DETERMINING THE CYTOTOXIC PROPERTIES OF A *FAGONIA INDICA* EXTRACT ON BREAST AND COLON CANCER

Amber Jade Lewis

Doctor of Philosophy

ASTON UNIVERSITY

October 2021

Thesis Summary

Fagonia indica is an herbaceous plant common to dry arid environments, including those across Eastern Europe and the Middle East. Traditional communities; particularly those in small villages of Pakistan, utilise *Fagonia indica* as an alternative medicine for a number of ailments, including breast cancer. Previous research has demonstrated several lines of antineoplastic activity from an aqueous extract of *Fagonia indica* against breast cancer cell lines, *in vitro*. Cytotoxicity in these cell lines was associated with activation of p53 and FOXO3a which were able to inhibit cell cycle progression and induce apoptosis in breast cancer. Despite this, there is still a limited understanding on the breadth of effects of *Fagonia indica* treatment, particularly against other aspects of tumorigenesis. The present study aimed to determine the mechanisms of action of an aqueous extract of *Fagonia indica* against phenotypically distinct breast and colon cancer cell lines. One of the most confounding issues in cancer treatment, is the acquisition of resistance mechanisms against commonly used chemotherapies. For the first time, this study also investigated the effect of *Fagonia indica* treatment against multi-drug resistant breast and colon cancer cell lines. An aqueous extract of *Fagonia indica* was able to induce cell death in wild-type and chemotherapy resistant breast and colon cancer cell lines, in correlation with a dysregulation of metabolism/ ATP production. Other associated mechanisms of action of *Fagonia indica* included down-regulation of VEGF, ICAM-1 and NF-kB expression, related to angiogenic and inflammatory processes. The effect of F*agonia indica* on chemotherapy resistant breast and colon cancer cell lines was associated with down-regulation of ABC transporters; ABCC4 and AGCG2. Investigation into the chemical properties of *Fagonia indica* revealed that a methanolic extract of *Fagonia indica* had increased total flavonoid, triterpene and antioxidant contents which was associated with increased antineoplastic activity *in vitro*. Further work is required to separate and identify individual compounds from this methanolic fraction for development into a therapeutic treatment.

Key words: Traditional medicine, VEGF, ABC transporter, flavonoid, triterpenoid.

Acknowledgements

Firstly, I would like to thank Aston University and Humanity First for funding and supporting this project. I would also like to thank Dr James Brown, Dr Alice Rothnie and Dr Alan Goddard for their unwavering support and for providing their knowledge and expertise throughout my PhD. Again, I would like to thank Dr Amtul Carmichael for providing the raw plant material.

A special thanks goes out to Dr Phillip Kitchen, Dr David Hardy and Dr Sukhvinder Phull for their assistance with various aspects of the project. I would also like to take the time to thank Dr Jiteen Ahmed and Dr Hayley Smith, for providing support with equipment and answering my many technical questions.

A particularly poignant thanks is owed to my laboratory partner and friend Dr Kiran Shabir and my fellow students; Dr Allan Cameron, Dr Laura Feather, Lois Grant, Ourania Gkanatsiou, James Gavin and John Linney, for all of their help and support, scientific or otherwise. Finally, I would like to thank my wonderful support system including my parents; Carol and Andrew Lewis, my grandma, Pearl Lewis, my brother; Mason Lewis and my partner; Sean Hawker, without whom I would never have had the motivation to apply myself.

List of contents

List of Figures

List of Tables

Abbreviations

ABC – ATP-binding cassette transporter

AKT – Protein kinase B

ATM - Ataxia telangiectasia mutated protein

ATP – Adenosine triphosphate

ATR - Ataxia telangiectasia and Rad3-related protein

AUF1- AU-rich element RNA- binding protein 1

BAX – BCL-2 associated protein X

BCA – Bicinchoninic acid

BCL2 – B cell lymphoma 2

BRCA1 – Breast cancer type 1 susceptibility protein

BRCA2 – Breast cancer type 1 susceptibility protein

CAPE – Caffeic acid phenethyl ester

CASP3 – Caspase 3

CASP9 – Caspase 9

cDNA – Complementary DNA

Chk1 – Checkpoint kinase 1

Chk2 – Checkpoint kinase 2

CMS – Consensus molecular subtype

CO_2 – Carbon dioxide

CRC – Colorectal cancer

DDR – DNA damage response

dH_20 – Distilled water

DMEM – Dulbecco's modified eagle medium

DMSO – Dimethyl sulfoxide

DNA – Deoxyribose nucleic acid

dNTP – Nucleotide triphosphate

EDTA – Ethylenediaminetetraacetic acid

EGFR – Epithelial growth factor receptor

EMT – Epithelial to mesenchymal transition

ER – Oestrogen receptor

FBS – Fetal bovine serum

GA – Gallic acid

HER2 – Human epidermal growth factor receptor 2

HBSS – Hanks balanced salt solution

ICAM1 – Intracellular adhesion molecule 1

LSB – Laemmli sample buffer

mAb – Monoclonal antibody

MAPK – Mitogen activated protein kinase

MDM2 – Mouse double minute 2 homolog

MTT – (3-(4, 5 -dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay

NaCl – Sodium chloride

NF-Kb – Nuclear factor kappa beta

NO – Nitric oxide

NR – Neutral red

PR – Progesterone receptor

QA – Quercetin acid

qPCR – Quantitative PCR

ROS – Reactive oxygen species

SDS – Sodium dodecyl sulphate

Ser – Serine

TBS – Tris buffered saline

TDX - Tomudex

TGF-β – Transforming growth factor receptor beta

Thr – Threonine

TLC – Thin layer chromatography

TNM – Tumour-node-metastasis acid

TRIS - Ethylenediaminetetraacetic

UA – Ursolic acid

UK – United Kingdom

UV – Ultraviolet

VEGF – Vascular endothelial growth factor

VEGFR – Vascular endothelial growth factor receptor

Wnt – Wingless-related integration site

YP1 – YO-PRO-1

5-FU – 5-Flourouracil

1 Chapter 1: Introduction

1.1 Cancer

Cancer is a term used to describe a wide array of diseases which arise due to uncontrolled division of normal body cells. Cancer is a worldwide health burden, with a significant load carried by Europe. In 2012 one-quarter of all new cancer cases were reported in the continent, despite only holding 9% of the world's population (Ferlay *et al*., 2018). In the UK alone, cancer is one of the leading causes of mortality with approximately 990 new cases diagnosed and 450 deaths, per day. There has been a significant rise in incidence levels over the past 30 years; due in part, to a rapidly ageing population. This is predicted to increase further, placing an unprecedented burden on a strained healthcare system (CRUK, 2019)

Cancer research has improved exponentially over the last millennia (Shastri, *et al*., 2019). The Edwin Smith papyrus; dating from 17th century B.C Egypt, is the oldest surviving trauma text and is accredited as containing the earliest medical observations of cancer (Van Middendorp, *et al*., 2010). In this ancient text, the writer concluded that the swelling of the breast was a grave disease with no known treatment available (Hajdu, 2011). The term 'carcinoma' came much later, termed by the Greek physician Hippocrates, to describe 'crab-like' tumours (Lakhtakia, 2014). However, it wasn't until 1902 that German scientist Theodor Boveri, first hypothesised the cellular processes which underpin the development of cancer (Boveri, 1902). Boveri made some pivotal insights which still relate to the current practice of cancer, including genomal instability and tumour suppressor genes (Hansford & Huntsman, 2014).

Cancer is primarily a disease of the genome. During homeostatic cellular replication, errors in the DNA can occur frequently and when left undetected, lead to the introduction of mutations into the genome of subsequent daughter cells (Graham & Sottoriva, 2017). Genomic mutations can provide genetic diversity into populations and act as a vector for natural selection. However, genomic instability can be damaging in diseases like cancer, which are formed due to an inactivation of DNA repair pathways, or aberrant activity of cellular processes, such as transcription and replication (Tubbs & Nussenzweig, 2017). Epigenetic changes can also

contribute to the formation of cancer, as epigenetic alterations in the genome can act as catalysts for genetic mutations. These epigenetic changes encompass events such as chromatin modification, DNA methylation and genomic imprinting (Iacobuzio-Donahue, 2009).

There have been several lines of research which demonstrate cancer as a multistep disease, whereby each step underlies a genetic alteration which drives the progressive transformation of normal cells into malignant cancer cells (Figure 1.1). Hanahan & Weinberg published a pivotal review demonstrating key 'shared hallmarks' which underpin the development of malignancy. These hallmarks were described as: the ability to evade apoptosis, an insensitivity to antigrowth signals, self-sufficiency in growth signals, sustained angiogenesis, an unlimited replicative potential and finally the ability to invade tissue and metastasise (Hanahan & Weinberg, 2000). This model was later expanded to include several additional hallmarks including reprogramming energy metabolism, evading immune response, development of a 'tumour environment', genome instability and inflammation (Hanahan & Weinberg, 2011).

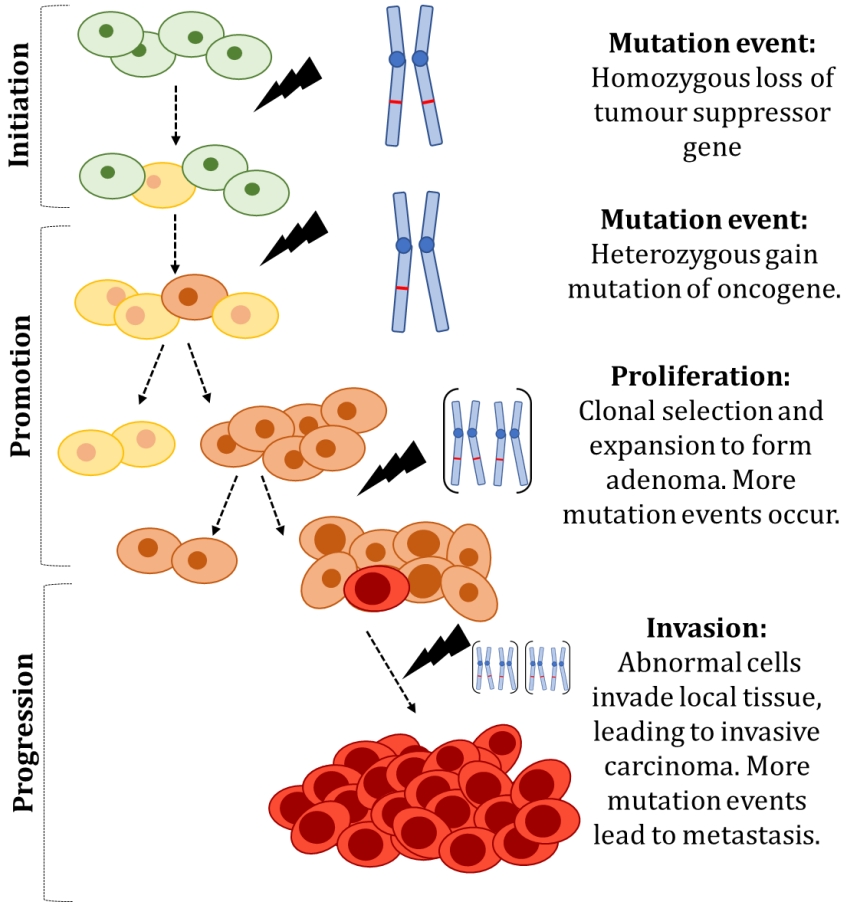

Figure 1.1: Molecular basis of multistep progression of invasive carcinoma. Malignant transformation occurs via a progressive process, whereby multiple 'rounds' of mutations are required. In this process, there will be an accumulation of multiple mutations. Loss of function mutations 'tumour suppressor genes' require homozygous mutations of both alleles, whereas acquisition of 'oncogene' mutations only require heterozygous mutations on one allele. Clonal selection occurs in subsets which have advantages mutations leading to an increase in aggressiveness and invasiveness. Information from (Kleinsmith, 2006).

1.1.1 Apoptosis and DNA damage repair pathways in cancer

DNA damage and checkpoint signalling pathways are typically the most mutated signalling cascades in eukaryotic cancers (Xu, *et al*., 2012). The DNA damage response pathway (DDR) is orchestrated by the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) serine kinase proteins. ATM and ATR are expressed in most tissues of the body and are key regulators of maintaining genome integrity. ATM and ATR respond to different forms of DNA damage, ATM responds primarily to double strand breaks, whereas ATR responds to any DNA damage which effects the integrity of replicating chromosomes (Maréchal & Zou, 2013). Upon activation, ATM and ATR mediate the DDR by phosphorylating serine (Ser) or threonine (Thr) residues of target proteins, including

downstream checkpoint kinases (Chk1 and Chk2), (Awasthi *et al*., 2015). This leads to an upregulation in pathways associated with cell cycle checkpoints, ultimately leading to cell cycle arrest and/or apoptosis.

Chk1 and Chk2 are functionally related Ser/Thr kinases, which upon activation relay signalling cascades associated with cell cycle checkpoints from ATM and ATR (Bartek & Lukas, 2003). Cell cycle suppression occurs through the downregulation of CDC25A and CDC25C phosphates by Chk1 and Chk2, which promotes progression of inhibitory phosphates (Lin *et al.,* 2017). Chk1 and Chk2 target different checkpoints of the cell cycle, with Chk1 targeting G_2 phase activation and Chk2 targeting G_1 phase activation. This mode-of-action has been demonstrated in therapeutic studies, whereby treatment with DNA damaging agents leads to activation of Chk1/Chk2 pathways, correlating to an arrest of the cell cycle at G_2 phase (Amico *et al*., 2003). Chk2 has also been shown to phosphorylate target protein p53, leading to its activation and subsequent role in regulating DNA repair and apoptosis (Pabla *et al*., 2007) (demonstrated in Figure 1.2). If these DDR mechanisms become defective it can result in the accumulation of genetic mutations and instability within the genome. This inability to repair damage to the DNA or inhibit cell cycle checkpoints, leads to the unregulated proliferation of damaged DNA (Choi *et al*., 2016).

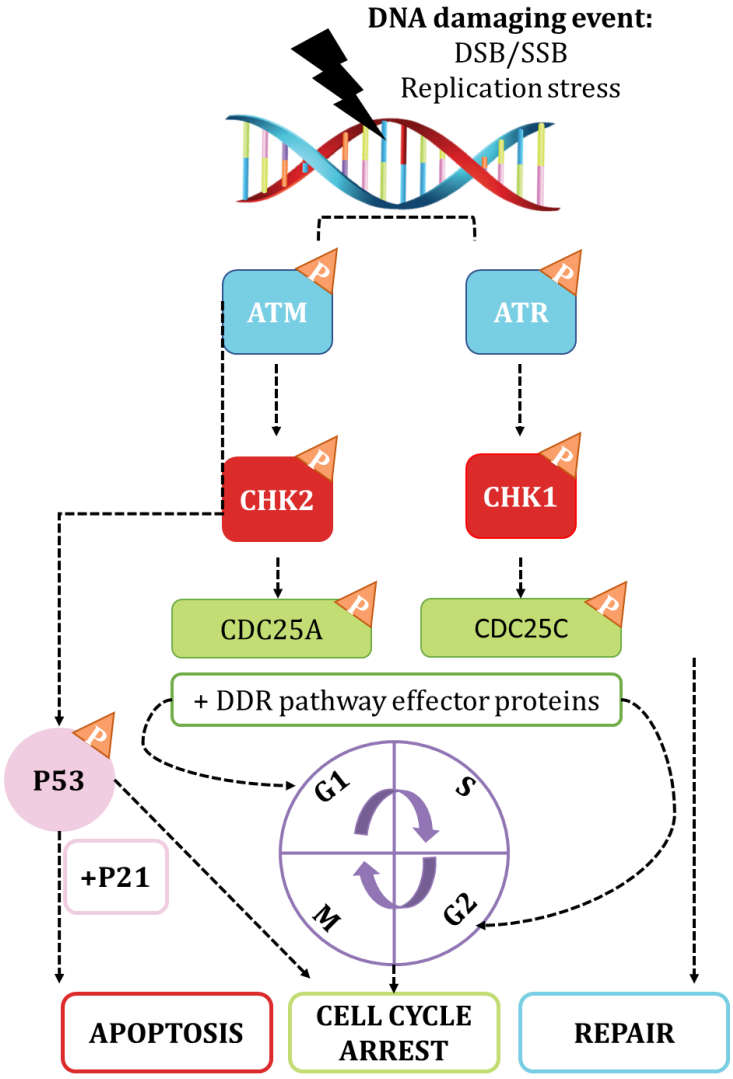

Figure 1.2: Schematic representation of DNA damage response pathway. A simplified diagram demonstrating the activation and downstream phosphorylation events of the ATM/ATR signalling cascade. Upon DNA damage (single/ double strand DNA breakages) or replicative stress, the ATM/ATR kinases are activated leading to the downstream phosphorylation of CHK1/CHK2. CHK1 and CHK2 relay signalling cascades of ATM/ATR by phosphorylation of target effector proteins (CDK's) leading to cell cycle arrest at G_1 and G_2 phase. ATM-CHK2 and ATR-CHK1 can also phosphorylate apoptotic and cell cycle regulator p53. Figure adapted from (Tšuiko et al., 2019)**;** (Pabla et al., 2007)**.**

Alongside the ability to promote cell cycle arrest, the DDR pathway can also induce apoptosis if damage to the DNA is beyond the capacities of cellular repair mechanisms (Enoch & Norbury, 1995). Apoptosis is heavily regulated by signalling pathways, striking balance between proapoptotic factors such as p53 and caspases against antiapoptotic factors such as BCL-2 and MDM2 (Choi *et al*., 2016). In healthy cells, p53 is expressed at very low levels and is generally unstable with a short life-span. This low protein expression is maintained by a continuous degradation of p53 by antiapoptotic regulator MDM2 (Moll & Petrenko, 2003).

This process of p53 degradation occurs in an autoregulatory feedback loop, with MDM2 binding to the transcriptional activation domain of p53 to inhibit its ability to activate target genes, whilst p53 activates the expression of MDM2 (Haupt *et al*., 1997). The p53 protein is activated under both intrinsic and extrinsic stresses by a variety of different post-translational modifications at several amino acid residues. These modifications can result in the phosphorylation, acetylation, methylation, ubiquitination or sumolation of the p53 protein (Harris & Levine, 2005). The nature of the post-translational modification of p53 and its subsequent activation, depends heavily on the origin of the stress signal. For example, DNA strand damage can activate either the ATM-CHK2 kinase or ATR-CHK1 kinase signalling pathways, leading to the phosphorylation of different amino acid residues on the same p53 protein (Appella & Anderson, 2001).

Once activated by post-translational modification, p53 accumulates in the nucleus of damaged cells and acts as a potent transcription factor, whereby it can activate transcription of multiple target genes associated with tumour suppressor functions (Xia *et al*., 2011). One of the main targets of p53 is p21, a cell cycle inhibitor with a major role in growth arrest following DNA damage. Research has demonstrated that induction of p53 results in the up-regulation of p21 and cell cycle arrest in cancer cells with wild-type p53 (Shangary *et al*., 2008). P53 also has transcriptional targets on genes which have proapoptotic effects, such as BAX and downstream targets cytochrome c and members of the caspase family (Katiyar *et al*., 2005). The protein also has inhibitory mechanisms on antiapoptotic genes such as BCL2, this has been demonstrated in *in vitro* studies which highlight imbalances in BAX/BCL2 in invasive carcinoma (Brambilla *et al*., 1998), (as demonstrated in Figure 1.3).

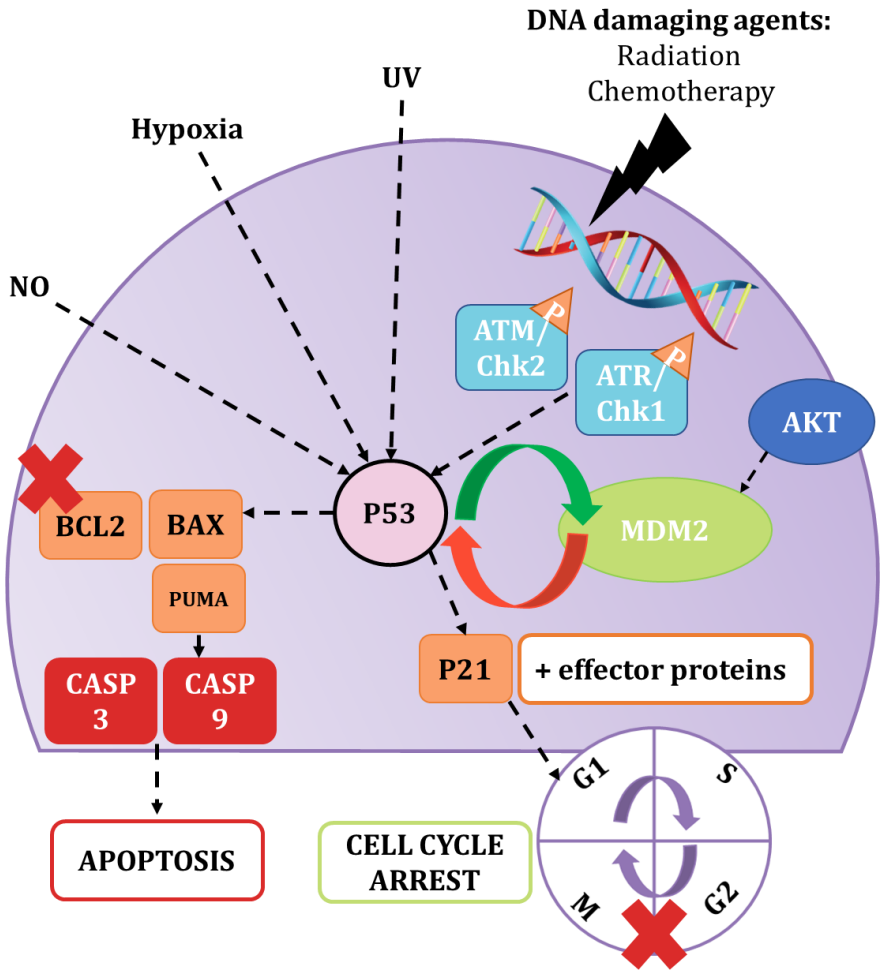

Figure 1.3: Schematic representation of the p53 signalling pathway. A simplified diagram demonstrating the activation and downstream signalling cascade of p53. Upon intrinsic/ extrinsic stresses, p53 is activated by post-transcriptional modifications. Upon activation p53 protein accumulates in the cell nuclei where it acts as a transcription factor for genes related to cell cycle arrest (p21) and apoptosis (PUMA, BAX, CASP3, CASP9). It also can inhibit transcription of antiapoptotic proteins such as BCL2. Information from (Subramanian, Jones, & Lal, 2013).

