Table 7.4.18 ANOVA for the effect of synthetic 6-bromoisatin on serum potassium level

Tests of Between-Subjects Effects

Dependent Variable: Potassium

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5.848[a]	12	.487	3.239	.026
Intercept	450.639	1	450.639	2995.026	.000
MICE	.828	9	.092	.612	.766
GROUP	2.873	3	.958	6.364	.008
Error	1.806	12	.150		
Total	587.500	25			
Corrected Total	7.654	24			

a. R Squared = .764 (Adjusted R Squared = .528)

Table 7.4.19 Tukey HSD for the effect of synthetic 6-bromoisatin on serum potassium level

Multiple Comparisons

Dependent Variable: Potassium
Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6BR0.025	6BR0.05	.5833	.22395	.093	-.0816	1.2482
	6BR0.1	.7238*	.21580	.026	.0831	1.3645
	C.AOM	-.3833	.22395	.360	-1.0482	.2816
6BR0.05	6BR0.025	-.5833	.22395	.093	-1.2482	.0816
	6BR0.1	.1405	.21580	.913	-.5002	.7812
	C.AOM	-.9667*	.22395	.005	-1.6316	-.3018
6BR0.1	6BR0.025	-.7238*	.21580	.026	-1.3645	-.0831
	6BR0.05	-.1405	.21580	.913	-.7812	.5002
	C.AOM	-1.1071*	.21580	.001	-1.7478	-.4664
C.AOM	6BR0.025	.3833	.22395	.360	-.2816	1.0482
	6BR0.05	.9667*	.22395	.005	.3018	1.6316
	6BR0.1	1.1071*	.21580	.001	.4664	1.7478

Based on observed means.

The error term is Mean Square(Error) = .150.
*. The mean difference is significant at the .05 level.

Table 7.4.20 ANOVA for the effect of synthetic 6-bromoisatin on serum sodium/ potassium

Tests of Between-Subjects Effects

Dependent Variable: NAK

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	300.830[a]	12	25.069	3.780	.012
Intercept	20304.629	1	20304.629	3061.324	.000
GROUP	154.352	3	51.451	7.757	.003
MICE	71.503	9	7.945	1.198	.372
Error	86.224	13	6.633		
Total	25671.540	26			
Corrected Total	387.054	25			

a. R Squared = .777 (Adjusted R Squared = .572)

Table 7.4.21 Tukey HSD for the effect of synthetic 6-bromoisatin on serum sodium/ potassium

Multiple Comparisons

Dependent Variable: NAK
Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6BR0.025	6BR0.05	-4.3833*	1.48690	.049	-8.7475	-.0191
	6BR0.1	-4.8958*	1.39087	.017	-8.9782	-.8135
	C.AOM	2.1667	1.48690	.489	-2.1975	6.5309
6BR0.05	6BR0.025	4.3833*	1.48690	.049	.0191	8.7475
	6BR0.1	-.5125	1.39087	.982	-4.5948	3.5698
	C.AOM	6.5500*	1.48690	.003	2.1858	10.9142
6BR0.1	6BR0.025	4.8958*	1.39087	.017	.8135	8.9782
	6BR0.05	.5125	1.39087	.982	-3.5698	4.5948
	C.AOM	7.0625*	1.39087	.001	2.9802	11.1448
C.AOM	6BR0.025	-2.1667	1.48690	.489	-6.5309	2.1975
	6BR0.05	-6.5500*	1.48690	.003	-10.9142	-2.1858
	6BR0.1	-7.0625*	1.39087	.001	-11.1448	-2.9802

Based on observed means.

The error term is Mean Square(Error) = 6.633.
*. The mean difference is significant at the .05 level.

Table 7.4.22 ANOVA for the effect of synthetic 6-bromoisatin on serum urea level

Tests of Between-Subjects Effects

Dependent Variable: Urea

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	24.187[a]	12	2.016	1.272	.336
Intercept	2060.342	1	2060.342	1300.350	.000
GROUP	3.037	3	1.012	.639	.603
MICE	19.974	9	2.219	1.401	.281
Error	20.598	13	1.584		
Total	2616.860	26			
Corrected Total	44.785	25			

a. R Squared = .540 (Adjusted R Squared = .116)

Table 7.4.23 ANOVA for the effect of synthetic 6-bromoisatin on serum creatinine level

Tests of Between-Subjects Effects

Dependent Variable: Creat

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	16.803[a]	12	1.400	1.541	.225
Intercept	4206.271	1	4206.271	4629.003	.000
GROUP	3.937	3	1.312	1.444	.275
MICE	9.562	9	1.062	1.169	.387
Error	11.813	13	.909		
Total	5294.000	26			
Corrected Total	28.615	25			

a. R Squared = .587 (Adjusted R Squared = .206)