Due to the critical role of p53 in preventing the passage of DNA damage to subsequent daughter cells, loss of p53 function is pivotal in the development of malignancy. As such, the tumour suppressor gene is mutated in over half of all human cancers (Liu, Song, & Xu, 2010). The majority of these mutations are missense mutations in the DNA-binding core domain of the protein. There are approximately 1,300 known cancer-related single amino acid change mutations in this core domain of p53 (Joerger & Fersht, 2007). The repercussions of p53 mutations are wide-ranging and highly dependent on the nature of the mutation and the tumour. A clinical study of p53 status in breast cancer revealed that loss of p53 protein was associated with a more aggressive phenotype, this alongside absence of progesterone receptor

was associated with a poorer prognosis. It was also determined that the mutations with the worst associated prognosis were missense mutations in the DNA-binding motif of the protein (Olivier *et al*., 2006). Due to the significant impact of p53 on tumour formation, it is important to consider p53 status during diagnosis and treatment of cancers.

1.1.2 Angiogenesis and migration

Angiogenesis is a mechanism in which new blood vessel formation occurs. This process is vital for the progression of tumours and involves the migration, growth and differenation of endothelial cells and results in the dissemination of metastases (Proniewska-Skretek & Mariak, 2004). Vascular growth is essential to support the large-scale growth of a tumour, and the process of promoting the formation of new blood vessels is often stimulated by angiogenic factors from the tumour itself (Weis & Cheresh, 2011). Typically, angiogenesis will be promoted by nutrient depletion and hypoxia within the tumour environment, which will trigger the release of cytokines and growth factors promoting an inflammatory and angiogenic response. When enough of these cells switch to an angiogenic state the tumour can expand exponentially and eventually shed metastatic cells (Folkman & Hanahan, 1991).

The vascular endothelial growth factor (VEGF) family have a vitally well-established role as angiogenic activators and propagators of inflammation in cancer (Nishida *et al*., 2006). VEGF-A plays a central role in endothelial cell proliferation, invasion, migration and survival of primary tumors via its interaction with the vascular endothelial growth factor receptor -2 (VEGFR-2). VEGF-A binding to tyrosine kinase receptor VEGFR2, causes phosphorylation and activation of AKT and p38/MAPK pathways, ultimately leading to apoptotic inhibition, and activation of proliferation and cellular migration (as illustrated in Figure 1.4) (K. E. Johnson & Wilgus, 2014). Overexpression of VEGF-A has been identified in several studies of human colorectal cancer (CRC) and is correlated with an increased risk of invasion and metastasis. Studies have demonstrated the importance of intracrine VEGF signaling mechanisms in CRC using siRNA knockdown of VEGF, depletion of intracellular VEGF and

VEGFR led to a marked reduction in migration and invasion in several CRC cell lines (Bhattacharya *et al*., 2017). Alongside VEGF, transforming growth factor beta (TGF-β) has been recognized as a significant regulator of angiogenesis in cancer and a potential molecular target (Jonker, 2014).

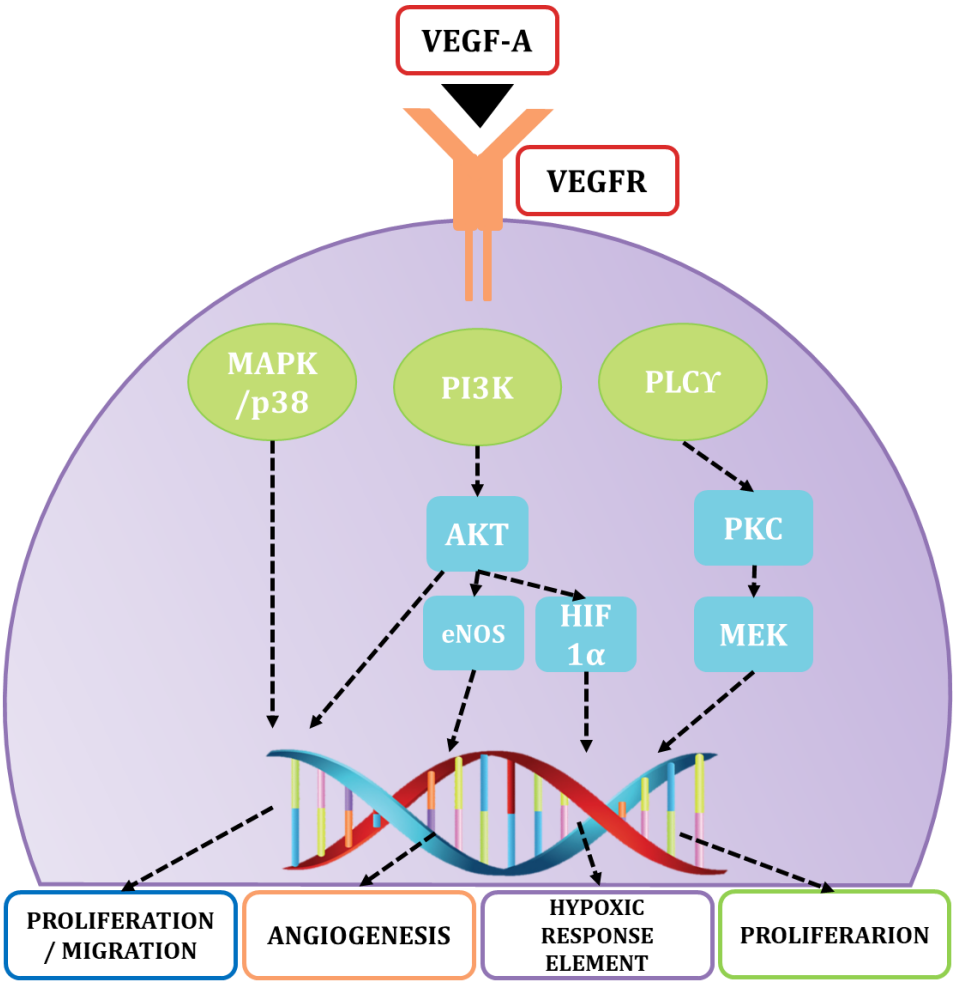

Figure 1.4 Simplified diagram of VEGF-A signalling in tumour cells. VEGF-A binds to tyrosine kinase VEGF2 receptor, leading to the phosphorylation and activation of signalling pathways. PI3K activates protein kinase B (Akt), which promotes transcription of genes associated with cell survival, angiogenesis and hypoxic response. PLCY activates MEK which leads to the transcription of genes associated with proliferation. The MAPK/p38 pathway can also induce proliferation and migration of epithelial cells, an important aspect of angiogenesis. Adapted from (Fallah *et al*., 2019).

There is a long-established link between expression of VEGF and intracellular adhesion molecule-1 (ICAM-1). ICAM-1 acts primarily as a mediator for vascular endothelium adhesion. VEGF has been shown to directly regulate the expression of ICAM-1 via the phosphatidylinositol 3 OH-kinase (PI3K)/AKT pathway in brain microvascular endothelial cells (Radisavljevic *et al*., 2000). The PI3K/AKT pathway has also been shown to induce

secretion of VEGF via hypoxia-inducible factor 1 (HIF-1) dependent and independent mechanisms (Karar & Maity, 2011). Aberrant expression of both VEGF and ICAM-1 has been used to predict clinical outcomes of patients with metastatic CRC. Studies identified a positive correlation between protein expression of VEGF and ICAM-1 in tissue from biopsied colon cancers compared against healthy controls (Yeh *et al*., 2019). As predicted, this relationship was significantly more frequent in tumors of increased size or with metastasis (Dymicka-Piekarska *et al.,* 2012).

In various disease states, VEGF has been revealed to link angiogenesis with inflammation. Scaldaferri *et al* (2009) demonstrated the capacity of VEGF to induce a pro-inflammatory phenotype via the induced expression of ICAM-1 and VCAM-1 in inflammatory bowel disease (IBD). The mechanism behind the induction of VEGF as a pro-inflammatory cytokine has been; in part, attributed to stimulation from the nuclear factor NF-kB signaling pathway. Expression of VEGF stimulated activity of NF-kB, resulting in an increase in protein levels of ICAM-1 and VCAM-1. Additionally, regulation of angiogenic factors such as VEGF by NF-kB stimulation have also been attributed to the progression of CRC (Wang *et al*., 2010).

1.1.3 Breast Cancer

Breast neoplasms are a spectrum of pathologies ranging from benign lesions to invasive carcinomas. In the UK, breast cancer remains one of the most frequently diagnosed cancers, with around 55,200 new cases diagnosed per year (CRUK, 2020.). Assuming that existing trends in terms of incidence and survival continue, by 2040 a quarter of the population of over 65's will be breast cancer survivors (Maddams *et al*., 2012). Early diagnosis of breast cancer has significantly improved with the introduction of the national breast screening programme. However, screening and the overtreatment of early-stage lesions encompasses a large cost burden on the NHS. Studies have indicated that the breast screening programme is only 'moderately likely' to be cost effective at a standard threshold (Pharoah *et al*., 2013). Future research needs for breast cancer include understanding genetic implications of the

development of cancer, the impact of lifestyle changes, chemosensitivity and understanding resistance (Eccles *et al*., 2013).

Alike other cancers, breast cancer tumorigenesis and the advancement of aggressive phenotypes, involves the stepwise accumulation of genetic mutation events. These will both activate growth signals providing proto-oncogenes and disrupt function of specific tumour suppressor genes (Lee & Muller, 2010). Although these mutations can occur in the genome spontaneously due to replicative errors, there are a number of risk factors associated with the onset of genetic mutations linked specifically to breast cancer development. Moderate risk factors include lifestyle choices such as hormone use, alcohol consumption, smoking and obesity, whereas factors associated with medical history of neoplastic disease or genetic predisposition present a significantly higher risk (Singletary, 2003). For example, the risk for women with atypia and a family history of breast cancer is 11 times higher than women without non-proliferative lesions or family history (Dupont & Page, 1985). There is also an increased risk for women carrying hereditary mutations in BRCA1 and BRCA2 genes. By the age of 70, BRCA1/2 carriers have a risk of developing breast cancer of 71 and 84%, respectively (Levy-Lahad & Friedman, 2007).

There are multiple subtypes of breast cancer depending on the origin site of tumour localisation. A large proportion of diagnosed cases are also made up of carcinoma *in situ*, a non-obligate, non-invasive precursor to malignancy which is confined within the basement membrane (Hong *et al*., 2018). The two most commonly diagnosed subtypes of invasive breast cancer include ductal carcinoma which makes up around 54% of all cases and lobular carcinoma which makes up 7% of cases, (as demonstrated in Figure 1.5), (Sibbering & Courtney, 2016). Histopathological analysis of the tumour biopsy will also allow the pathologist to assign a stage to the carcinoma according to the tumour-node-metastasis (TNM) staging system. As the name implies, stages are assigned based on tumour size and relation to surrounding structures, the number of involved lymph nodes and the presence of metastasis

(Cserni *et al*., 2018). All of these factors depict the range in severity of the tumour and reflect the likely prognosis.

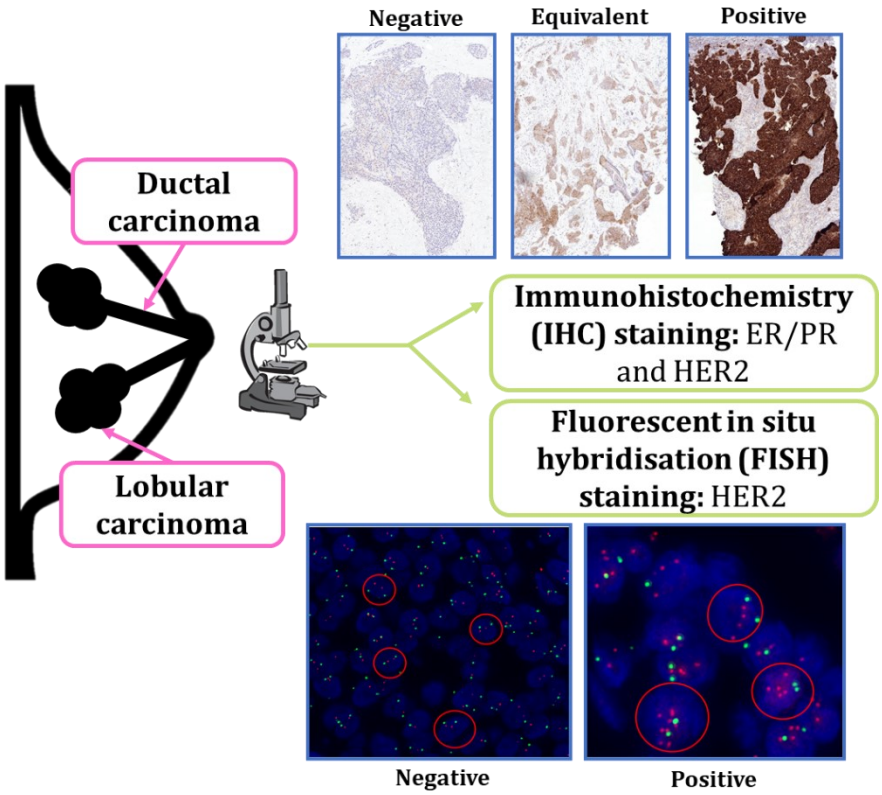

Figure 1.5: Schematic of invasive breast cancer subtypes and molecular phenotyping. The most common subtypes of invasive breast cancer are ductal carcinoma and lobular carcinoma. After diagnosis a biopsy of the tumour will be sent to a histopathology consultant for molecular phenotyping. Hormone receptor status ER/PR will be determined using IHC. HER2 receptor status is determined using IHC, those with equivalent staining will be sent for fluorescent in situ hybridisation (FISH), so determine overexpression of the allele.

Alongside the staging system described above, breast cancers are also characterised based on their molecular phenotypes. Elevated expression of receptors, such as oestrogen (ER) and progesterone (PR) hormone receptors and the human epidermal growth factor receptor 2 (Her2), are identified using immunohistochemical staining in routine pathological screening in breast cancer and can be used as targets for personalised treatment (Figure 1.5), (Timmer *et al*., 2017). Approximately 75% of breast cancers are positive for ER/ PR (Yersal & Barutca, 2014). In classical activation of the receptor, oestrogen entering the cell binds to ER, which subsequently dissociates, undergoing a series of conformational changes, before binding to

upstream oestrogen response elements (ERE), (McDevitt *et al*., 2008). This binding alters the transcription of a number of tumour-promoting genes, such as ABCA3, NRIP1, etc (Lin *et al*., 2004) (Figure 1. 6). Discovery of hormone receptor overexpression in breast cancer led to the development of Tamoxifen, a successful long-term adjuvant antihormonal therapy (Jordan *et al*., 2011). Prior to the development of Tamoxifen, ER status was considered a poor prognostic factor (Berry *et al*., 2006), however, treatment of ER overexpression with antihormonal therapy has led to a marked reduction in recurrence and mortality in pre-menopausal women (Perez *et al*., 2014). Women treated with Tamoxifen for 5 years have a significantly reduced rate of recurrence during the treatment period and the following decade (Davies *et al*., 2013).

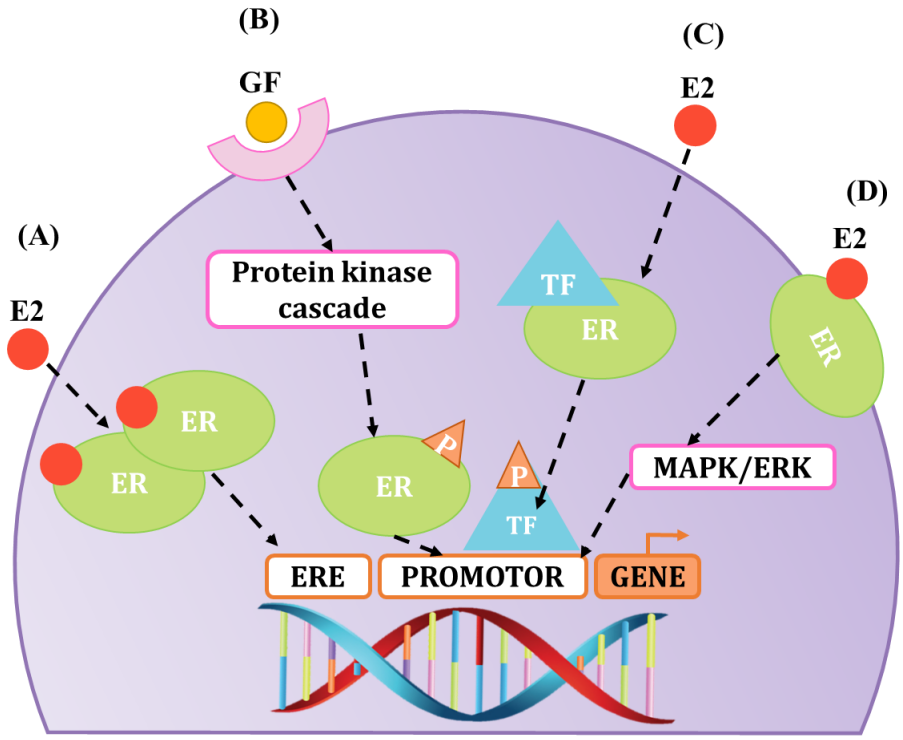

Figure 1.6: Schematic of ER activation pathways. (a) ER-dependent activation, nuclear initiation of oestrogen occurs through ER signalling leading to an activation of transcription via ERE. (b) ER-independent activation, growth factors activate protein kinase cascades at the membrane leading to phosphorylation of nuclear ERs at ERE. (c) Similar to (a) with nuclear-initiated activation of ER signalling, mediated instead by transcription factors (TR) irrespective of ERE binding (d) Membrane initiated ER-dependent activation, cytoplasmic activation occurs via secondary messenger systems and transcription factors. Figure adapted from (Cui, Shen, & Li, 2013).

Human epidermal growth factor receptor 2 (HER2), is a member of the human epidermal factor (EGFR) family of transmembrane tyrosine kinases, localised on the long arm of chromosome 17 (17q12-21). These receptors initiate downstream kinase signalling pathways which activate genes associated with cell growth, differentiation and survival (Harbeck, 2013). Approximately, 20-25% of all breast cancers will test positive for HER2 amplification, these cancers typically have a lower rate of disease-free survival and display an additional resistance to chemotherapy agents (M. C. Johnson, 2010). The most recent ASCO/CAP update advises oncologists to request HER2, IHC/FISH testing for every primary, recurrent and metastatic tumour as a basis to pursue a HER2 targeted therapy (Wolff *et al*., 2018).

Breast tumours which exhibit overexpression of the HER2 receptor or oncogene are treated with adjuvant trastuzumab (Herceptin). Trastuzumab is an approved humanised recombinant monoclonal antibody (mAb) therapy which binds to the extracellular domain of the HER2 protein (Sorlie *et al*., 2001). Trastuzumab has multiple modes of action, including inhibition of HER2 signalling, extracellular domain shedding, inhibition of tumour angiogenesis and cytotoxic properties in DNA repair and damage mechanisms (Triulzi *et al*., 2018). Since its development, a combination therapy of trastuzumab and chemotherapy for the duration of one year has become gold standard treatment for HER2 positive breast cancer. An 11-year follow up study of women with HER2 positive early-stage breast cancer treated with adjuvant trastuzumab for one year, demonstrated a 24% increase in disease-free survival events and a 26% reduction for risk of death (Cameron *et al*., 2017).

A small subset of breast cancer; approximately 15% of all cases, fail to express any of the three commonly amplified breast cancer related receptors (ER, PR and HER2) (Gierach *et al*., 2010). These are summarised in Table 1.1. Triple negative breast cancer (TNBC) is particularly problematic as it does not respond to targeted therapy and is also associated with a younger age of onset, more advanced stage at diagnosis, a higher mitotic index and the inherited breast cancer associated genes (Rampurwala *et al*., 2016). As such, TNBC remains the most difficult breast cancer subtype to treat, with cytotoxic chemotherapy being the gold

standard care. Even then, these tumours are more likely to relapse during remission, metastasise and have a reduced overall survival. Therefore, it remains critical to develop novel treatment options which are effective against this subset of breast cancers (Garrido-Castro *et al*., 2019).

Breast cancer Subtypes	**ER/PR Status**	**HER2 Status**	**EGFR/ Ki67**
Luminal A	Positive	Negative	Ki67 Low
Luminal B	Positive	Negative	Ki67 High
Her2 Positive	Positive/ Negative – Low level	Positive	-
Basal-like/ Triple Negative	Negative	Negative	EGFR
Normal-like/ Triple Negative	Negative	Negative	Negative

Table 1.1: Summary of the biological sub-types of breast cancer. Subclassification of breast cancer types based on molecular phenotyping of hormone and growth factor receptor status, (Yersal & Barutca, 2014).

1.1.4 Colon Cancer

Colorectal cancer (CRC); sometimes referred to as colon cancer or bowel cancer, is a disease of the large intestine and rectum. CRC is the fourth most commonly diagnosed cancer in the UK (approx. 11%) and is the second largest contributor to cancer related mortality (George *et al.,* 2018). Scientific reasoning for this high mortality rate comes from the generally late-stage of diagnosis, with around 70% of cases remaining asymptomatic until reaching an advanced disease state (Maida *et al*., 2017). In the UK, faecal occult blood test screening (FOBt) is available biennially for adults aged 60-74, to aid in the detection of early stage CRC and improve overall survival rates (Dodd *et al*., 2019). Diet and gut microbiome structure are closely linked and are both highly associated with the development of colorectal cancer, particularly in Westernised countries (Song & Chan, 2017). Future research into prevention in especially low-income areas, would focus on education and changes to healthy lifestyles and combatting treatment resistance (Arnold *et al*., 2017).

Colorectal cancer cannot be defined as a singular disease, instead distinct subtypes can be classified based on the biological origin of disease, original cell type and molecular subtypes (Fessler & Medema, 2016). In terms of anatomical distribution, the rectum, sigmoid colon and caecum are the three most affected areas of the colon in both males and females. There is also some evidence to suggest differences in molecular phenotypes and subsequent prognosis between right sided (ascending) colon cancer (RCC) and left sided (descending) colon cancer (LCC) (He *et al*., 2018). In general, LCC patients had an increased overall survival and marginally improved cancer-related outcomes, however they were also more likely to have metastasis to the liver or lungs. In terms of cellular site of origin, adenocarcinoma is a colon cancer originating from epithelial cells lining the surfaces of the colon or rectum, which make up around 95% of all CRC (Thrumurthy *et al*., 2016).

The colorectal cancer Subtyping Consortium used gene expression data to produce a unified classifying system for CRC, known now as the Consensus Molecular Subtypes (CMS). The outcome of this was four distinct groups summarised in Figure 1.7. The four CMS classifications were based on molecular information such as mutations, copy number alterations, methylation, post-translational modifications and biological datasets (Thanki *et al*., 2017). Consensus molecular subtype 1 (CMS1) is summarised as immunogenic and hypermutated. Hypermutation in CMS1 occurs due to defected DNA mismatch repair mechanisms and high-level microsatellite instability (MSI-H), due to germline mutations or somatic inactivation in these same gene types. This frequently corresponds to mutations in cell cycle regulator and proto-oncogene BRAF. There is also evidence of strong immune activation in this subset with evidence of natural killer (NK) cell, T-helper 1 and cytotoxic T cell infiltration within the tumour environment (Müller *et al*., 2016). This subset typically responds poorly to treatment with standardised CRC 5-FU-based chemotherapy, but due to the significant immune response is a good candidate for targeted immunotherapy (Gatalica *et al*., 2016).

Consensus molecular subtype 4 (CMS4), also has an established immune presence, however this tends to be associated more with complement activated inflammation (Thanki *et al*., 2017). The CMS4 subtype has a notably increased expression in genes associated with epithelial to mesenchymal transition (EMT), alongside evidence of matrix remodelling, stromal invasion and angiogenesis within the tumour (Müller *et al*., 2016). Some of these aggressive characteristics of CMS4 have been attributed with increased transforming growth factor receptor beta (TGF-β) signalling. Disrupted TGF-β signalling can be induced by aberrant epithelial and stromal cells and has a range of repercussions in terms of tumorigenesis, inflammation, apoptosis and cellular growth (Itatani *et al*., 2019). TGF-β is also heavily associated with inducing EMT, causing epithelial cells to lose their tight cellular junctions in favour of a migratory invasive phenotype, typically associated with a poorer prognosis (Fessler *et al*., 2016).

In comparison to CMS4 and CMS1, Consensus molecular subtype 2 (CMS2) is poorly immunogenic with no known dendritic cell recruitment. Instead CMS2 is characterised by Wnt-β-catenin pathway activation, epithelial growth factor receptor (EGFR) amplification and vascular endothelial growth factor (VEGF) expression (Thanki *et al*., 2017). In fact, CMS2 cancers generally accumulate a larger degree of copy number oncogene gains and tumour suppressor losses than any other subtype (Müller *et al*., 2016). Wnt signalling is well characterised in colorectal cancer, as it leads to the stabilisation of β-catenin in the cytosol for translocation to the nucleus, and subsequent transcription of genes associated with the cell cycle and proliferation such as, cyclin D1 and CD44 (Lugli *et al*., 2007). Some studies have suggested that although the majority of CMS2 tumours respond fairly well to chemotherapy (Mooi *et al*., 2018), targeting the tumour with Wnt antagonists could also be a promising treatment avenue (Albuquerque & Pebre Pereira, 2018).

Similarly, Consensus molecular subtype 3 (CMS3) is also poorly immunogenic, however displays evidence of increased metabolic dysregulation than CMS2 (Thanki *et al*., 2017). Characterisation of CMS3 with increased metabolic reprogramming is strongly linked with

mutations in Kirsten rat sarcoma viral oncogene homolog (KRAS). These mutations can have an expansive range of effects on energy metabolism, including alternations in glycolysis, glutamine utilisation and fatty acid and lipid metabolism (W. Wang *et al*., 2019). CMS3 is closely associated with the 'Warburg effect' a notion whereby cancer cells favour glycolysis rather than oxidative phosphorylation. Mutated KRAS supports growth of the tumour by accelerating glucose uptake and reprogramming glucose intermediates into the pentose phosphate pathway (PPP) (Aguilera & Serna-Blasco, 2018).

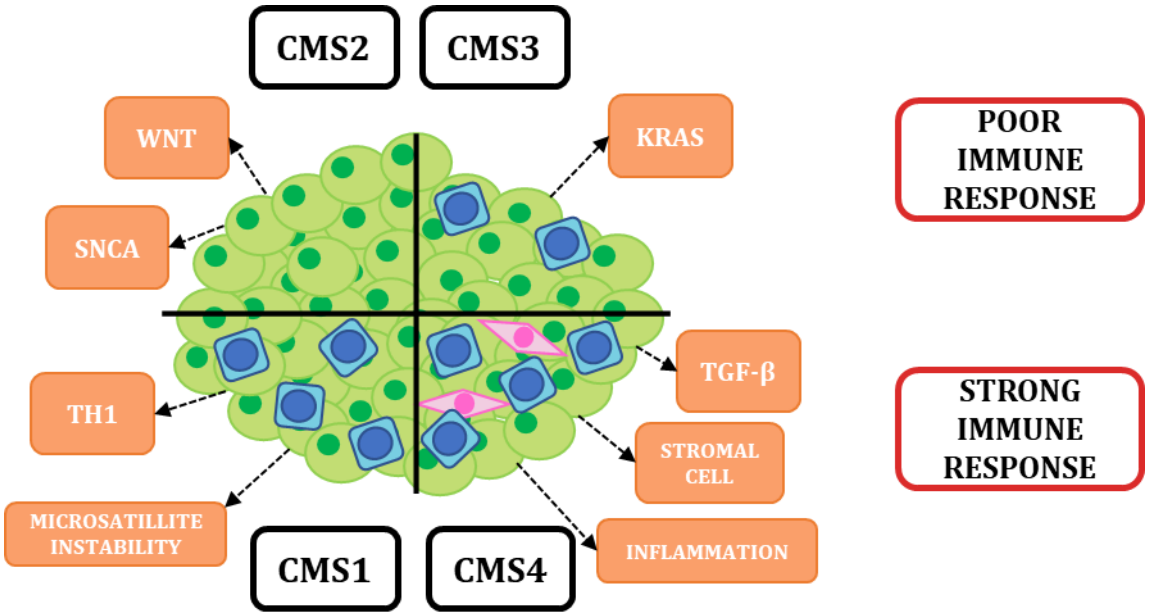

Figure 1.7: Schematic representation of the four distinct classifications of the Colorectal cancer (CRC) Consensus molecular subtypes (CMS). Both CMS1 and CMS3 display distinctly high levels of infiltration immune components (blue), whereas CMS3 and CMS2 do not. CMS1 is characterised by infiltration with T-helper 1 (Th1) cells in the tumour environment and microsatellite instability in DNA repair mechanisms. CMS4 is prone to a higher level of epithelial to mesenchymal transition (EMT) due to excessive TGF-β signalling. The microenvironment of CMS4 tumour has a high level of stromal cells (pink) and cancer-associated fibroblasts (not shown). CMS2 is prone to copy number variations and has the greatest gain of oncogenes and loss of tumour suppressor functions. It is characterised by excessive Wnt-β-catenin signalling and SNCA expression. Finally, CMS3 is associated with metabolic dysfunction and reprogramming via mutations in KRAS. Figure adapted from (Roelands *et al*., 2017; Singh *et al*., 2019).

Alongside these aforementioned CRC subtypes, are a group of unassigned tumours which cannot be classified to one specific CMS group. In this 'intermediate' group, the tumours have mixed phenotypes of multiple CMS subtypes or/and display intertumoral heterogeneity (W. Wang *et al*., 2019). Despite the introduction of these highly assembled sub-sets of CRCs, these classifications have yet to be harnessed in patient treatment. Outside of surgery, adjuvant

chemotherapy is recommended as standard clinical practice. Choice of cytotoxic agent for the treatment of colorectal cancer usually includes 5-fluorouracil (5-FU), a combination therapy with 5-FU and the 5-FU prodrug capecitabine (Gustavsson *et al*., 2015). Due to the vast molecular landscape of CRC and the rise of resistant phenotypes, there is a real necessity for novel chemotherapy agents in the treatment of this disease.

1.1.5 Multi-drug resistant cancer

Standard treatment of primary and metastatic tumours; including those of the breast and colon, typically require treatment with a cytotoxic agent, such as chemotherapy. In the majority of these cases several drugs will be utilised simultaneously to reduce potential of remission and the development of resistant populations (Szakacs *et al*., 2006). Drug resistance to single chemotherapy compounds occurs almost universally among cancers and drug tolerance to chemotherapy has been well documented in literature, notably as one of the 'largest emerging issues' currently challenging cancer research (Housman *et al*., 2014). Tumours will initially display susceptibility to the novel chemotherapy agent, over time resistance will ensue due to subsequent acquisition of additional mutation events. Resistance to treatment often emerges as a result of selection pressure leading to the expansion of sub-clonal populations of tolerant cells (Dagogo-Jack & Shaw, 2018). This heterogeneity has been described in recent literature, where exome sequencing has highlighted the occurrence of novel mutations and structural variations among sub-populations of primary tumour cells (Zabrosky, *et al*., 2018).

Decades of utilising chemotherapeutic agents in the pursuit of remission has revealed underlying issues with the current regime. For example, drugs need to be given at the maximum tolerated dose to be effective and avoid resistance, secondly, combination therapies are also required for similar reasons (L. Wang & Bernards, 2018). Chemotherapeutic resistance can be either intrinsic or acquired; intrinsic resistance is present before selection pressure of the cytotoxic compound, whereas acquired resistance develops during the treatment process (Menchón, 2015). Multi-drug resistance (MDR) occurs when cross-

resistance to several cytotoxic agents occurs, despite being structurally or functionally unrelated (Ullah, 2008). There are several mechanisms in which multi-drug resistance can occur including: altered drug targets, decreased uptake of the drug, aberrant sphingolipid metabolism, inhibition of required apoptosis pathways, drug inactivation, altered cell signalling events, compartmentalisation and overexpression of efflux pumps (Huang & Hung, 2009; Kartal-Yandim *et al*., 2016) (these are summarised in Figure 1.8).

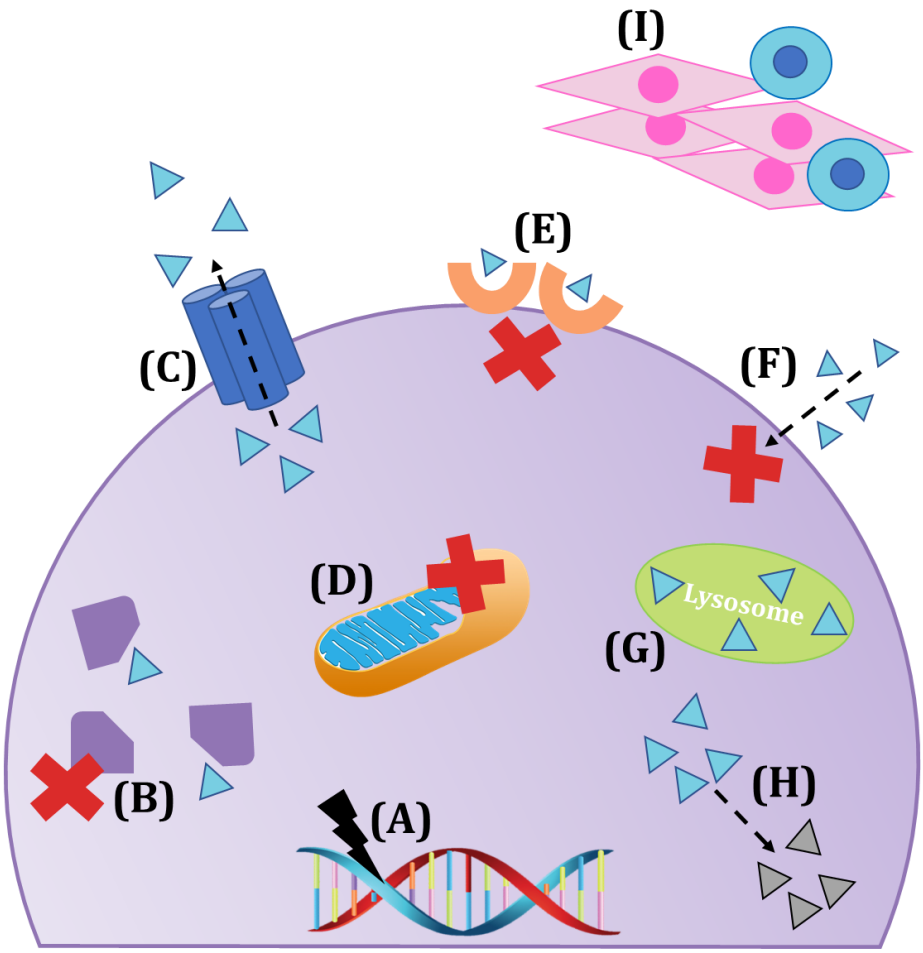

Figure 1.8: Schematic representation of molecular mechanisms of multidrug resistance (MDR) in cancer. There are 8 major mechanisms of MDR. (A) Enhanced DNA repair mechanisms or the ability to overcome drug induced apoptotic mechanisms. (B) Mutation of specific binding site on drug target. (C) Over-expression of ATP-dependent efflux pumps. (D) Inactivation of proapoptotic pathways e.g.: BLC2 (credit: CC0 public domain). (E) Mutation of binding domain on cellular targets. (F) Decreased efflux of drug into the cell. (G) Compartmentalisation of drug into lysosomes. (H) Increased intracellular metabolism of drug into an inactive form. (I) Tumour microenvironment including, tumour associated fibroblast and macrophages. Adapted from (Li et al., 2018).

1.1.6 ATP-binding cassette transporter proteins (ABC)

There are four distinct classes of membrane bound transporter proteins, these include: ion channels, transporters, aquaporins and ATP-dependent efflux pumps (Vasiliou *et al*., 2009). ATP-binding cassette (ABC) transporters are a form of ubiquitous membrane protein, essential for the transportation of a diverse range of substrates across the lipid bilayer (Hollenstein *et al*., 2007). ABC-transporters have a prominent role in human physiology with evidence of their action in almost every cell type (van der Deen *et al*., 2005). ABC transporter proteins were originally isolated in the 1970's from gram-negative bacteria, identified as a system driven by the hydrolysis of ATP, which did not require co-activation with calcium or magnesium (Berger & Happel, 1974). Since the discovery of this class of membrane transporter, entire families of structurally and functionally related subunits have been identified (Higgins *et al*., 1986). Currently, there are approximately 48 characterised ABC transporters in humans, which are divided into seven sub-categories based on their sequence and protein organisation (ABCA-ABCG) (Tsukamoto *et al*., 2017).

ABC transporters are organised into multiple subunits (demonstrated in Figure 1.9), including two hydrophilic nucleotide-binding domains (NBD) located at the cytoplasmic surface of the membrane and two hydrophobic transmembrane domains (TMD), which together produce the substrate translocation pathway (Jones & George, 2002). In eukaryotic cells these exporter units are either made of a single polypeptide chain which encompass all four functional units, or the transporters are assembled as homo/heterodimers (Wilkens, 2015). The NBD domain is highly conserved across species and is the site of ATP hydrolysis, whereas the TMD has been shown to be highly variable across families and is the proposed site of substrate binding (Saurin *et al*., 1999). Binding and hydrolysis of ATP to ADP, induces a conformational change in the protein from an inward to outward-facing alignment, allowing movement of the substrate across the membrane (Sokolov *et al*., 2019; Yu *et al*., 2015). Aberrant expression of one or several ABC transporters leads to the increased exportation of chemotherapeutic compounds and their metabolites through the lipid bilayer.

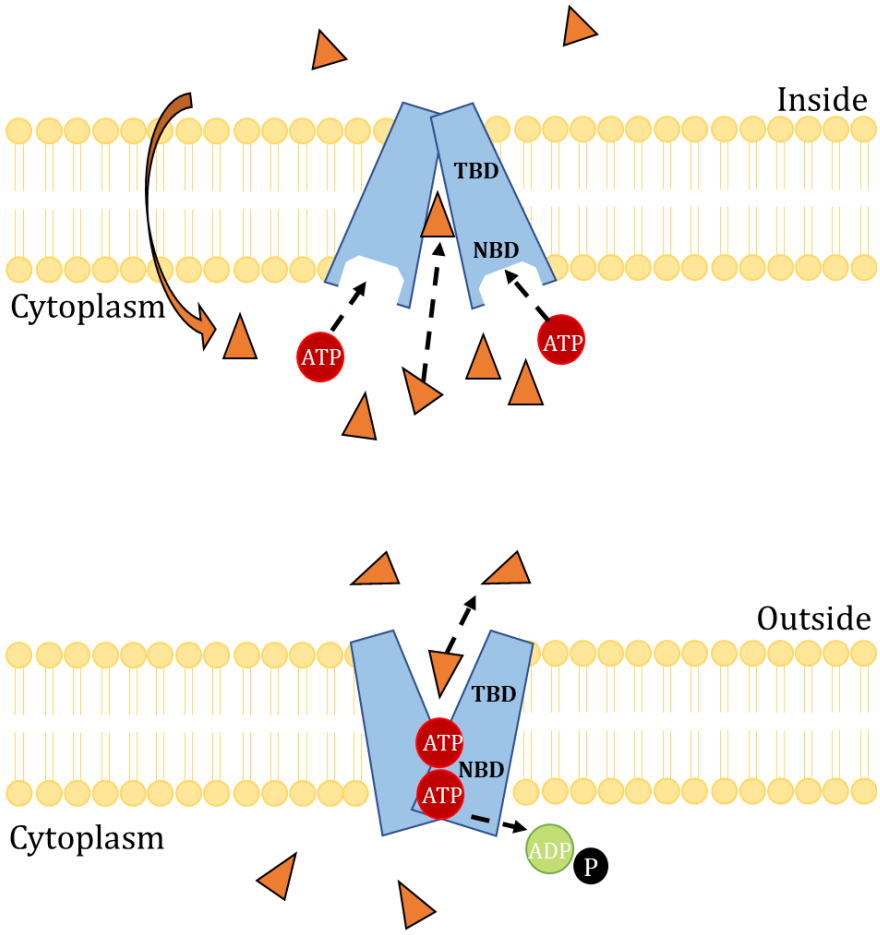

Figure 1.9: Mechanistic action and function of ATP-binding cassette (ABC) transporters. ABC transporters are made up of two distinct domains, transmembrane domain (TMD) and nucleotide binding domains (NBD). The TMD contains the substrate binding site, whereas the NBD contains the ATP binding site for hydrolysis (ATP – ADP + P). The 'switch' model of ABC-meditated transport, begins in the 'inside' mode whereby the substrate binds to the binding-site in the TMD initiating a conformational change to allow ATP binding to the NBD. Subsequently, ATP binding causes a conformational change in the NBD causing a 'closed' state formation. This change in the NBD causing the TMD to rotate to an 'outside' state. The outside state allows substrate translocation, the hydrolysis of ATP causes the dissolution of this outside 'open' state. Adapted from (Dermauw & Van Leeuwen, 2014).

Among these efflux transporters, P-glycoprotein (ABCB1), multidrug resistance protein 1-4 (MRP 1-4/ ABCC1-4) and breast cancer resistance protein (BCRP/ ABCG2) are most frequently associated with impeding the efficacy of several chemotherapeutic agents (Tan *et al*., 2018). Overexpression of ABCB1, ABCC1 and ABCG2, has been directly correlated with an increased risk of chemotherapy failure and multidrug resistance. Overexpression of ABCB1, ABCC1 and ABCG2 in tumour cells led to the cross-resistance of methotrexate and mitoxantrone, the extent of which was associated with cell type and treatment length (Meng *et al*., 2018). Inhibiting the expression of ABC transporters in human cell lines expressing

MDR, can result in a reversal of the resistant phenotype. For example, inhibiting the expression of ABCC4 enhanced the accumulation of intracellular drug concentration by 150% in acute myeloid leukaemia (Drenberg *et al*., 2016). Further studies inhibiting ABCC4 in colorectal cancer cell lines demonstrated an increase in intracellular accumulation and cellular sensitivity of treatment with 5-FU (Chen *et al*., 2017). Similarly, in triple negative breast cancer cell line MDA-MB-231, inhibition of ABCC4 led to a marked reduction in subsequent development of spontaneous metastasis when compared to high endogenous ABCC4 levels (Kochel *et al*., 2017).

Chemosensitizer is a term coined to describe a group of molecules which are able to improve the sensitivity of chemotherapeutic agents via interaction with ABC transporters. These molecules offer an avenue to overcome multi-drug resistance in cancer treatment and have therefore became a promising area of research (Limtrakul, 2007). Studies have identified an ability of some naturally synthesised molecules to accumulate within the tumour site and act as modulators of chemotherapy (Guestini *et al*., 2017). There is evidence that isolated metabolites from plants, including alkaloids, phenols and triterpenoids, have potent inhibitory effects against ABC transporters (Wink *et al*., 2012). Plant triterpenoids have become a potent candidate for ABC inhibition in cancer therapeutics (Yan *et al*., 2014). Triterpenoids have both direct and indirect mechanisms of controlling the expression and functionality of transporter proteins, which consequently inhibit the ability to transport substrates. Natural and synthesised triterpenoids were evaluated for their inhibitory potential against ABCC1 transfected mouse T-lymphoma cell lines. The natural compounds demonstrated a much higher inhibitory effect on ABCC1 (Ramalhete *et al.,* 2016). Some commonly isolated plant phytochemicals including oleanolic and ursolic acid, have demonstrated significant inhibition of ABC-mediated transport (Tan *et al*., 2013). Further studies, have shown a direct inhibitory effect of oleanolic acid, independent of changes to mRNA expression (Braga, *et al*., 2007).