Table 7.4.24 ANOVA for the effect of synthetic 6-bromoisatin on serum calcium level

Tests of Between-Subjects Effects

Dependent Variable: Calcium

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.104[a]	12	.009	2.156	.092
Intercept	99.829	1	99.829	24866.627	.000
GROUP	.011	3	.004	.902	.467
MICE	.085	9	.009	2.364	.077
Error	.052	13	.004		
Total	124.373	26			
Corrected Total	.156	25			

a. R Squared = .666 (Adjusted R Squared = .357)

Table 7.4.25 ANOVA for the effect of synthetic 6-bromoisatin on serum total protein level

Tests of Between-Subjects Effects

Dependent Variable: Protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	34.673[a]	12	2.889	.343	.963
Intercept	44385.344	1	44385.344	5275.992	.000
GROUP	10.302	3	3.434	.408	.750
MICE	32.510	9	3.612	.429	.896
Error	109.365	13	8.413		
Total	55621.000	26			
Corrected Total	144.038	25			

a. R Squared = .241 (Adjusted R Squared = -.460)

Table 7.4.26 ANOVA for the effect of synthetic 6-bromoisatin on serum albumin level

Tests of Between-Subjects Effects

Dependent Variable: Albumin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	20.783[a]	12	1.732	.665	.756
Intercept	16438.802	1	16438.802	6316.567	.000
GROUP	8.668	3	2.889	1.110	.380
MICE	18.668	9	2.074	.797	.626
Error	33.832	13	2.602		
Total	20776.000	26			
Corrected Total	54.615	25			

a. R Squared = .381 (Adjusted R Squared = -.191)

Table 7.4.27 ANOVA for the effect of synthetic 6-bromoisatin on serum globulin level

Tests of Between-Subjects Effects

Dependent Variable: Globulin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	11.648[a]	12	.971	.499	.881
Intercept	6800.378	1	6800.378	3492.457	.000
GROUP	1.187	3	.396	.203	.892
MICE	10.229	9	1.137	.584	.789
Error	25.313	13	1.947		
Total	8425.000	26			
Corrected Total	36.962	25			

a. R Squared = .315 (Adjusted R Squared = -.317)

Table 7.4.28 ANOVA for the effect of synthetic 6-bromoisatin on red cell count

Tests of Between-Subjects Effects

Dependent Variable: Redcell

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	82.248[a]	12	6.854	.509	.880
Intercept	1637.977	1	1637.977	121.579	.000
GROUP	11.000	3	3.667	.272	.845
MICE	67.950	9	7.550	.560	.810
Error	215.560	16	13.473		
Total	2132.271	29			
Corrected Total	297.808	28			

a. R Squared = .276 (Adjusted R Squared = -.267)

Table 7.4.29 ANOVA for the effect of synthetic 6-bromoisatin on blood hemoglobin level

Tests of Between-Subjects Effects

Dependent Variable: Hemoglobin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	11266.165[a]	12	938.847	1.650	.191
Intercept	393978.225	1	393978.225	692.611	.000
MICE	8680.641	9	964.516	1.696	.187
GROUP	1875.787	3	625.262	1.099	.385
Error	7394.796	13	568.830		
Total	471421.000	26			
Corrected Total	18660.962	25			

a. R Squared = .604 (Adjusted R Squared = .238)

Table 7.4.30 ANOVA for the effect of synthetic 6-bromoisatin on hematocrit (Hct)

Tests of Between-Subjects Effects

Dependent Variable: Hct

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.004[a]	12	.000	1.128	.419
Intercept	3.679	1	3.679	13299.998	.000
GROUP	.001	3	.000	1.564	.249
MICE	.002	9	.000	.991	.493
Error	.003	12	.000		
Total	4.604	25			
Corrected Total	.007	24			

a. R Squared = .530 (Adjusted R Squared = .060)

Table 7.4.31 ANOVA for the effect of synthetic 6-bromoisatin on mean corpuscular volume (MCV)

Tests of Between-Subjects Effects

Dependent Variable: MCV

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6.978[a]	12	.582	.465	.901
Intercept	43074.552	1	43074.552	34409.242	.000
GROUP	.811	3	.270	.216	.883
MICE	5.686	9	.632	.505	.845
Error	15.022	12	1.252		
Total	53846.000	25			
Corrected Total	22.000	24			

a. R Squared = .317 (Adjusted R Squared = -.366)