1.2 Plant-based medicine

Plant-based remedies have been utilised in the management of human disease over centuries. The investigation of cultural medicine from traditional communities has proved beneficial in the discovery of novel pharmacological therapies (Qureshi, *et al*., 2016). According to the World Health Organisation, approximately 80% of the population in developing countries rely on plant-based traditional medicines as their primary healthcare (WHO, 2002). These treatments are considered safe, cost effective and are easily accessible, which is vital in deprived communities or communities with limited access to modern treatment facilities. Plant materials, whether as a pure compound or standardised extract, provide a breadth of opportunities for drug discovery due to the unmatched chemical diversity (Sasidharan, *et al*., 2010). A number of isolated bioactive phytochemicals from plant materials are considered to be effective alternatives to their chemically synthesized counterparts (Rashid, *et al*., 2013). The importance of plant-use in pharmacological development is apparent in western countries, where over 25% of commercially available drugs are based on plants and their derivatives (Principe, 2005).

There are currently four classes of widely available anti-cancer drugs derived from compounds isolated from plants, these include vinca alkaloids, epipodophyllotoxins, taxanes and campototchecins. Vinca alkaloids are a type of microtubule-targeting agent (MTA), which function by interfering with mitotic division and inhibiting cellular replication (Martino *et al*., 2018). Vincristine is a commonly utilised chemotherapeutic agent and a member of the vinca alkaloid class of plant-derived neoplastic drug (López-Gómez *et al*., 2018). The compound was originally isolated from *Catharanthus roseus* and has been used to treat a number of cancers including those of the breast and colon (Daniyal *et al*., 2019). Taxanes are a widely used class of plant-derived chemotherapy, which evert their action via the disruption of β-tubulin triggering cell cycle arrest during mitosis and inducing the intrinsic apoptotic pathway. Paclitaxel is a form of taxane chemotherapy obtained from the pacific yew tree, used routinely in the treatment of breast and ovarian cancer (Weaver, 2014; Bernardo *et al*., 2017). Paclitaxel

also induces mitotic-related apoptosis, via the promotion of the spindle assembly checkpoint during mitosis, preventing chromosome segregation (Waters *et al*., 1998) (As shown in Figure 1.10).

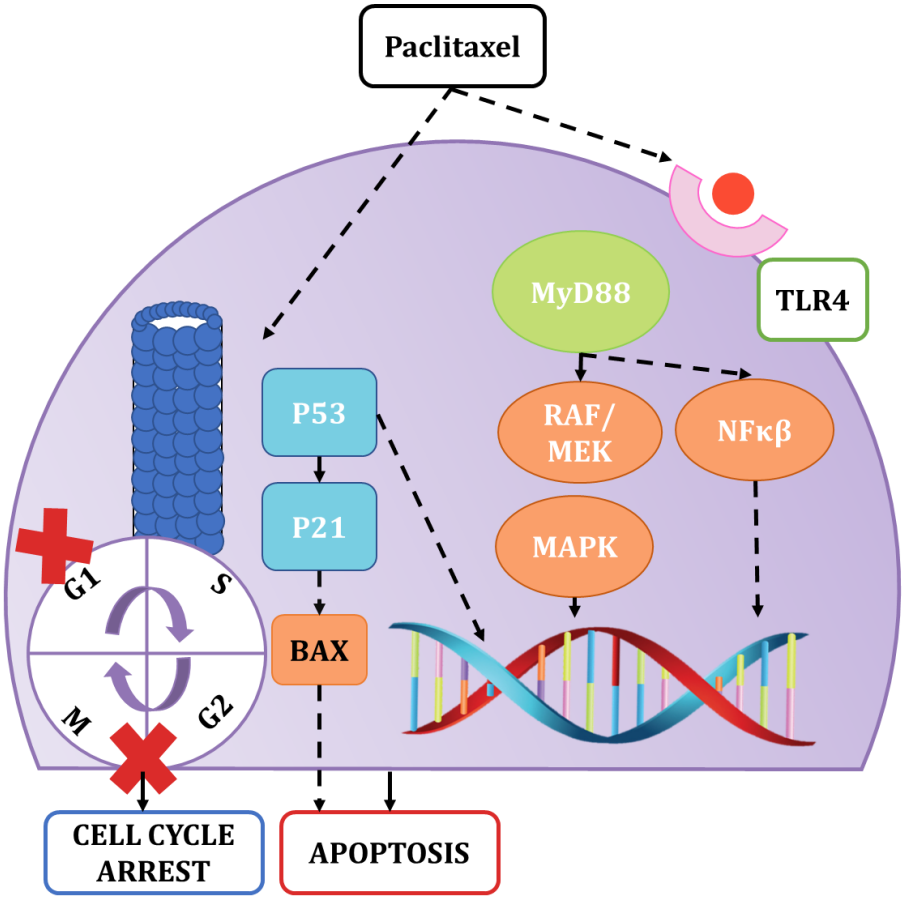

Figure 1.10: A simplified schematic of the mode-of-action of Paclitaxel (Taxol). Paclitaxel targets microtubules during cellular division leading to arrest at both G2/M and G0/G1 phase. Paclitaxel can activate apoptosis via induction of p53 through cell cycle arrest or through activation of RAF/MEK signalling pathways leading to the transcription of apoptotic relating genes. This also activates pathways involved in immunomodulation and inflammation. Adapted from (Kampan *et al*., 2015).

1.2.1 Fagonia indica

Fagonia indica is a small, spiny undershrub belonging to the family Zygophyllaceae (Manandhar, 1956). *Fagonia indica* is found growing mostly on calcareous rocks, in warm arid regions of most continents (Beier, 2001). It is widely distributed in dry areas such as Algeria, Cyprus, Egypt, Iran, Morocco, Pakistan and Saudi Arabia (Ali, *et al*., 2008). Morphological studies of *Fagonia indica* describe the plant to be branched, glandular, with annual or perennial shrublets. The leaves are uni or lower trifoliate and the flowers are pink, spathulate and obtuse (Anil *et al*., 2012). As is typical of perennial plants, there is a

relationship between the reproductive effort of *Fagonia indica* and its size, which varies based on site, moisture and soil (Shaukat *et al*., 2012). The medical properties of *Fagonia indica* are well documented in folklore and exist today in alternative practice in traditional communities.

1.2.2 Traditional uses of *Fagonia indica*

Indigenous medical knowledge of *Fagonia indica* is particularly prevalent in Pakistani culture, with traditional medicines being used by the greater population (Qureshi *et al*., 2009). Due to the diverse geology and climate, Pakistan has an extensive population of medicinal plant species. Over time, the use of plant materials in medical practices in rural Pakistan has become a staple in regional culture and local heritage (Adnan *et al*., 2014). *Fagonia indica* is used in several regions of Pakistan, however its use is most widely documented in the Punjab Province region of Northern Pakistan. Cholistan is part of the Great Indian Desert and one of the largest deserts of Pakistan, it lies within the Punjab province, here *Fagonia indica* is used by local healers for heat burn, kidney stones, diarrhoea and jaundice (Ahmed *et al*., 2014). Local inhabitants; including practising herbalists (Hakeem), ethno-veterinarians and midwives, of the Thal desert of the Punjab province, have also recorded using *Fagonia indica* as an extract or infusion to treat hepatitis, skin disorders, digestive ailments and cancers (Shaheen *et al*., 2014). Sultana *et al* (2006) gathered indigenous knowledge from local women of the remote villages of district Chakwal. The local name for *Fagonia indica* here is Dhamian/ Dhumia and is regularly used in powder form for both gastrointestinal and dermatological irritations. Other studies in nearby regions have demonstrated similar knowledge of local inhabitants on the use of *Fagonia indica* in the treatment of dermatological diseases (Sultana *et al*, 2006) (Qureshi & Raza Bhatti, 2008).

Indigenous use of *Fagonia indica* varies greatly between communities in Northwest Pakistan. In the Lakki Marwat District a shade-dried extract is administered orally for the treatment of cardiovascular, dermatological and gynaecological complaints. In this region *Fagonia indica* has a fidelity rate of 88%, demonstrating the preference of this species as a medicinal option

(Ullah, *et al*., 2014). In the rural District of Dera Ghazi Khan, the local population boil the extract for the treatment of vomiting, dysentery, asthma, liver illness, typhoid and dermatological irritations, the plant is said to have a bitter taste. In some cases, healers will prepare a paste to be applied on tumours or other swellings of the neck (Gulshan *et al*., 2012). During this investigation, researchers determined that *Fagonia indica* had become an endangered species in this specific area due to over-reliance and overuse, special care is now required for conservation. *Fagonia indica* is also utilised as a medicinal plant in the Frontier Region in Bannu, where 2-3 tablespoons are administered orally as a purgative treatment (Adnan *et al*., 2014).

1.2.3 Phytochemical composition of *Fagonia indica*

The reported medicinal properties of *Fagonia indica* can be attributed to its vast active phytochemical make-up. Qualitative phytochemical analysis of *Fagonia indica* has identified the presence of several molecules, including, alkaloids, flavonoids, terpenoids, saponins, tannins, sterols and glycosides (Qureshi *et al*., 2016). Preliminary screening of *Fagonia indica* extract indicated a significantly higher proportion of active principles: saponins, tannins and cardiac glycosides (Khalik *et al*., 2000; Perrone *et al*., 2007). Further investigation revealed the highest abundance of active phytochemicals were located in the stems, leaves, flowers, fruits and shoot of the plant, providing a rationale for a 'whole plant' dried extract (Anil *et al*., 2012). Research into the medical capacity of *Fagonia indica* has attributed bioactivity to the vast triterpenoid content. Triterpenoids are an established metabolite of Isopentenyl pyrophosphate, originating from the formulation of six isoprene units in the mevalonate pathway of isoprenoid precursor biosynthesis (Mahato, Sarkar, & Poddar, 1988). These isoprene units are produced and distributed ubiquitously in plant species in the form of free triterpenoids and saponins.

1.2.4 Cytotoxicity of *Fagonia indica*

In indigenous culture *Fagonia indica* is claimed to be a successful 'remedy' for early stage tumours, particularly of the breast (Rahman, 1986). Soomro & Jararey (2003) investigated the effect of *Fagonia indica* on experimentally produced subcutaneous tumours in albino rats. In this study, the extract was prepared by soaking the sectioned plant in boiling water for 24 hours and administered orally in 5 mL increments. Rats treated with extract had a marked improvement in overall survival, for females this increased from 21-57 days to 55-118 days, whilst in male streatment increased survival from 10-27 days to 39-98 days. There are no details regarding the concentration of plant extract used in this study, which is vital in determining an optimal range for growth inhibition. Said *et al* (2014) prepared an extract of *Fagonia indica* by soaking ground plant material in ethanol and resuspending in 90% aqueous methanol and hexane. Cytotoxicity of these fractions was measured against instar larvae of brine shrimp at various concentrations (10-1000 μg/mL). After 24 hours there was a significant reduction in percentage mortality in the test organisms with the hexane fraction of *Fagonia indica*.

Cytotoxic effects of *Fagonia indica* have also been demonstrated in *in vitro* studies on breast and colon cancer cell lines (Shebab, *et al*., 2011). In this study, MCF-7 and HCT cell lines were treated with alcoholic, ethyl-acetate and butanolic fractions of *Fagonia indica*. All of the extracts exhibited marked antitumor activity against both cell lines, with the ethyl-acetate extract displaying the lowest IC_{50} against both MCF-7 and HCT cells. Healthy rats administered with the alcoholic extract showed no signs of mortality, morbidity or behavioural changes, suggesting minimal toxicity to healthy cells. Other studies have demonstrated marked antitumor activity of *Fagonia indica* against MCF-7 breast cancer cell line. Ali *et al* (2014) produced an alcoholic extract of *Fagonia indica* which reduced percentage cell survival by 50% after treatment with 1 mg/mL of extract at 24 hours. Farheen *et al* (2015) examined non-polar constituents from *Fagonia indica*, all fractions and sub-fractions inhibited growth of HT29 colorectal cancer cells. Further research isolated two novel compounds from

Fagonia indica fractions, which displayed 51.5% and 48.3% growth inhibition at 6.25 and 12.5 μM/mL concentrations against H29 colorectal cancer cells (Farheen *et al*., 2015).

Lam *et al* (2012) investigated the mechanisms of extract cytoxicity in MCF-7 and MDA-MB-231 breast cancer cells. These *Fagonia indica* treated cell lines were more susceptible to cell cycle modulation, with a significant increase of cells remaining in G_1 phase, and a parallel reduction of cells entering G_2 phase 24 hours after extract treatment. A blockade of the cell cycle at the G1/S phase, prevented cells from becoming committed to the cell cycle and inhibited cellular proliferation; this corresponded to reduced growth in the cancer cell lines. This was accompanied by a corresponding increase of annexin V binding, used as an indicator of cellular apoptosis. Typically, such cell cycle arrest would be initiated by activation of a DNA damage response; which was demonstrated by increased levels of ϒ-H2AX. H2AX becomes rapidly phosphorylated to ϒ-H2AX upon double strand DNA breakage. Lam *et al* (2012) suggested that *Fagonia indica* extract treatment instigated cytotoxic events by inducing double strand DNA breakages, stabilising the p53 protein, increasing the expression of transcriptional factors, subsequently triggering cell cycle arrest and apoptosis. P53 is one of the most extensively studied tumour suppressor proteins, namely for its ability to inhibit progression of tumours by provoking transient or permanent growth arrest (Stegh, 2012). In the current study the significant increase in the p53 expression was followed by an elevated expression of transcriptional targets, p21 and BAX.

Interestingly, Lam *et al* (2012) were able to demonstrate that loss of cell viability post-treatment with *Fagonia indica* was not solely dependent on stabilisation of p53. Knockdown of p53 induced a significant reduction in extract induced cytoxicity, however the effects of the *Fagonia indica* extract were not fully alleviated. This would imply a presence of factors independent of p53 and its subsequent activation of transcriptional factors. These 'alternative factors' have been attributed to an increase in forkhead box protein 3a (FOXO3a) expression. FOXO3 has numerous modes of action, including regulating apoptosis, initiating DNA damage response pathways and triggering cell cycle arrest (Hu *et al*., 2017). FOXO proteins

are able to initiate apoptotic pathways by increasing expression of pro-apoptotic genes. Previous studies have demonstrated an interaction between FOXO3 and runt-related transcription factor 1 (RUNX), a crucial component in the induction of pro-apoptotic proteins such as BCL-2-like protein 11 (BIM). RUNX is frequently lost in gastric cancers resulting in a down-regulation of FOXO3 mediated apoptosis. Activation of FOXO's also play an important role in controlling the onset of cell-cycle arrest. FOXO3 controls transcription of a number of cell-cycle regulators; such as p27 and cyclin D1, vital players in cell cycle progression. In breast cancers this pathway is typically inhibited via oncogenes in the P13K/AKT pathway. Notch Homolog 1 translocation-associated protein (NOTCH1) is a prevalent oncogene in breast cancer, which inhibits FOXO3 interactions with Ataxia-Telangiectasia mutated gene (ATM), a crucial component of the DNA damage response pathway. It would be beneficial to investigate both the expression of the inhibitory oncogenes and transcription regulators of FOXO3a in extract-treated cell lines, to determine whether FOXO3 expression is a direct target of *Fagonia indica*.

Waheed *et al* (2012), used a biological activity guided fractionation approach to isolate and identify active compounds from a crude plant extract of *Fagonia indica*. A steroidal saponin was isolated from an ethanol fraction of *Fagonia indica* and assayed against MCF-7 and MDA-MB-468 breast cancer cell lines and Caco-2 colon adenocarcinoma cell line to determine its cytotoxic potential. There was a significant reduction in cellular proliferation in both MDA-MB-468 and Caco-2 cells after treatment with the isolated saponin after 24 hours. Treatment with the saponin caused induction of apoptosis in both cell lines, demonstrated by PARP cleavage and DNA fragmentation. Waheed *et al* (2012) and Lam *et al* (2012) also found that treatment with *Fagonia indica* was not toxic to HUVEC (human umbilical vein endothelial cells), U937, or HMEpC cells, indicating some level of selectivity between healthy and malignant cells.

1.2.5 Other medicinal properties of *Fagonia indica*

Alongside the aforementioned cytotoxicity of *Fagonia indica*, there has been several experimental works demonstrating a range of medicinal effects including, but not limited to: antioxidant, analgesic, antimicrobial, antidiabetic and anti-inflammatory (Shehab *et al*., 2011). It is important to consider these additional medicinal properties of *Fagonia indica*, as some of the affected pathways such as metabolism and cellular signalling, could also be involved in cytotoxic mechanisms of the plant in cancer.

1.2.6 Gastric diseases

Traditional practitioners have long detailed the use of plant materials as anti-spasmodic agents in rural communities in the middle east. Tanira *et al* (1996) screened plants with the potential to relax smooth muscle of the small intestine. Jejunum tissue from male rabbits was removed from the small intestine and mounted on thermostatically controlled organ baths and subjected to spontaneous rhythmic contractions. Treatment with *Fagonia indica* extract caused an irreversible inhibition of jejunal contractions, demonstrating its anti-spasmodic potential. Guinea-pig ileum was treated with acetylcholine and dose-response curves were constructed with and without plant agonists. *Fagonia indica* was able to inhibit acetylcholine contractions at a concentration of 1 mg/mL. *Fagonia indica* affected gastrointestinal transmit time by 59.9%, in comparison to the standard treatment morphine which delayed transmit by 76%. This study demonstrates the potential of *Fagonia indica* in inhibiting muscular contraction, whilst particularly reducing the effects on gastrointestinal transmit time.

Peptic ulcer disease is characterised by a breakdown in the protective mechanisms of the gastrointestinal mucosa, accompanied by an inflammatory cell infiltration and coagulation necrosis of the stomach and/or small intestine. Management of the disease typically relies on drugs which inhibit the secretion of gastric acid or eradicate infection of *H. pylori* (Sung *et al*., 2009). Madhy *et al* (2015) investigated the effect of a whole plant extract of *Fagonia indica* on ethanol induced gastric ulcerations in male albino rats. Treatment with *Fagonia indica* for 14 days, was sufficient in significantly reducing a number of gastric lesions when

compared with an untreated control. *Fagonia indica* had a determined gastric ulcer preventative index of 89%, highlighting the potential of a whole plant extract to prevent gastric ulceration *in vivo*.

1.2.7 Liver diseases

Ethnomedicines have played a pivotal role in the development of therapies to manage various liver disorders, particularly in remote regions of the developing world. Several studies have demonstrated the effectiveness of many herbal medicines in treating infectious and non-infectious liver diseases, such as hepatitis and liver cirrhosis (Daniyal *et al*., 2019). Azam *et al* (2018) demonstrated a potential immunoregulatory role of *Fagonia indica* in hepatotoxicant induced mice liver injury models. Those mice administered with the plant extract displayed a significantly reduced accumulation of abnormal liver serum markers, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and globulin, when compared to the group with induced liver injury alone. At the macroscopic level, morphological analysis revealed a reversal of damaged liver architecture upon treatment with *Fagonia indica*, this indicates a hepatoprotective mechanism as treatment reduced effects of induced liver injury. Expression analysis also demonstrated that treatment with *Fagonia indica* significantly downregulated proinflammatory and fibrosis markers IL-1β, IL-6, TNF-α, TNF-β, collagen-1α and α-SMA, revealing a potential immune regulatory mechanism.

Other researchers have induced liver injury in albino rats using Carbon tetrachloride (CCL4), to study dose-responses of *Fagonia indica*. Bagban *et al* (2012) found that treatment with CCL4 resulted in a significant increase in serum AST, ALT, ALP, bilirubin and cholesterol, which was subsequently reversed upon treatment with a methanolic extract of *Fagonia indica*. Microscopic evaluation on sectioned liver tissues revealed that treatment with 400 mg per kg of *Fagonia indica* was able to normalise abnormal histological architecture seen in the rats with induced liver injury. In a later study, rats with or without CCl4 induced liver injury were treated with *Fagonia indica* (Shebab *et al*., 2015). Groups which were pre-treated 14 days

prior to the induction of liver injury showed significantly lower serum levels of ALT and AST. Again, this was supported by histological analysis which showed that pre-treated tissues demonstrated features of hepatocyte renewal and regeneration, with only minor depletion of glycogen stores. Antioxidant activity of *Fagonia indica* was also investigated in this study, all extracts demonstrated potent free radical scavenging activity with the highest observed in the ethanolic and methanolic extracts.

1.2.8 Diabetes

Diabetes mellitus is a metabolic disease characterised by an increase in glucose concentrations or relative insulin deficiency (Moradi *et al*., 2018). Many species of plants from around the world have been investigated for their antidiabetic capabilities, *Fagonia indica* has gained some traction in this area. Islet cells from the pancreas of mice were isolated to determine the effect of characterised triterpenoid glycosides from *Fagonia indica* on *in vitro* insulin secretion. In this study, 'compound 8' a pre-determined triterpenoid, induced potent insulin secretion during high glucose levels, but not at basal levels (Kanwal *et al*., 2017). Further studies have noted a hyperglycaemic effect of *Fagonia indica* in mice. Treatment with a crude *Fagonia indica* extract induced significant reductions in blood glucose over the course of 5 hours (Mahdy *et al*., 2015).

1.2.9 Burns and wound healing

Due to the increased demand of topical burn gel in middle Eastern countries and the interest and accessibility of *Fagonia indica* as a dermatological treatment, Kadhim *et al* (2014) conducted a study investigating the effect of crude extract formulas. Burns were administered to rats using a heated aluminium stamp, the animals were then subdivided into three groups to be treated with a commercial product, a 0.5% w/w crude *Fagonia indica* extract or a control. *Fagonia indica* treatment resulted in a 95% reduction in wound size, alongside dermal infiltration of inflammatory cells and fibrous tissue formation examined by histopathological staining (Kadhim *et al*., 2014).

1.3 Aims and hypothesis

The aims of the project were to determine the cytotoxic potential of extracts of *Fagonia indica* on wild type and chemotherapy resistant human breast and colon cancer cell lines and to elucidate the mechanisms of action. A secondary aim was to isolate and identify bioactive extracts and compound(s) of *Fagonia indica*. The hypothesis is that *Fagonia indica* extract will demonstrate cytotoxicity against human breast and colon cancer, including multi-drug resistant clones.

1.4 Objectives

The specific objectives of this PhD project were to:

1) Determine the cytotoxic potential of *Fagonia indica* extracts against wild-type and chemotherapy resistant human breast and colon cancers *in vitro.*
2) Elucidate to potential mechanisms of action of *Fagonia indica*, in relation to apoptosis, inflammation and angiogenesis.
3) To produce an extract of *Fagonia indica* with a high triterpenoid content
4) Conduct a pilot study to determine the effect of *Fagonia indica* on biochemical markers in human serum.

2 Chapter 2: Materials & Methods

2.1 Materials

The following solvents were purchased from Fisher & co: ethanol, methanol, dichloromethane, chloroform, butanol, ethyl acetate and hexane.

The following chemicals were purchased from Sigma: glacial acetic acid, sodium chloride, trisaminomethane base (TRIS), tritox-100, ethylenediaminetetraacetic acid (EDTA), protease inhibitors, sodium dodecyl sulphate (SDS), glycerol, sodium phosphate anhydrous, β-mercapotoethanol, sulphuric acid, ferric chloride, iron chloride, vanillin, sodium nitrite, sodium hydroxide, ammonium molybdate, dimethyl sulfoxide (DMSO) and ammonium hydroxide.

All other chemicals, consumables and equipment were purchased from Sigma unless stated otherwise.

Dried *Fagonia indica* extract was kindly gifted by Prof Amtul Carmichael.

2.2 *Fagonia indica* extract preparation

An aqueous extract of *Fagonia indica* was produced following the protocol described by Lam *et al* (2012). In short, 20 g of dried plant material was soaked in 500 mL of dH_20 for 5 hours at 70 ºC with constant agitation. Following this, the extract was filtered to remove solid material before being subjected to a liquid-liquid partition with 3 x equal volumes of culture-grade hexane. The aqueous phase was collected and freeze-dried under vacuum for 3 days, before being stored at 4 ºC.

Solvent extractions of *Fagonia indica* were produced by soaking 20 g of dried plant material in 500 mL of 75% methanol, butanol or dichloromethane for 5 hours at room temperature under constant agitation. Similarly, extracts were filtered to remove solid material before removal of the solvent under rotary evaporation. The extracts were then collected and freeze-dried under vacuum for 3 days and stored at 4 ºC.

For cell-viability assays (NR and MTT), a series of stock solutions between 0.5-2.5 mg/ mL of *Fagonia indica* extracts were prepared using 10% FBS supplemented DMEM media.

2.3 Cell Culture

2.3.1 Revival of cells from liquid nitrogen

Cells were retrieved from storage in liquid nitrogen and thawed in a 37 ºC water bath. These cells were resuspended in 5 mL of relevant culture media and transferred into a 25 cm^2 culture flask (Appleton Woods, UK). These were inspected regularly using a Motic AE2000 light microscope (X10 magnification).

2.3.2 Maintenance of cell lines

Both the resistant and wild-type (wt) cell lines were cultured in 75 cm^2 culture flasks (Appleton Woods, UK) and maintained at 37ºC in a 5% CO_2 humidified incubator. Once approximately 70-80% confluent, cells were passaged by aspirating the media and washing the flasks with 10 mL of Hank's Balanced Salt Solution (HBSS) (Sigma), followed by a 5-minute incubation with 2 mL of ETDA-Trypsin (Sigma, UK) solution at 37 ºC. Detached cells were immediately resuspended in 8 mL of the appropriate culture medium (Table 2.1), to inactivate the trypsin. Resuspended cells were for 5 minutes to form a pellet, which were subsequently resuspended into 10 mL of fresh culture medium. As a routine, cells were passaged in a split ratio of either 1:3 or 1:4, into the relevant culture vessel.

Cell line	Culture medium and growth conditions	Origin
MCF7-WT	DMEM – high glucose (Sigma, UK) (100 µ/mL FCS and 100 mg/ mL penicillin/streptomycin)	University of Wolverhampton
H630-WT	DMEM – high glucose (Sigma, UK) (100 µ/mL FCS and 100 mg/ mL penicillin/streptomycin)	University of Wolverhampton
H630-5FU	DMEM – high glucose (Sigma, UK) (100 µ/mL FCS and 100 mg/ mL penicillin/streptomycin) 10 µM 5-fluorouracil (Sigma, UK)	University of Wolverhampton
H630-GM	DMEM – high glucose (Sigma, UK) (100 µ/mL FCS and 100 mg/ mL penicillin/streptomycin) 1 µM gemcitabine (Santa Cruz biotechnology)	University of Wolverhampton
MDA-MB-231-WT	DMEM – high glucose (Sigma, UK) (100 µ/mL FCS and 100 mg/ mL penicillin/streptomycin)	University of Wolverhampton
MDA-MB-231-PAC	DMEM – high glucose (Sigma, UK) (100 µ/mL FCS and 100 mg/ mL penicillin/streptomycin) 10 nM paclitaxel (Santa Cruz biotechnology)	University of Wolverhampton
MDA-MB-231-GM	DMEM – high glucose (Sigma, UK) (100 µ/mL FCS and 100 mg/ mL penicillin/streptomycin) 100 nM gemcitabine (Santa Cruz biotechnology)	University of Wolverhampton
RKO-WT	DMEM – high glucose (Sigma, UK) (100 µ/mL FCS and 100 mg/ mL penicillin/streptomycin)	University of Wolverhampton
RKO-TDX	DMEM – high glucose (Sigma, UK) (100 µ/mL FCS and 100 mg/ mL penicillin/streptomycin) 2 µM tomudex (Tocris, UK)	University of Wolverhampton

Table 2.1. Cell lines, culture and growth conditions for the present study. WT = wild-type, 5FU = f-fluorouracil, GM = gemcitabine, PAC = paclitaxel, TDX = tomudex.

2.4 YO-PRO-1 staining

YO-PRO-1 (YP1) is a nuclear marker which is able to bind directly to the DNA of apoptotic cells via associated loss in membrane integrity (Fujisawa *et al*., 2014). In brief, cells were seeded in 12 well plates (Costar, UK) at a density of $2.5x10^6$ cells per well and left to incubate overnight at 37ºC. After seeding, cells were treated with 1 mg/mL of *Fagonia indica* extract or 1 µM gemcitabine; as a positive control, or a vehicle control, for 24 hours. Cells were then stained with 5 µM of YO-PRO-1 (Life Technologies®, UK) for a 30-minute incubation at 4°C in the dark. After incubation, cells were washed three times in high glucose DMEM medium before imaging using a EVOS fluorescence microscope. Excitation and emission wavelengths for YP1 were 535 nm and 617 nm, respectively.

2.5 Cell viability

2.5.1 Cell viability assay - (MTT)

The MTT (3-[4,5-dimethylthizaol-2-yl]-2,5 diphenyl tetrazolium bromide) colorimetric assay is based on the conversion of MTT into formazan crystals via mitochondrial pathways. In the majority of cell populations total mitochondrial activity is relative to the number of viable cells, as such the assay can be used to as a broad measure of drug cytotoxity *in vitro*. The MTT assay was performed as described by Mosmann, (1983.) In brief, cells in an exponential growth phase were seeded in a flat bottomed 96-well plate (Costar, UK) at a density of $1x10^4$ cells per well. Cells were left to adhere overnight, after which the media was replaced with an equal volume of test media. Cells were treated with either the relevant inhibitor (Table 2.2), *Fagonia indica* extract (0-2.5 mg/mL), or gemcitabine (1-10 µM); as a positive control, for 24-72 hours, prior to the addition of 0.5 mg/mL MTT (Sigma, UK) and incubation for 2 hours at 37 ºC. Cells were lysed and formazan product solubilised with 100 µL DMSO. Absorbance was measured at an absorbance of 570 nm using a Thermoscientific Multiscan GO microplate reader. All experiments were; at minimum, performed in triplicate and repeated three times. Data was analysed as a percentage of an untreated control.

Drug treatments (5-FU, TDX, GM and PAC) were made in stock solutions with DMSO. Final DMSO concentrations in the test media was >0.1%.

Inhibitors/activators used in MTT assays				
Inhibitor/ Activator	**Target**	**Concentration**	**Manufacturer**	**Reference**
Apatanib	VEG-FA	1 µM	Sigma, UK	Kim & Suh, 2017; Lin *et al*., 2017
SC-79	Akt activator	10 µM	Tocris, UK	Yu, Luo & Wen, 2019
SB203580	Akt and p38 MAP kinase	10 µM	Tocris, UK	Han *et al*., 2018
Caffeic acid phenethyl ester (CAPE)	NF-κB	1 µM	Tocris, UK	Chuu *et al*., 2012
Z-VAD-FMK	Pan Caspase	50 µM	Tocris, UK	Mousavi *et al*., 2009
MK571	ABCC1	50 µM	Sigma, UK	Low *et al,* 2020

Table 2.2. Inhibitors/ activators used in the current study

2.5.2 Cell viability assay – Neutral red

Cell viability was determined using the Neutral red colorimetric assay as described by Repetto, del Peso, & Zurita, (2008). In brief, cells were seeded in a flat-bottomed 96-well plate (Costar, UK) at a density of 1 x10^4 cells per well and left to adhere overnight at 37 ºC. Cells were treated with plant extract (0-2.5 mg/mL) or gemcitabine (10 µM) for 24-72 hours, before addition of neutral red reagent (40 µg/mL) and incubation at 37°C for 2 hours. Cells were lysed and the neutral red was solubilised using 100 µL neutral red destain solution (50% ethanol, 49% dH20 and 1% glacial acetic acid). Absorbance was measured at a wavelength of 540 nm using a Thermoscientific Multiskan GO microplate reader. All experiments were; at minimum, performed in triplicate and repeated three times. Data was analysed as a percentage of an untreated control.

2.6 Luminescent ATP assay

Intracellular ATP was measured using the Luminescent ATP Detection Assay Kit (ab113849, Abcam, UK), following the manufacturers protocol. In brief, cells were seeded into sterile

white 96-well plates (Sigma, UK) at a density of 1 x 10^4 cells per well and left to adhere overnight at 37°C. Cells were treated with 1 mg/mL *Fagonia indica* extract or low-glucose DMEM, as a positive control, for 24 hours. Luminescence was measured on an Orion II luminescent microplate reader and intracellular ATP levels were measured as a percentage of the control. All experiments were, at minimum, performed in triplicate and repeated three times.

2.7 Real time quantitative PCR.

2.7.1 RNA Extraction

RNA was extracted using the Isolate RNA mini kit (Isolate, UK). As per the manufacturer's instructions, cell pellets were resuspended in 450 µL of Lysis Buffer R before being transferred to Spin Columns R1 and centrifuged for 2 min at 10000 g. An equal volume of 70% ethanol was added to the filtrate before subsequent transfer to Spin Columns R2 and centrifugation at 10000 g for a further 2 minutes. Columns were then washed with 500 µL of Wash Buffer AR and spun for 1 minute at 10000 g, before a second wash step with 700 µL of Wash Buffer BR and a further centrifugation step for 1 minute at 10000 g. Washed columns were then spun for a further 3 minutes to ensure removal of all wash buffer reagents at 10000 g. To elute the isolated RNA sample, 30-80 µL of RNase-free water was added to the Spin Column R2 membrane, before a final spin at 6000 g for 1 minute into a 1.5 mL DNA elution tube. Following this, RNA concentrations were measured using the NanoDrop™1000 spectrophotometer before being stored at -80 ºC.

2.7.2 Reverse Transcription

RNA was reversed transcribed using the Precision nanoScript ™2 Reverse Transcription kit (Primerdesign, UK), to produce cDNA. The protocol follows the supplied instructions from the manufacture. In brief, RNA samples were diluted to a concentration of 500 ng in RNase-free water, to a final volume of 9 µL. To each sample, 1 µL of Oligo-DT primer was added prior to a 5-minute incubation at 65 ºC. Following incubation, samples were immediately placed on ice to allow primers to anneal. 10 µL of pre-prepared 'mix' containing 5 µL of 4X

nanoScript2 Buffer, 1 µL of 10 mM dNTP mix, 1 µL of nanoScript2 enzyme and 3 µL of RNase/ DNase-free water, was added to each sample. Samples were placed in a thermocycler under the following conditions: 20 min at 42 ºC, 10 min at 75 ºC and a hold temperature of 4 ºC, before finally being diluted 1:10 and stored at -20 ºC.

2.7.3 Quantitative Polymerase Chain Reaction (qPCR)

Prior to PCR set up a 'master mix' was prepared containing 10 µL of PrecisionPLUS 2x qPCR mastermix with SYBR green (Primerdesign, UK), 3 µL of RNase/DNase-free water and 1 µL of both forward and reverse primers (Table 2.3). For each independent reaction, 5 µL of cDNA and 15 µL of master mix was added to each well in triplicate. Gene expression levels were determined using the PikoReal™ 96 (Thermo Scientific, UK) and analysed using the PikoReal™ Software 2.2. Conditions of the PikoReal™ thermal cycler were as follows, 10 minutes at 95 ºC, 15 seconds at 95 ºC and 1 minute at 60 ºC (40 cycles), and cooling of 40 ºC for 10 seconds. All PCR set-up was conducted under sterile conditions using a PCR workstation (UVP). Each experiment was; at minimum, conducted in triplicate and repeated three times. Data was presented as relative gene expression compared against an untreated control.

Primers used in qPCR		
Gene (primers)	**Manufacturer**	**Primer sequence**
Actin	Invitrogen	F: CTGGAACGGTGAAGGTGACA R: AAGGGACTTCCTGTAACAATGCA
YWHAZ	Invitrogen	F: CCGCCAGGACAAACCAGTAT R: CCGCCAGGACAAACCAGTAT
VEGF-A	Invitrogen	F: CTACCTCCACCATGCCAAGT R: GCAGTAGCTGCGCTGATAGA
NFκB- P65	Invitrogen	F: CCTGCTTCTGTCTCTAGGAGGTA R: TAAGCAGAAGCATTAACTTCTCTGGA
ICAM-1	Invitrogen	F: GACTCCAATGTGCCAGGCTT R: TAGGTGCCCTCAAGATCTCG
ABCC4	Invitrogen	F: TGTGGCTTTGAACACAGCGTA R: CCAGCACACTGAACGTGATAA
ABCG2	Invitrogen	F: CAGGAGGCCTTGGGATACTT R: TATAGAGGCCTGGGATT
AUF-1	Invitrogen	F - GGTGGTTTTGGTGAGGTGGAATC R -CCCACGCCTCTTATTGGTCTTGT

Table 2.3. RT-PCR primer sequences used in the current study.

2.8 Wound scratch assay

Cellular migration of cells was assessed using a standard wound scratch assay, as described by Koblinski *et al* (2005). Briefly, cells were seeded in 6-well plates at a density of 1 x10^6 cells per well and left to adhere serum-depleted overnight. A straight single-line scratch wound was introduced into each monolayer using a sterile 20 µL pipette tip, before washing with HBSS. Cells were treated with 1 mg/mL of *Fagonia indica* extract and 5 µg/mL mitomycin (Sigma, UK); to prevent cellular replication, over a time course of 24 hours. Images were taken on an EVOS microscope (X10 magnification) and analysed using ImageJ. Each experiment was; at minimum, conducted in triplicate and repeated three times. Data was analysed and presented as percentage wound closure against a 0-hour timepoint.

2.9 Colony formation assay

The capacity for a single cell to proliferate was determined using a colony formation assay, as described by Crowley *et al* (2016). In brief, cells were treated for 24 hours with 2 mg/mL of *Fagonia indica*, 1 µM gemcitabine; as a positive control, or a vehicle control. Cells were then

seeded into 24 well plates (Costar, UK) at a density of 200-400 cells per mL depending on optimisation and incubated for 7 days. The colonies were fixed with 100% methanol for 20 mins before staining with 0.1% crystal violet solution (Sigma, UK). Visible colonies of >50 cells were counted manually using a transparent film and a Motic AE2000 light microscope (X4 magnification). Each experiment was; at minimum, conducted in triplicate and repeated three times. Data was analysed and presented as percentage change in colony formation compared to an untreated control.

2.10 Calcein-AM assay

ABC membrane transporter (ABCC1) inhibitory potential of an aqueous extract of *Fagonia indica* in wild-type and chemotherapy resistant breast and colon cell lines was determined using a Calcein-AM assay, adapted from a similar assay by Tiberghien & Loor, (1996). In brief, cells were seeded in black, clear bottom 96-well plates (Sigma, UK) at a density of 2.5 x 10^4 cells per well and left to adhere overnight at 37 ºC. Cells were then treated with either 2 mg/mL *Fagonia indica*, 50 µM of MK571; as a positive control, or a DMSO vehicle control for 30 minutes. 1 µM of light protected Calcein-AM solution (Sigma, UK), was added to each well. Fluorescence was detected immediately at constant time intervals (1 mins) up to 30 minutes at an excitation/ emission wavelength of 485/ 520 nm on an Omega fluorescent microplate reader. Data was analysed as change in fluorescence from 0-minutes.

2.11 Phytochemical Screening tests

2.11.1 Salkowski's test

A Salkowski's test was performed to indicate the presence of terpenoids, as described by Rana & Saklani (2017). In brief, 10 mg/ mL of each *Fagonia indica* extract was dissolved in 2 mL of chloroform, followed by 3 mL of concentrated sulphuric acid in 1 mL increments. The formation of reddish-brown interface demonstrated the presence of terpenoids.

2.11.2 Froth test

A Froth test was performed to indicate the presence of saponins, as described by Rana & Saklani (2017). In brief, 10 mg of each *Fagonia indica* extract was dissolved in 20 mL of distilled water. The solution was shaken in a graduated cylinder for 15 minutes. The formation of a 1 cm layer of foam lasting for 10 minutes indicated the presence of saponins.

2.11.3 Keller Killiani test

A Keller Killiani test was performed to indicate the presence of cardiac glycosides, as described by Rana & Saklani (2017). In brief, 10 mg of each *Fagonia indica* extract was dissolved in 1 mL of glacial acetic acid and cooled over ice. After cooling, 3 drops of ferric chloride were added, slowly. To this solution, 2 mL of concentrated sulphuric acid was added dropwise to the side of the test tube. Formation of a reddish-brown ring at the junction of the two layers of solution indicated the presence of cardiac glycosides.

2.11.4 Flavonoid screening test

Flavonoid screening was performed, as described by Rana & Saklani (2017). In brief, 4 mL of 1% ammonium hydroxide was added to 0.5 mL of 10 mg/mL *Fagonia indica* extract, followed by 1 mL of concentrated sulphuric acid. Appearance of a yellow colour indicated the presence of flavonoids.

2.11.5 Ferric chloride test - tannin

A Ferric chloride test was performed for the presence of tannins, as described by Rana & Saklani (2017). In brief, 10 mg of *Fagonia indica* was dissolved in 10 mL of distilled water and filtered. 1 % aqueous iron chloride solution was added dropwise to the filtered solution. The appearance of a green, purple or blue colour indicated the presence of tannins.

2.11.6 Ferric chloride test – phenols

A Ferric chloride test was performed for the presence of phenols, as described by Rana & Saklani (2017). In brief, 10 mg of *Fagonia indica* extract was dissolved in 2 mL of distilled water. To this solution, 10% ferric chloride solution was added dropwise. The appearance of a green colour indicated the presence of phenols.

2.12 Thin layer chromatography

A small amount of each dried *Fagonia indica* extract and 10 µM Ursolic acid; as a positive control, was solubilised in its extraction solvent and applied 1.5 cm from the bottom of a silica gel, aluminium backed TLC plate (Fisher Scientific, UK), cut to 6cm by 10cm (W x H). An ethyl acetate: methanol (90:10) mobile phase was used to separate the compound mixtures. After the solvent front had migrated to within 2 cm of the top of the plate the solvent was removed by air drying and the compounds stained by submerging the plate in vanillin-sulphuric acid solution (Sigma, UK) and heating on a hot plate at 60°C until compounds were visible. Triterpenes were visible as reddish-brown bands.

2.13 Phytochemical quantification

2.13.1 Vanillin-sulphuric acid assay

A vanillin-sulphuric acid assay for total triterpenoid content was carried out as described by Tan *et al* (2014). In brief, 0.3 mL of 10 mg/mL aqueous, methanolic and butanolic *Fagonia indica* extracts were mixed with 0.3 mL 8% (w/v) ethanolic vanillin solution and 3 mL of 72% (v/v) sulphuric acid. The solution was mixed and incubated at 60°C for 15 minutes before being cooled on ice for 10 minutes. The absorbance was measured at 560 nm using a Thermoscientific Multiscan GO microplate reader against a blank solvent cuvette. Ursolic acid (UA) (Sigma, UK) was used as a standard and UA equivalents were calculated using a standard graph. The experiment was; at minimum, conducted in triplicate and repeated three times. Values were expressed as equivalent of UA in mg per g of dry extract.

2.13.2 Phosphomolybdate assay

A phosphomolybdate assay for total antioxidant capacity was carried out as described by Khatoon *et al* (2013). In brief, 0.3 mL of 10 mg/mL aqueous and methanolic *Fagonia indica* extract was combined with 3 mL of phosphomolybdate solution (0.06 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). After combining, the solution was incubated at 95°C for 90 minutes. The samples were left to cool to room temperature before measuring the absorbance of the solution at 695 nm against a solvent blank cuvette using a

Thermoscientific Multiscan GO microplate reader. Quercetin acid (QA) was used as a standard and QA equivalents were calculated using a standard graph. The experiment was conducted; at minimum, in triplicate and repeated three times. Values were expressed as equivalent of QA in mg per g of dry extract.

2.13.3 Folin-Ciocalteu assay

The total phenolic content of the individual plant extracts was determined using the Folin-Ciocalteu method, as described by Aryal *et al* (2019). In brief, 1 mL of 1 mg/mL aqueous and methanolic *Fagonia indica* extract was mixed with 2.5 mL of 10% (w/v) Folin-Ciocalteu reagent (Sigma). After 5 minutes, 2.0 mL of 75% (w/v) Na_2CO_3 was added to the solution and incubated with constant agitation at 50° C for 10 minutes. The solution was then cooled and the absorbance measured at 765 nm against a solvent blank using a Thermoscientific Multiscan GO microplate reader. Gallic acid (GA) (Sigma, UK) was used as a standard and GA equivalents were calculated using a standard curve. The experiment was conducted in triplicate and the values were expressed as equivalent of GA in mg per g of dry extract.