Table 7.4.32 ANOVA for the effect of synthetic 6-bromoisatin on mean corpuscular hemoglobin (MCH)

Tests of Between-Subjects Effects

Dependent Variable: MCH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.454[a]	12	.038	.897	.573
Intercept	4550.648	1	4550.648	107918.518	.000
GROUP	.161	3	.054	1.270	.329
MICE	.294	9	.033	.775	.643
Error	.506	12	.042		
Total	5656.000	25			
Corrected Total	.960	24			

a. R Squared = .473 (Adjusted R Squared = -.054)

Table 7.4.33 ANOVA for the effect of synthetic 6-bromoisatin on mean corpuscular hemoglobin concentration (MCHC)

Tests of Between-Subjects Effects

Dependent Variable: MCHC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	157.875[a]	12	13.156	1.543	.261
Intercept	1919610.263	1	1919610.263	225199.607	.000
MICE	82.224	9	9.136	1.072	.460
GROUP	61.617	3	20.539	2.410	.134
Error	76.716	9	8.524		
Total	2269063.000	22			
Corrected Total	234.591	21			

a. R Squared = .673 (Adjusted R Squared = .237)

Table 7.4.34 ANOVA for the effect of synthetic 6-bromoisatin on white blood cell count

Tests of Between-Subjects Effects

Dependent Variable: Whitecell

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	39.743[a]	12	3.312	1.365	.299
Intercept	471.932	1	471.932	194.486	.000
GROUP	28.940	3	9.647	3.976	.035
MICE	12.288	9	1.365	.563	.803
Error	29.119	12	2.427		
Total	608.030	25			
Corrected Total	68.862	24			

a. R Squared = .577 (Adjusted R Squared = .154)

Table 7.4.35 Tukey HSD for the effect of synthetic 6-bromoisatin on white blood cell count

Multiple Comparisons

Dependent Variable: Whitecell
Tukey HSD

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6BR0.025	6BR0.05	-.6500	.89936	.886	-3.3201	2.0201
	6BR0.1	2.0000	.84128	.135	-.4977	4.4977
	C.AOM	.3600	.94326	.980	-2.4404	3.1604
6BR0.05	6BR0.025	.6500	.89936	.886	-2.0201	3.3201
	6BR0.1	2.6500*	.84128	.037	.1523	5.1477
	C.AOM	1.0100	.94326	.713	-1.7904	3.8104
6BR0.1	6BR0.025	-2.0000	.84128	.135	-4.4977	.4977
	6BR0.05	-2.6500*	.84128	.037	-5.1477	-.1523
	C.AOM	-1.6400	.88805	.300	-4.2765	.9965
C.AOM	6BR0.025	-.3600	.94326	.980	-3.1604	2.4404
	6BR0.05	-1.0100	.94326	.713	-3.8104	1.7904
	6BR0.1	1.6400	.88805	.300	-.9965	4.2765

Based on observed means.

The error term is Mean Square(Error) = 2.427.
*. The mean difference is significant at the .05 level.

Table 7.4.36 ANOVA for the effect of synthetic 6-bromoisatin on neutrophil number

Tests of Between-Subjects Effects

Dependent Variable: Neutrophils

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	11.031[a]	12	.919	.963	.525
Intercept	55.659	1	55.659	58.332	.000
GROUP	6.398	3	2.133	2.235	.137
MICE	4.149	9	.461	.483	.860
Error	11.450	12	.954		
Total	83.010	25			
Corrected Total	22.482	24			

a. R Squared = .491 (Adjusted R Squared = -.019)

Table 7.4.37 ANOVA for the effect of synthetic 6-bromoisatin on lymphocytes number

Tests of Between-Subjects Effects

Dependent Variable: Lymphocytes

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	14.660[a]	12	1.222	1.310	.324
Intercept	180.175	1	180.175	193.160	.000
GROUP	7.982	3	2.661	2.852	.082
MICE	8.729	9	.970	1.040	.464
Error	11.193	12	.933		
Total	238.430	25			
Corrected Total	25.854	24			

a. R Squared = .567 (Adjusted R Squared = .134)

Table 7.4.38 ANOVA for the effect of synthetic 6-bromoisatin on monocytes number

Tests of Between-Subjects Effects

Dependent Variable: Monocytes

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.243[a]	12	.020	.676	.744
Intercept	.625	1	.625	20.855	.001
GROUP	.093	3	.031	1.029	.417
MICE	.179	9	.020	.664	.726
Error	.330	11	.030		
Total	1.240	24			
Corrected Total	.573	23			

a. R Squared = .425 (Adjusted R Squared = -.203)

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