2.13.4 Dowd assay

The total flavonoid content of the individual plant extracts was determined using the Dowd method, as described by Aryal *et al* (2019). In brief, 1 mL of 1 mg/mL aqueous or methanolic *Fagonia indica* extract was mixed with 0.3 mL of 5% (w/v) sodium nitrite and 0.3 mL of 10% (w/v) aluminium chloride. After 6 minutes, 1 mL of 1 M sodium hydroxide was added to the reaction solution before being made up to 10 mL with dH20. Finally, absorbance was measured at 510 nm against a solvent blank using a Thermoscientific Multiscan GO microplate reader. Quercetin (QA) was used as a standard and equivalents were calculated using a standard curve. The experiment was conducted in triplicate and the values were expressed as equivalent of QA in mg per g of dry extract.

2.14 Reactive oxygen species (ROS) detection.

2.14.1 DCFDA/ H2DCFDA Cellular ROS assay

Intracellular ROS was measured using the DCFDA/ H2DCFDA Assay Kit (ab113851, Abcam, UK), following the manufacturers protocol. In brief, cells were harvested and seeded in a black, clear bottom 96-well microplate at a density of 25,000 cells/ per well and incubated overnight to adhere. After 24 hours, culture medium was removed and replaced by 100 µL 1X Buffer. The buffer was removed and replaced with 100 µL per well of diluted DCDFA staining solution. The microplate was incubated with the diluted DCFFA solution for 45 minutes, at 37 ºC in the dark. Afterwards, the staining solution was removed and cells were treated with 1 mg/mL aqueous and methanolic *Fagonia indica* extract or TBHP as a positive control for 4 hours. The plate was measured immediately at the end point on an Omega fluorescent microplate reader at an excitation and emission wavelength of 485/535 nm. Each experiment was; at minimum, conducted in triplicate and repeated three times. Data was analysed and presented as fold-change of intracellular ROS against an untreated control.

2.14.2 ROS/ Superoxide detection assay

ROS and superoxide were measured using the ROS/ superoxide Assay Kit (ab139476, Abcam, UK), following the manufacturers protocol. In brief, cells were harvested and seeded in black, clear bottom 96-well microplates at a density of 20000 cells/ per well and left to adhere overnight at 37 ºC, to ensure 80% confluency on the day of the experiment. After 24 hours, culture medium was removed and cells were washed with 1X Wash Buffer. The cells were then treated with 1 mg/mL of aqueous and methanolic *Fagonia indica* extract or TBHP as a positive control, in addition to 0.1 mL ROS/ Superoxide detection for 60 minutes at 37°C in the dark. The plates were then measured immediately without removal of the detection mix on an Omega fluorescent microplate reader using standard fluorescein excitation and emission (488/520 nm) and rhodamine excitation and emission filter sets (550/610 nm). Each experiment was; at minimum, conducted in triplicate and repeated three times. Data was analysed and presented as fold-change of intracellular ROS/ superoxide against an untreated control.

2.15 Statistical analysis

Data are expressed as the mean ± SEM, for at least three independent experiments undertaken in triplicate, unless stated otherwise. General data analysis was carried out in Microsoft Excel and GraphPad Prism 7.0 software. Statistical tests used are as follows: a student t-test was used when comparing the mean of two independent groups (pairwise comparison), when comparing more than two groups or undertaking pairwise comparisons a one-way ANOVA was used with the relevant post-hoc test.

3 Chapter 3 – *Fagonia indica* breast cancer

3.1 Introduction

Breast cancer is the most common cancer affecting women and the second most commonly diagnosed cancer worldwide (Parsekar *et al*., 2021). Cancer incidence and mortality projections predict that breast cancer will remain amongst the most commonly diagnosed malignancies in 2035 (Smittenaar, *et al*, 2016). Breast cancer incidence and survival rates vary dramatically depending on geographical location. Countries in Western Europe often have higher incidence and survival rates, due to both an association with an increased prevalence of lifestyle risk factors and access to higher quality medical interventions (Dafni *et al*, 2019). On a global scale, environmental and socioeconomic disparities have led to significant variations in breast cancer incidence (Bray, McCarron, & Parkin, 2004). International comparison of diagnosis and survival can aid in the determination of underlying risk factors, effects of natural and medical interventions and can serve as important indicators of treatment success.

Non-metastatic, locally advanced breast cancer accounts for up to twelve-times more cases in developing countries; including those in the Middle East (Iqbal *et al.,* 2010). Confounding issues in breast cancer diagnosis and survival amongst Middle Eastern countries include, greater socioeconomic imbalance, late and advanced stage of diagnosis, cultural and religious implications and limited access to health and supportive care (Haidari *et al.*, 2020). In

Pakistan, use of unconventional therapies for breast cancer is widespread. Traditional herbal medications are amongst the most widely utilised alternative therapy for breast cancer, as these treatments are perceived to be more accessible, safer and inexpensive (Malik, Khan, & Khan, 1999). Traditional herbal medications of this nature are typically administered to treat multiple disease conditions, as they contain a multitude of chemically active phytochemicals (Engel *et al*., 2016).

Fagonia indica is a prime example of a medicinal plant; native to several Middle Eastern and European countries, which is traditionally grown and administered therapeutically by local healers and practitioners. The uses of *Fagonia indica* range dramatically depending on individual tribes and communities, however its medicinal potential as an alternative treatment for breast cancer is well established (Hussain, Zia, & Mirza, 2007). The popularity of *Fagonia indica* lies in the limited side effects and tolerability of treatment, when compared to chemotherapies. Lam *et al* (2012) and Ullah *et al* (2014), demonstrated the usefulness of *Fagonia indica* as a cytotoxic agent against breast cancer, demonstrating the ability of a whole plant extract to initiate apoptosis *in vitro*.

A primary characteristic of a successful chemotherapeutic agent is the inhibition of proliferation and the induction of apoptosis, whilst minimising the damage to surrounding healthy tissue. Initial studies have indicated that treatment with *Fagonia indica* induced growth arrest in breast cancer cell lines in relation to the induction of p53 and downstream targets, p21, BAX and PARP cleavage (Lam *et al*., 2012). *Fagonia indica* was also able to induce apoptosis independently of p53 activation, which was partially attributed to activation of the FOXO3a transcription factor, involved in the regulation of several downstream genes related to apoptosis and cell cycle progression (Sunters *et al*., 2003). Despite this, there is still a very limited understanding in regards to the mechanisms of action of the active components of *Fagonia indica*.

Activation of p53, FOXO3a and other pro-apoptotic proteins can be activated/ inhibited in the cross talk between p38/MAPK and Akt pathways (Figure 3.1.1). Therapies have targeted these

signalling pathways in the treatment of cancer. Resveratrol; a natural polyphonic compound, induced autography *in vitro* via the inhibition of proliferation and activation of p53 in association with p38 and Akt pathways (Wang *et al*., 2018). In this study, targeted inhibition of p38 and activation of Akt reversed the anti-tumour effect of resveratrol. These pathways target cellular functions outside of proliferation and apoptosis, which are key indicators of tumour progression. For example, Akt activation mediates cellular energy metabolism, one such method is by increasing glycolytic and oxidative metabolism which bolsters ATP production (Robey & Hay, 2009). Another important downstream target of Akt is VEGF; a key factor in tumour angiogenesis, which has been shown to be secreted upon phosphorylation of Akt (Liang *et al*., 2007). Although p38 is primarily considered to be a tumour suppressor protein it has some dual functions, particularly in relation to inflammation. Both Akt and p38 pathways can induce the formation of the NF-κB transcription factor complex (Saha, Jana, & Pahan, 2007).

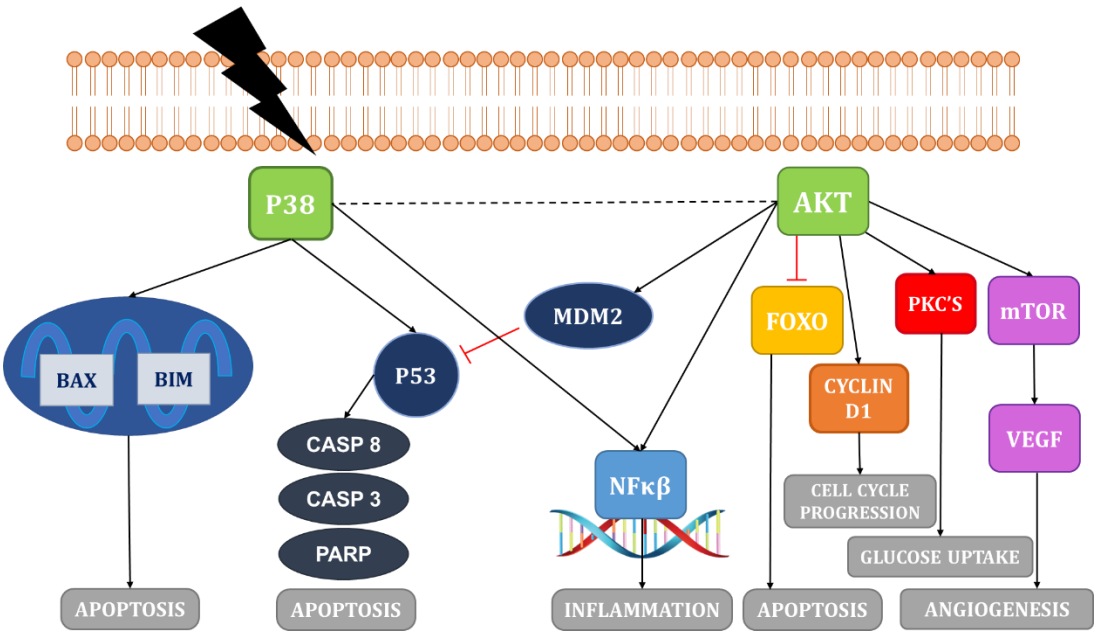

Figure 3.1.1: Simplified schematic representation of P38/ Akt signalling pathways in cancer. P38 MAPK (Mitogen activated protein kinase)/ Akt pathways are intrinsically linked and are both involved in cellular proliferation and apoptosis. Activation of P38/ MAPK pathway can occur upon environmental stress to the cell. P38 has a dual role in tumorigenesis, as it activates downstream targets involved with the induction of cell cycle arrest and apoptosis, whilst also activating the assembly of the inflammatory transcription factor NF-κB. Akt is a frequently overexpressed in breast cancer and has several targets associated with increased metabolism, proliferation, angiogenesis, cell cycle progression

and inflammation. Akt also inhibits several proteins associated with apoptosis, including P53 and FOXO proteins. CASP = Caspases.

Despite overall survival rates of breast cancer improving significantly over the past decade, response rates to chemotherapies remain suboptimal due to the acquisition of subsequent mutations leading to multidrug resistance (Kuo, 2007). Several studies have identified the overexpression of ABC transporters as a major contributor to multidrug resistance and failure of chemotherapy in cancer (Sun *et al*., 2012). This overexpression has also been noted in induced resistant strains of *in vitro* cancer cell lines. Liu *et al* (2005), found that 16 ABC transporters were overexpressed in MDR cell lines of MCF-7. In this study ABCG2 had the greatest fold increase and was very likely to contribute to the MDR in MCF-7 resistant cell lines. Interestingly, naturally occurring flavonoids present in food and herbal products have been shown to reverse resistance to mitoxantrone in MCF-7 cells, resulting in increased cellular accumulation of mitoxantrone and cytotoxicity (Zhang, Yang, & Morris, 2004). There have been no studies on the effect of *Fagonia indica* extracts, or extract derived compounds on drug resistant breast cancers.

The primary aims of this chapter were to establish the cytotoxic capabilities of a whole plant extract of *Fagonia indica* in breast cancer *in vitro* and to demonstrate whether treatment disrupts the ability of the cancer cells to proliferate and migrate. The breast cancer cell lines MDA-MB-231 and MCF-7 were used. These cell lines were selected because although both are considered to be aggressive examples of invasive ductal breast carcinoma, they differ greatly in their respective phenotypic and genotypic backgrounds. MCF-7 is a luminal-type hormone dependent breast cancer, positive for the overexpression of progesterone, estrogen, and human epidermal growth factor 2 (HER2) receptors, whereas MDA-MB-231 is basal-type and triple negative in regards to hormone and growth factor receptor status. Furthermore, to follow on from previous studies which highlighted a role of p53 and FOXO3a in extract induced cytotoxicity, this chapter aimed to elaborate in greater depth the potential mechanisms of action of *Fagonia indica*. There was a particular focus on members of the Akt and p38 pathways, VEGF and NF-κB; in order to obtain a wider perspective from those previously

established by Lam *et al* (2012). For the first time, this chapter also explored the ability of *Fagonia indica* to induce cell death in chemotherapy resistant/ multidrug resistant clones.

3.2 Materials and methods

3.2.1 Extract preparation

An aqueous extract of *Fagonia indica* was prepared as described in section 2.2.

3.2.2 MTT assay

The effect of an aqueous extract of *Fagonia indica* on MCF-7, MDA-MB-231 and MDA-MB-231-GM cell viability was determined using the MTT assay as described in section 2.5.1. In brief, cells were seeded at $1x10^4$ prior to treatment with 0-2.5 mg/mL of aqueous *Fagonia indica* extract for up to 72 hours, prior to analysis using MTT. The effect of pathway inhibitors and activators on extract treatment was determined using varying concentrations of selective inhibitors; Z-VAD-FMK, SC79, SB203580, CAPE, Apatanib and MK571 for 48 hours, alongside treatment with 1-1.5 mg/mL of *Fagonia indica* prior to MTT analysis (as described in further detail in section 2.X). The ability to restore sensitivity of chemotherapeutic agent; gemcitabine, was determined by pre-treating resistant MDA-MB-231 cell lines for 24 hours with 1 mg/mL *Fagonia indica* prior to treatment with either 1µ M of gemcitabine, prior to MTT analysis.

3.2.3 NR assay

The effect of an aqueous extract of *Fagonia indica* on MCF-7 and MDA-MB-231 cell viability was determined using the NR assay as described in section 2.5.2 In brief, cells were seeded at $1x10^4$ prior to treatment with 0-2.5 mg/mL of aqueous *Fagonia indica* extract for up to 72 hours, prior to analysis using NR.

3.2.4 YO-PRO-1 staining

The effect of an aqueous extract of *Fagonia indica* extract inducing apoptosis in MCF-7 and MDA-MB-231 breast cancer cells was determined using YO-PRO-1 fluorescent microscopy, as described in section 2.4.

3.2.5 ATP assay

The level of intracellular ATP produced in MDA-MB-231 and MCF-7 breast cancer cells was detected using the luminescent ATP assay kit as detailed in section 2.6.

3.2.6 RNA extraction, cDNA production and qPCR

The effects of aqueous *Fagonia indica* extract treatment on VEGF, Caspase-8, AUF1, ICAM-1, NF-kB, ABCC4 and ABCG2 mRNA expression in MCF-7 and MDA-MB-231 breast cancer cells was determined by real-time qPCR as outlined in sections 2.7.1, 2.7.2 and 2.7.3. A total of 500 ng of extracted RNA per treatment was reversed transcribed into cDNA, prior to qPCR analysis. YHWAZ and Actin were both used as housekeeping genes.

3.2.7 Colony formation assay

The effect of *Fagonia indica* on MCF-7 and MDA-MB-231 colony formation was detected using the colony formation assay as described in section 2.9. To determine the effect of VEGF signalling in *Fagonia indica*, MCF-7 and MDA-MB-231 cells were pre-treated alongside 1 μM of Apatanib for 24 hours prior to seeding the colony formation assay.

3.2.8 Scratch wound assay

The effect of *Fagonia indica* on wound healing and migration was determined on MCF-7 and MDA-MB-231 breast cancer cells after 24 hours as described in section 2.8.

3.2.9 Calcein-AM assay

To determine whether an aqueous extract of *Fagonia indica* could inhibit the activity of ABCC1, the level of cellular efflux between wild type and paclitaxel/ gemcitabine resistant MDA-MB-231 cells was measured using the Calcein-AM fluorescent microplate assay, as detailed in section 2.10.

3.3 Results

3.3.1 An aqueous extract of *Fagonia indica* induces apoptosis and inhibits proliferation of breast cancer lines MCF-7 and MDA-MB-231

An aqueous extract of *Fagonia indica* was prepared and tested for its cytotoxicity using the MTT assay, to determine its ability to reduce breast cancer viability in two phenotypically

distinct cancer cell lines. Treatment with F*agonia indica* reduced cell viability in a time and concentration dependent manner in MCF-7 and MDA-MB-231 wild type cells (Figure 3.3.1). After 24 hours of treatment with 2.5 mg/mL *Fagonia indica* extract, cell viability of MCF-7 cells was significantly reduced by 32% ±6.55 ($p<0.01$), which increased to 48.2% ±2.32 ($p<0.01$) after 72 hours. In MDA-MB-231 cells there was a significant reduction in cell viability after treatment with 2.5 mg/mL *Fagonia indica* for 24 hours of 31% ±1.51 ($p<0.01$), which similarly increased to 41% ±4.10 ($p<0.05$) after 72 hours.

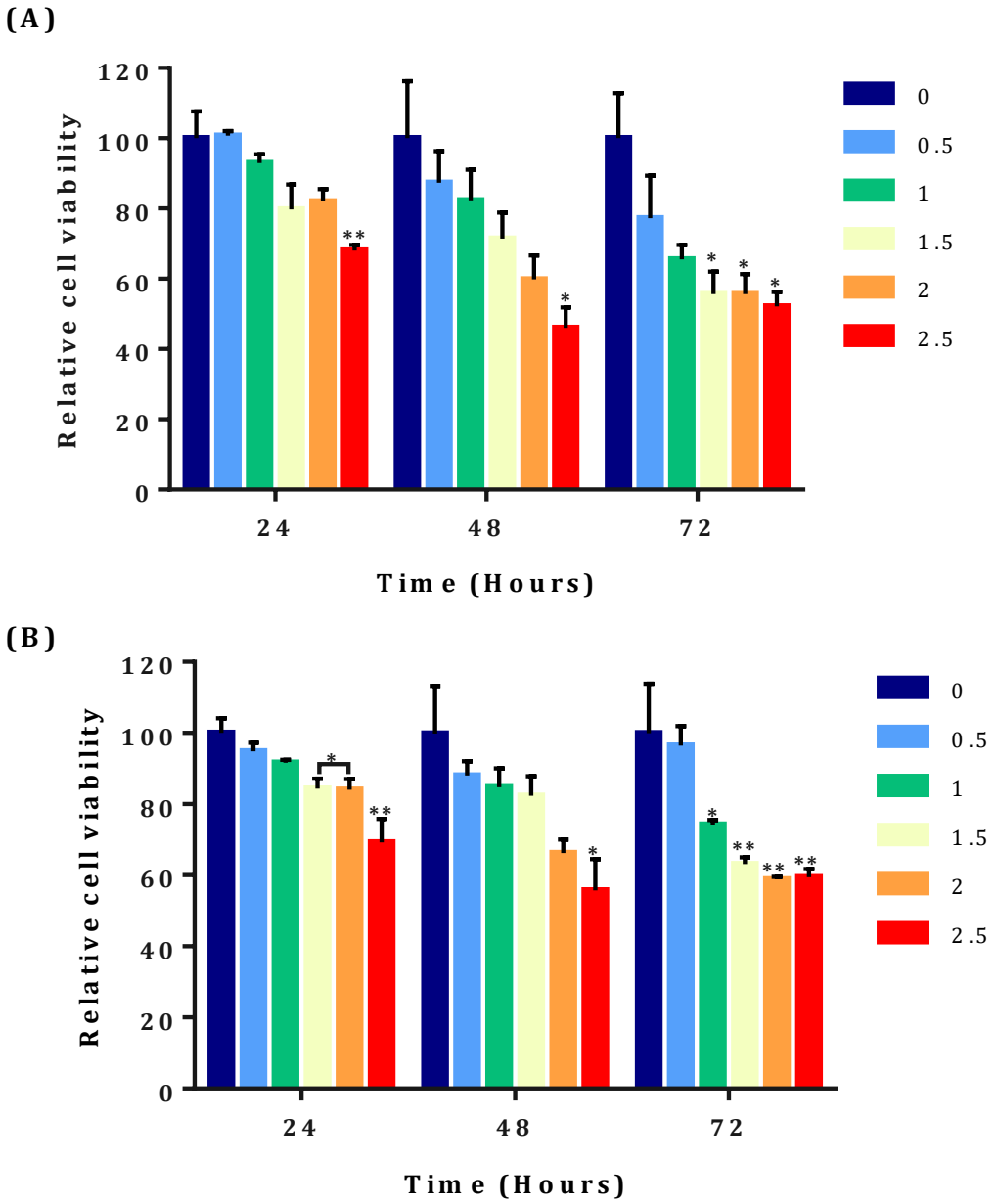

Figure 3.3.1: *Fagonia indica* extract treatment reduces *in vitro* cell viability of MDA-MB-231 and MCF-7 breast cancer cell lines. (A) MDA-MB-231 and (B) MCF-7 cells were treated with 0-2.5 mg/mL aqueous *Fagonia indica* extract for 24-72 hours. Cell viability was determined as a percentage of an untreated DMSO vehicle control using MTT assay. Data denoted * ($p<0.05$) and ** ($p<0.01$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM

Extract induced cytotoxicity was also determined using the NR assay (Figure 3.3.2). In MCF-7 cells, cell viability was not significantly reduced after 24 hours, but increased to 42.5% ±1.79 ($p<0.01$) after 72 hours of 2.5 mg/mL *Fagonia indica* treatment (Figure 3.3.2B). Using NR, MDA-MB-231 cells were more sensitive to *Fagonia indica* extract, with a 41% ±2.94

(p<0.01) loss in cell viability after 24 hours, increasing to 61.3% ±4.41 (p<0.0001) at 72 hours (Figure 3.3.2A).

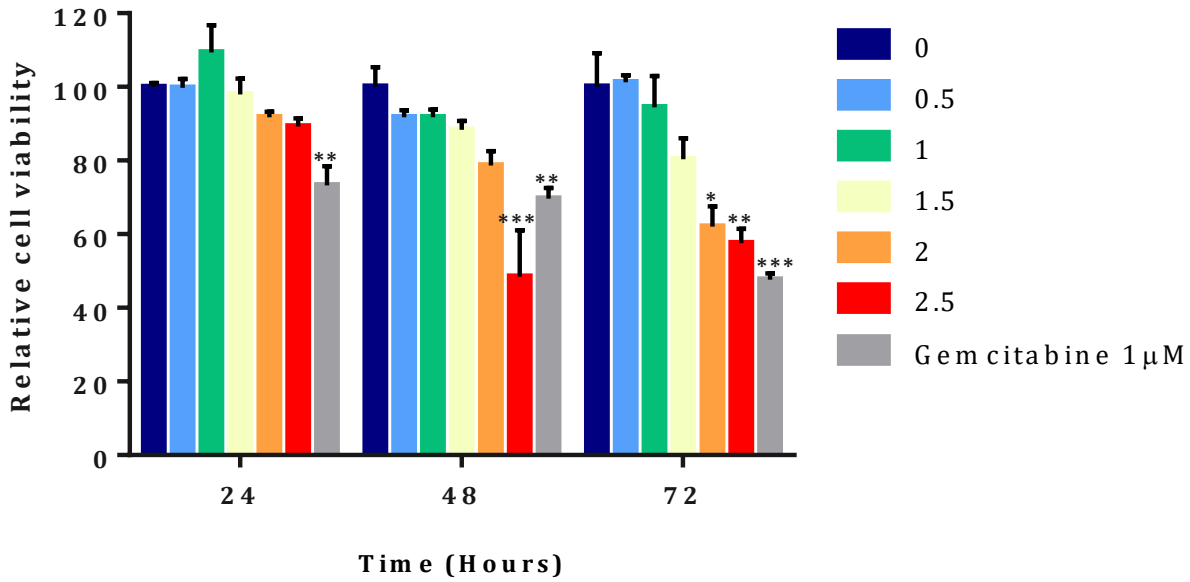

Figure 3.3.2: *Fagonia indica* extract treatment reduces *in vitro* cell viability of MDA-MB-231 and MCF-7 breast cancer cell lines. (A) MDA-MB-231 and (B) MCF-7 cells were treated with 0-2.5 mg/mL aqueous *Fagonia indica* extract or 1 µM of Gemcitabine for 24-72 hours. Cell viability was determined as a percentage of an untreated DMSO vehicle control using the NR assay. Data denoted * (p<0.05), ** (p<0.01), *** (p<0.001) and # (p<0.0001) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

Aberrant metabolism is a key feature of tumorigenesis with high intracellular ATP levels and increased glycolysis being synonymous with tumour development. Intracellular ATP levels were measured in *in vitro* breast cancer cell lines; MDA-MB-231 and MCF-7 (Figure 3.3.3). Extract treatment at 1 mg/mL over 24 hours induced a significant reduction in both MDA-MB-231and MCF-7 intracellular ATP levels by 46.02% (p>0.05) and 64.93% (p>0.01), respectively, when compared to an untreated control group.

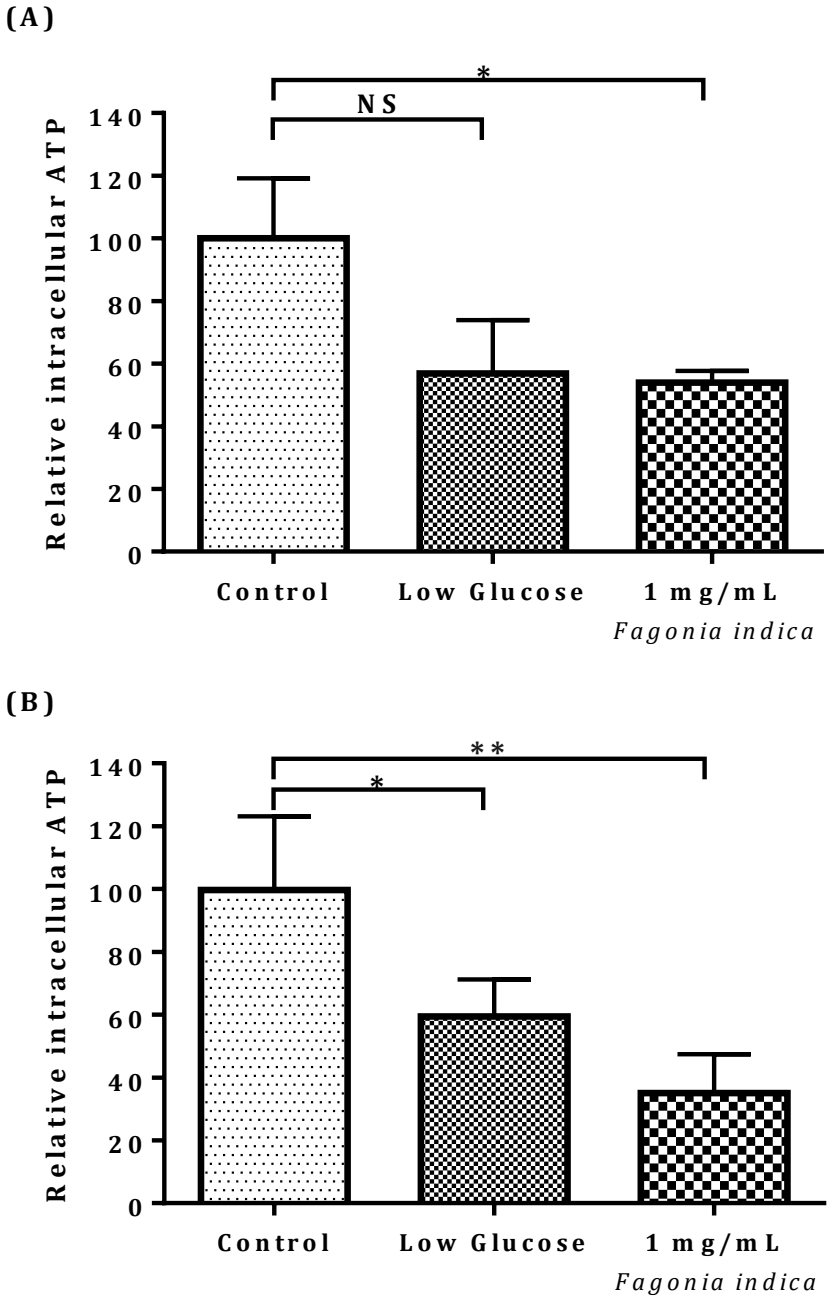

Figure 3.3.3: *Fagonia indica* extract treatment reduces *in vitro* intracellular ATP concentrations in MDA-MB-231 and MCF-7 breast cancer cell lines. (A) MDA-MB-231 and (B) MCF-7 cells were treated with 1 mg/mL aqueous *Fagonia indica* extract or low glucose supplemented DMEM for 24 hours prior to analysis of intracellular ATP with a luminescent ATP assay. ATP concentration was determined as a percentage of an untreated control. Data denoted * ($p<0.05$) and ** ($p<0.01$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

The caspases are a family of distinct protease enzymes with a key role in initiating programmed cell death. Pan-caspase inhibition by z-VAD-FMK (IC_{50}= 40nM), prevents apoptosis and reverses therapeutic cytotoxicity in multiple cell types. Inhibition of pan-caspases did not significantly reduce cell viability in untreated MDA-MB-231 cells (Figure 3.3.4A). In addition, mRNA expression of Caspase 8 in MDA-MB-231 cells was determined using qPCR (Figure 3.3.4B). In MDA-MB-231 cells expression of Caspase 8 was not significantly affected after treatment with 1mg/mL *Fagonia indica* for 6 hours. Inhibition of pan-caspases with z-VAD-FMK did not significantly affect cell viability in MCF-7 cells (Figure 3.3.5A), however, there was a significant 3.9-fold increase ($p<0.01$) in mRNA expression of Caspase 8 in MCF-7 cells after 6 hours of treatment with 1 mg/mL *Fagonia indica* extract (Figure 3.3.5B).

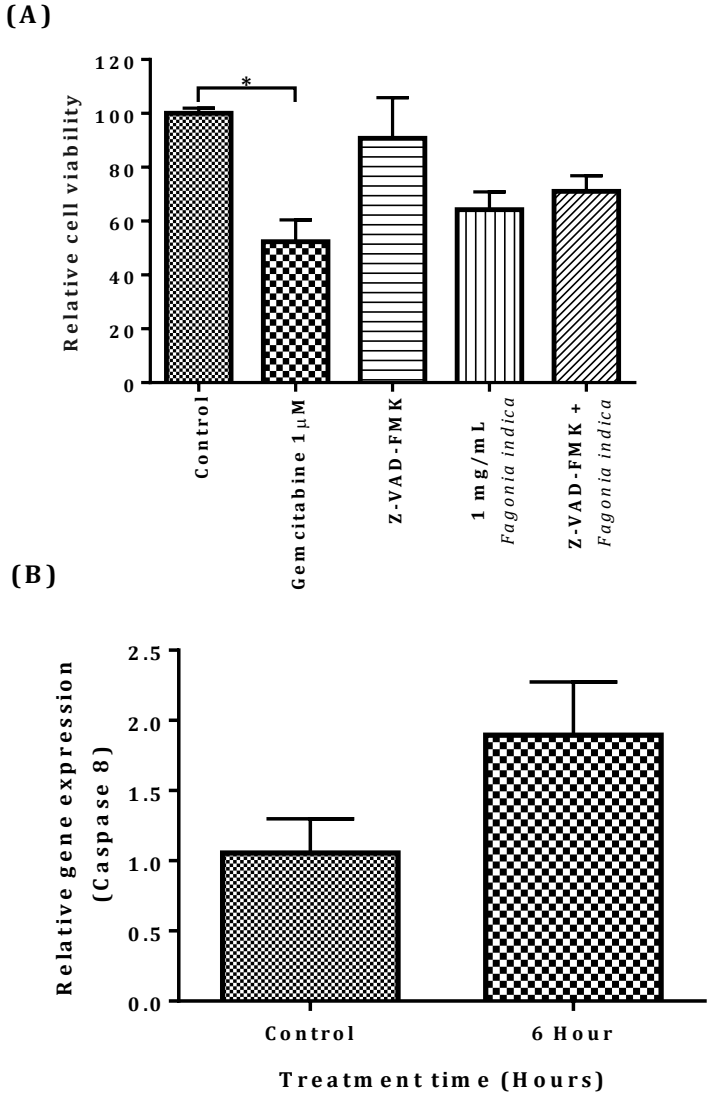

Figure 3.3.4: *Fagonia indica* extract treatment induces expression of Caspase 8 in MDA-MB-231 breast cancer cell line. (A) MDA-MB-231 cells were treated with 1 mg/mL aqueous *Fagonia indica* extract and/or 50 μM of pan-caspase inhibitor Z-VAD-FMK. 1 μM of Gemcitabine was used as a positive control. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted * ($p<0.05$) was significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test (B) Gene expression of Caspase 8 was determined on MDA-MB-231 breast cancer cell lines. Pellets were collected and mRNA extracted after 6 hours of treatment with1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control, subject to a student t-test to determine statistical significance. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

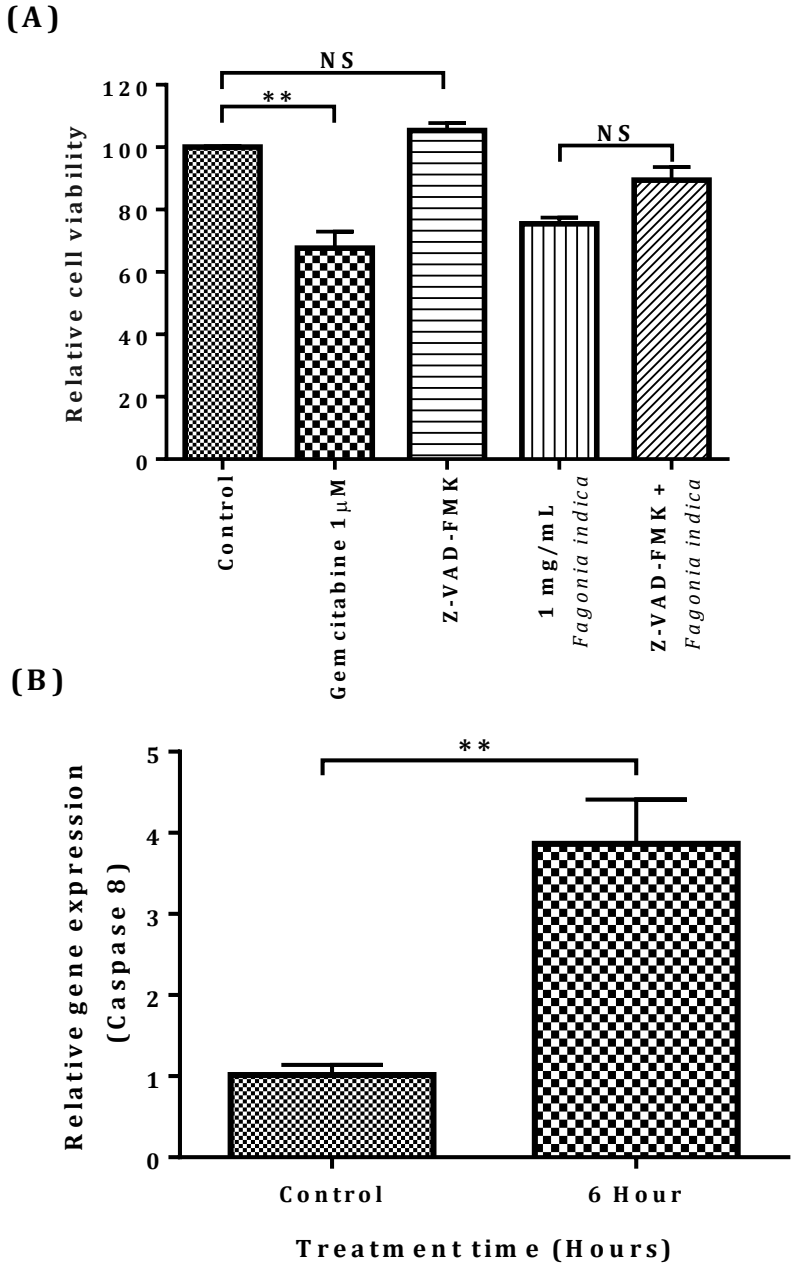

Figure 3.3.5: *Fagonia indica* extract treatment induces expression of Caspase 8 in MCF-7 breast cancer cell line. (A) MCF-7 cells were treated with 1 mg/mL aqueous *Fagonia indica* extract and/or 50 µM of pan-caspase inhibitor Z-VAD-FMK. 1 µM of Gemcitabine was used as a positive control. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted ** ($p<0.01$) were significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test (B) Gene expression of Caspase 8 was determined on MCF-7 breast cancer cell lines. Pellets were collected and mRNA extracted after 6 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted ** ($p<0.01$) were significant compared to the untreated control analysed by unpaired student t-test. Data denoted as NS were not significant when compared to an untreated control. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

The AU-rich element RNA-binding protein 1 (AUF1), is an RNA-binding protein, which is able to stabilise and destabilise RNA transcripts associated with several key cancer related genes. As the MDA-MB-231 breast cancer is triple negative for hormone and growth factor receptor status (ER, PR and HER2), AUF1 expression was evaluated using RT-PCR as a potential alternative mechanism of action for *Fagonia indica*. In MDA-MB-231 cells, treatment with 1 mg/mL of *Fagonia indica* for 6 hours significantly reduced expression of AUF1 to 0.049 ($p<0.01$) of an untreated control (Figure 3.3.6).

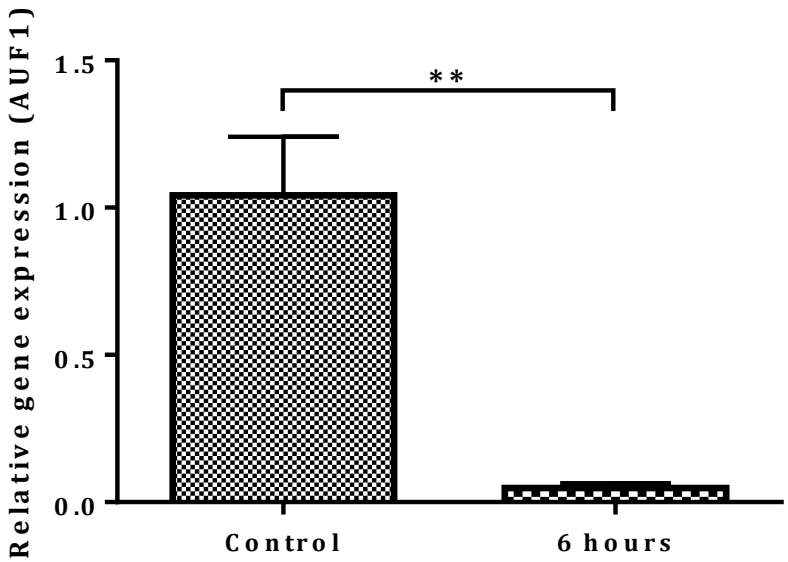

Figure 3.3.6: *Fagonia indica* extract treatment reduces expression of AUF1 in MDA-MB-231 breast cancer cell line. Gene expression of AUF1 was determined on MDA-MB-231 breast cancer cell lines. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted ** ($p<0.01$) was significant compared to the untreated control analysed by students t-test.. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

P38 mitogen activated protein kinase (MAPK) signalling is activated upon stress-stimuli from the environment and is directly associated with inhibition of cell cycle progression and induction of apoptosis. SB203580 (SB20) is a selective ATP-competitive inhibitor of p38 MAP kinase (IC_{50} = 34 nM) and was used to inhibit activation of p38 signalling. Inhibition of p38 MAPK did not significantly affect cell viability in untreated MDA-MB-231 cells, nor did it significantly impact *Fagonia indica* induced cell death (Figure 3.3.7A). When activated, p38 MAPK can indirectly inhibit oncogene Akt. Akt activator SC79 induces a conformational change in the plecktrin homology region of Akt which enhances phosphorylation and cytosolic signalling. In MDA-MB-231 cells, treatment with SC79 alone did not significantly affect cell viability in untreated cells, however activation of Akt alongside *Fagonia indica* treatment was able reduce cell viability by a further 23.3% ($p<0.05$) (Figure 3.3.7B). In MCF-7 cells, treatment with SB20 did not significantly affect cell viability in untreated or *Fagonia indica* treated cells (Figure 3.3.7C), nor did Akt activation with SC79 (Figure 3.3.7D).

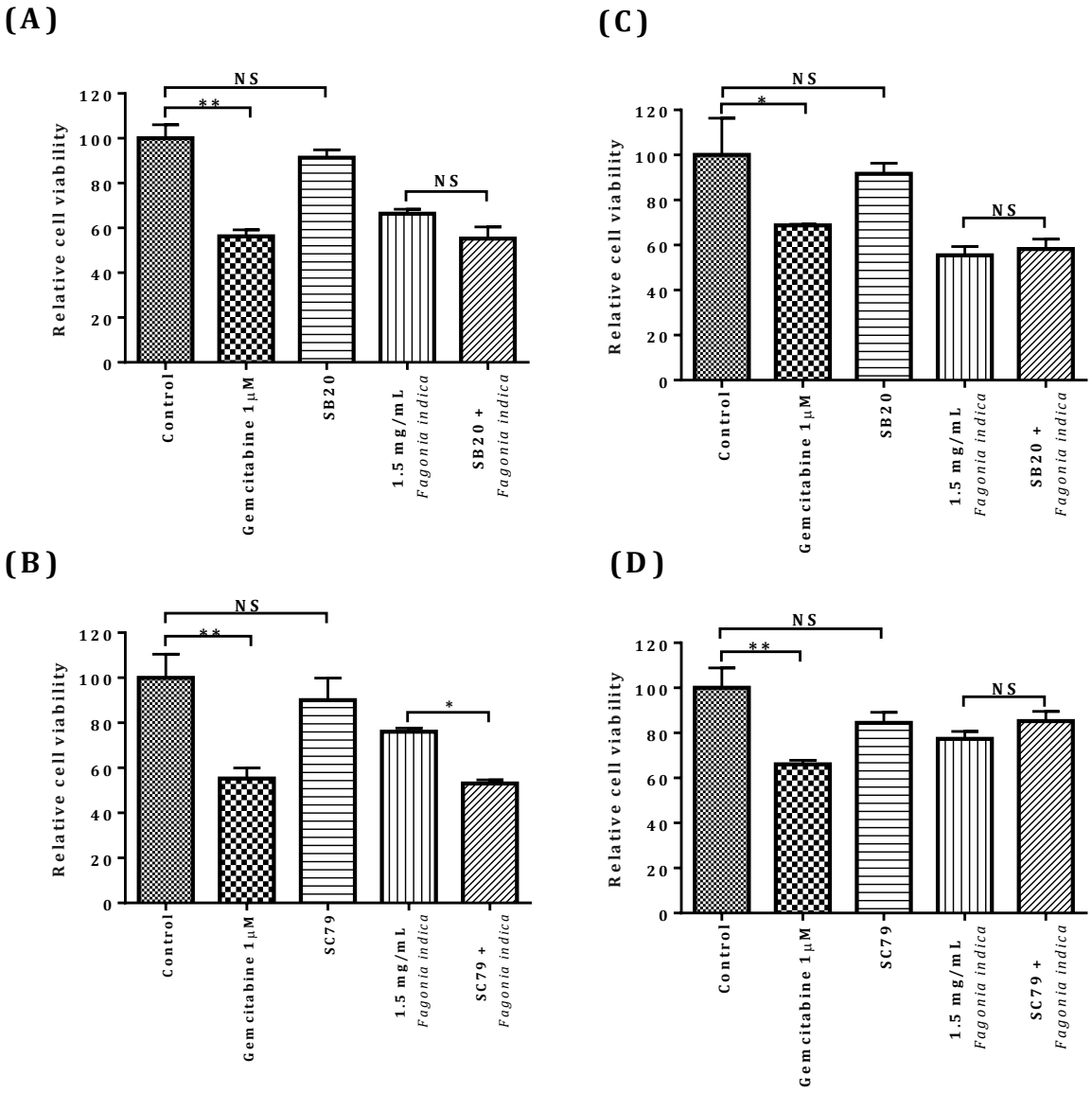

Figure 3.3.7: Effects of p38 MAP kinase inhibition and AKT activation on extract induced MDA-MB-231 and MCF7 breast cancer cytotoxicity. (A) MDA-MB-231 and (C) MCF7 cells were treated with 1.5 mg/mL of aqueous extract and 1 μM of SB203580 (SB20) p38 MAP kinase inhibitor for 48 hours. (B) MDA-MB-231 and (D) MCF7 cells were treated with 1.5 mg/mL of aqueous extract and/or 1 μM of SC79 Akt activator for 48 hours. 1 μM Gemcitabine was used as a positive control. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted as * ($p<0.05$) and ** ($p<0.01$), was statistically significant compared to the untreated DMSO vehicle control, analysed using one-way ANOVA with Sidaks's multiple comparison test. All data points are representative of three independent experiments, performed in triplicate and are expressed as mean ± SEM.

YO-PRO-1 (YP1) staining is used as an early marker of apoptosis, as due to its relatively large size it is only able to enter the cell when the membrane integrity has been jeopardized. YP1 fluorescence staining was used to detect early apoptosis in MCF-7 and MDA-MB-231 breast cancer cell lines treated with 1.5 mg/mL *Fagonia indica* for 24 hours (Figure 3.3.8). After treatment with *Fagonia indica* extract, YP1 fluorescence was increased in both MCF-7 and MDA-MB-231 cell lines, in comparison to an untreated control.

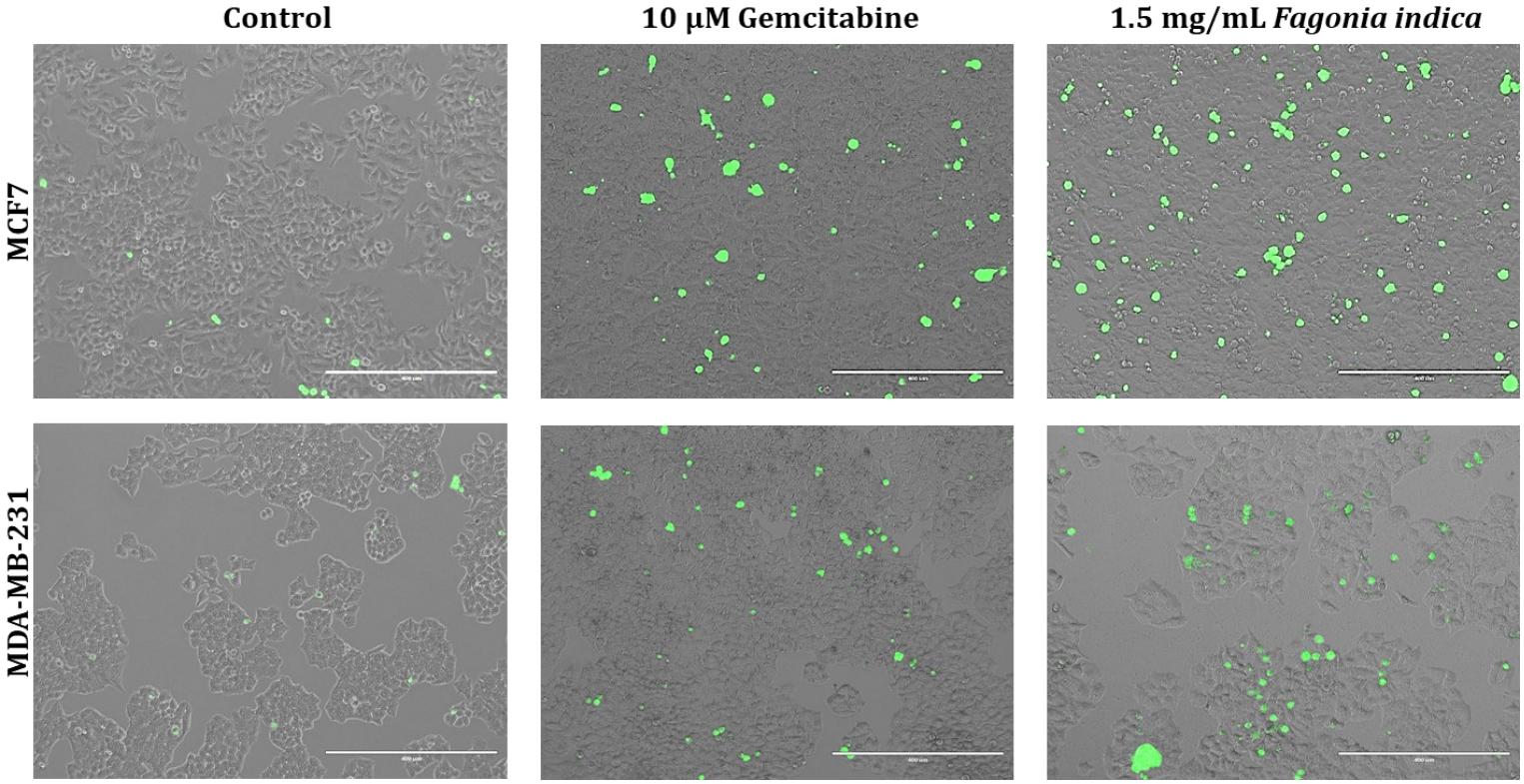

Figure 3.3.8: Yo-pro-1 staining of apoptotic MCF-7 and MDA-MB-231 breast cancer cells. MCF-7 (top) and MDA-MB-231 (bottom) cells were treated with 1.5 mg/mL aqueous *Fagonia indica* extract for 24 hours prior to staining with Yo-pro-1. 10 µM Gemcitabine was used as a positive control. Images were captured on an EVOS fluorescent microscope at 10X magnification. All images are representative of N=3. Scale bar = 400

3.3.2 An aqueous extract of *Fagonia indica* inhibits cologenic formation, wound healing and migration of breast cancer lines MCF-7 and MDA-MB-231

The effect of *Fagonia indica* treatment on the proliferation from a single cell, colony formation and migration in MDA-MB-231 and MCF7 cell lines was investigated. After treatment with *Fagonia indica* extract for 24 hours, cells were seeded and colony formation was assessed after one week. Extract treated MDA-MB-231 and MCF-7 cells formed fewer colonies than the control group. In MDA-MB-231 cells, colony formation was significantly reduced by 80.1% ($p<0.01$) in cells seeded at 200 cells per well, compared to an untreated control (Figure 3.3.9B). Colony formation was also significantly reduced in MCF-7 cells treated with *Fagonia indica* by 66.3% ($p<0.01$) seeded at 200 cells per well (Figure 3.3.10B).

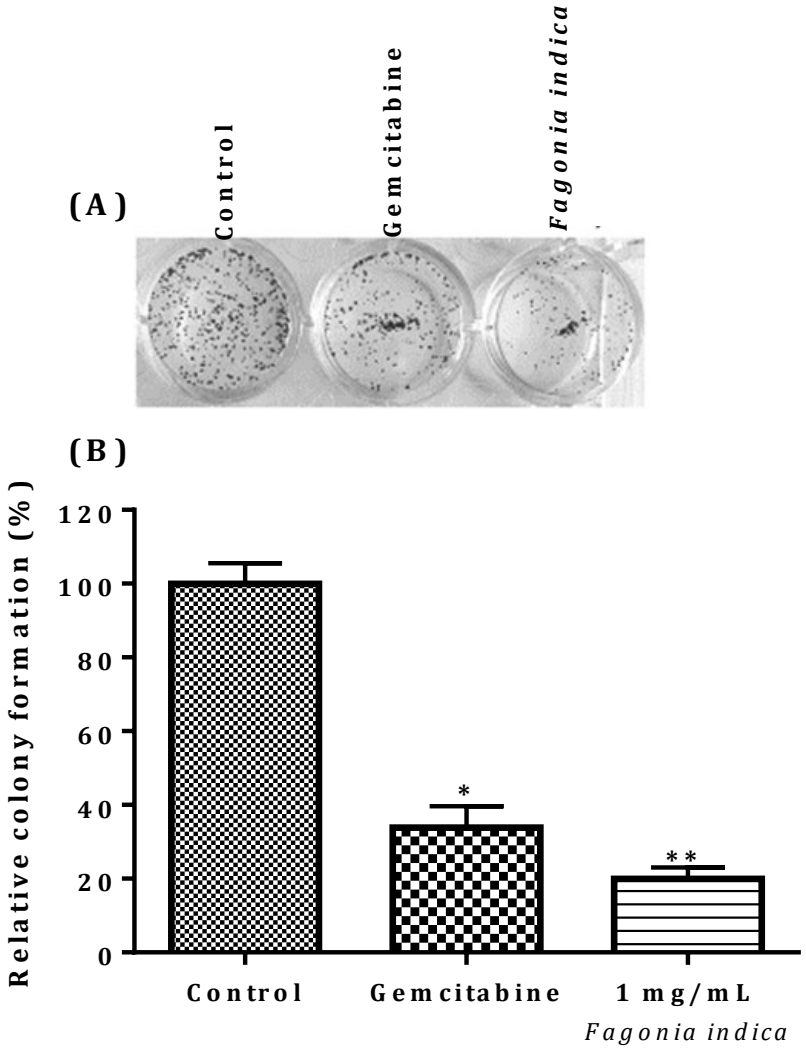

Figure 3.3.9: *Fagonia indica* treatment inhibits proliferation from a single cell of MDA-MB-231 breast cancer cell lines. MDA-MB-231 cells were pre-treated with 1.5 mg/mL aqueous Fagonia indica or 1 μM Gemcitabine for 24 hours and seeded at a density of 200 cells per well in a 24 well plate for 1 week. Colonies of 50> cells were stained using 0.01% crystal violet stain and counted using an EVOS microscope (A). (B) Colony formation was measured as a percentage of an untreated DMSO vehicle control. Data denoted * ($p<0.05$) and ** ($p<0.01$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. Data denoted as NS were not significant when compared to an untreated control. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

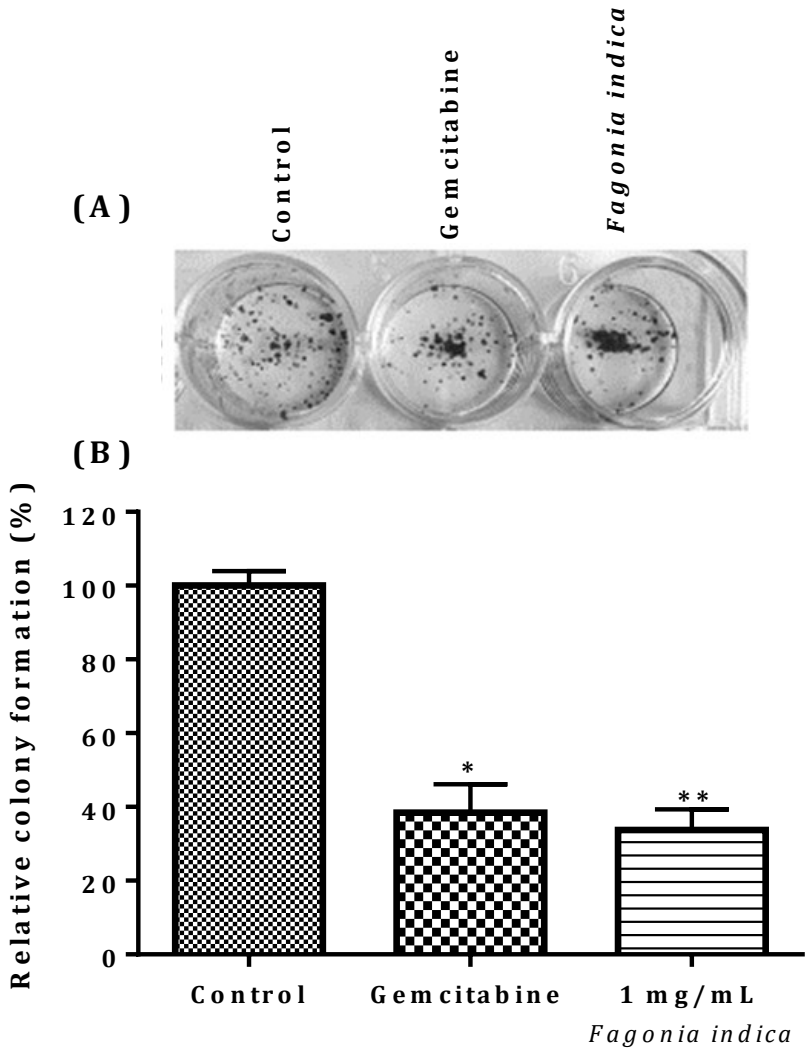

Figure 3.3.10: *Fagonia indica* treatment inhibits proliferation from a single cell of MCF-7 breast cancer cell lines. MCF-7 cells were pre-treated with 1.5 mg/mL aqueous *Fagonia indica* or 1 μM Gemcitabine for 24 hours and seeded at a density of 200 cells per well in a 24 well plate for 1 week. Colonies of 50> cells were stained using 0.01% crystal violet stain and counted using an EVOS microscope (A). (B) Colony formation was measured as a percentage of an untreated DMSO vehicle control. Data denoted * ($p<0.05$) and ** ($p<0.01$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

In addition, a scratch wound assay was used to determine whether *Fagonia indica* extract inhibited the migration of MDA-MB-231 and MCF-7 breast cancer cells. After 24 hours, wound healing of untreated MDA-MB-231 cells was 64.7%, which was significantly reduced in *Fagonia indica* extract treated wounds to 32.2% ($p<0.05$) (Figure 3.3.11). In MCF-7 cells, percentage wound healing was not significantly changed between the untreated control and *Fagonia indica* treated cell lines after 24 hours (Figure 3.3.12).

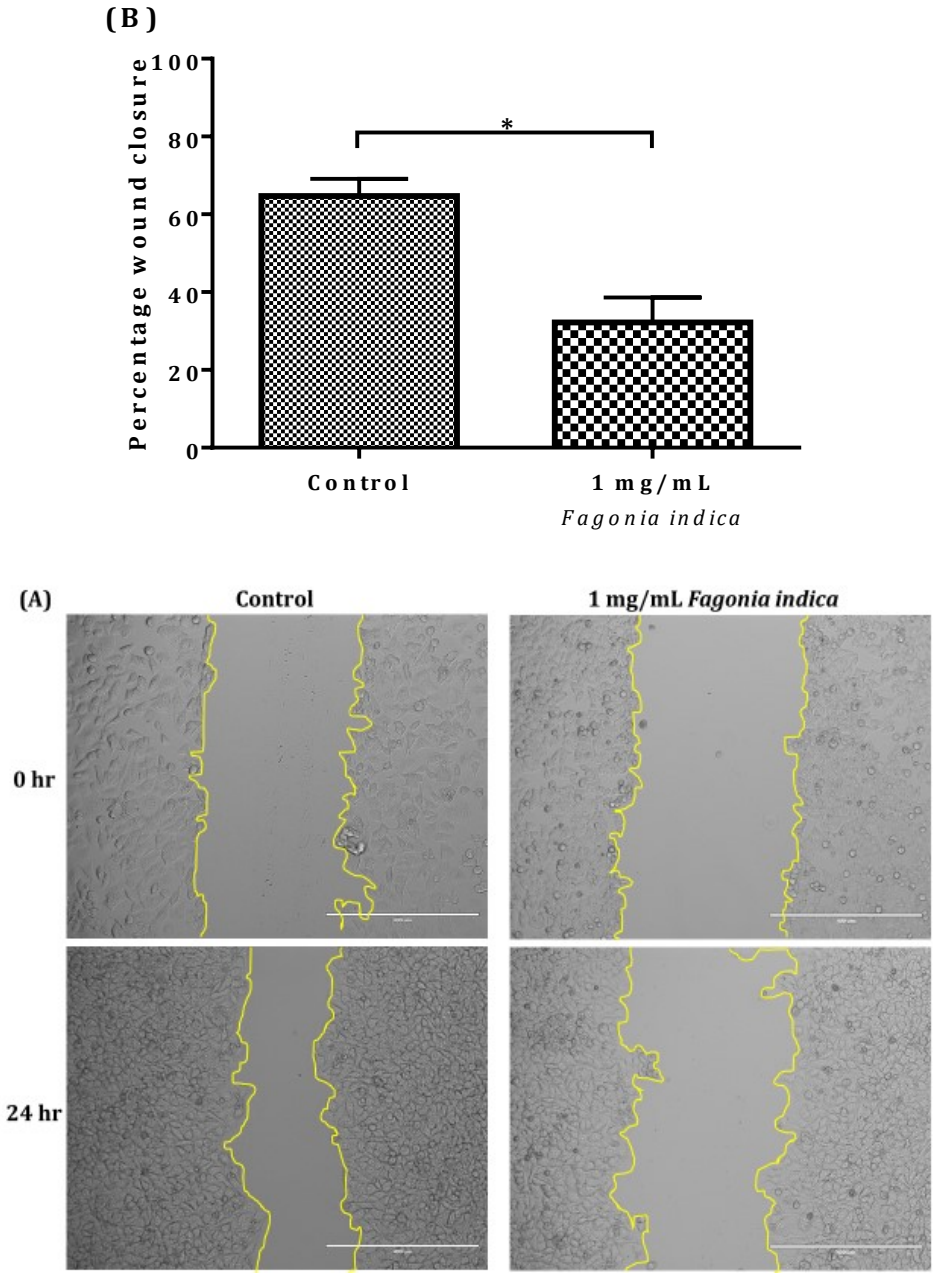

Figure 3.3.11: *Fagonia indica* treatment inhibits migration of MDA-MB-231 breast cancer cell lines. MDA-MB-231 cell monolayers were scratched with a 20 µL pipette tip and treated with 1 mg/mL of aqueous *Fagonia indica* for 24 hours. Wound closure was captured with an EVOS microscope at 0 hour and 24 hours timepoints at x10 magnification (A). (B) Wound closure was determined using ImageJ software and is displayed as percentage wound closure after 24 hours. Data denoted * ($p<0.05$) were significant compared to an untreated control analysed by unpaired students t test. All data is

representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

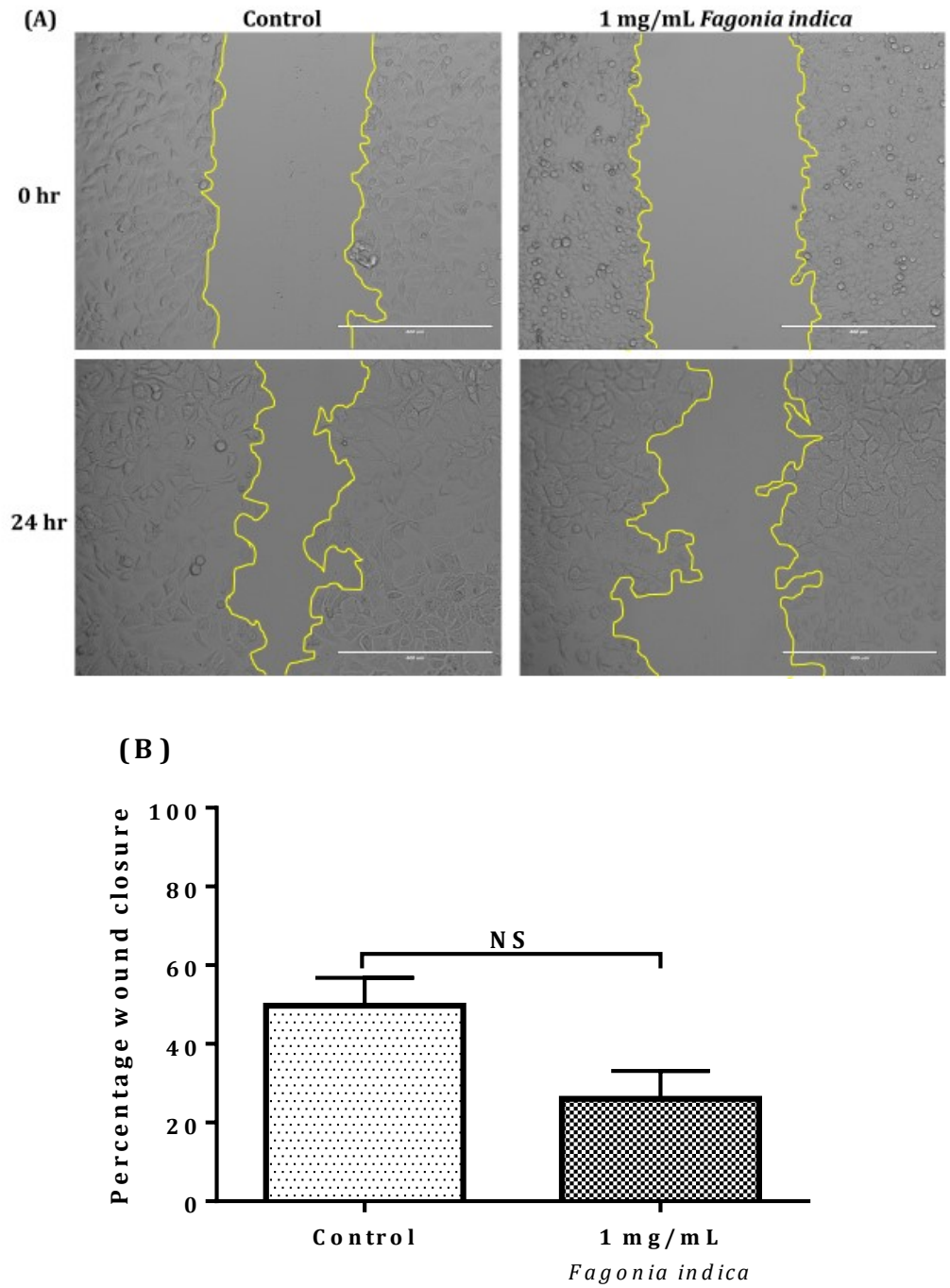

Figure 3.3.12: *Fagonia indica* treatment inhibits migration of MCF-7 breast cancer cell lines. MCF-7 cell monolayers were scratched with a 20 µL pipette tip and treated with 1 mg/mL of aqueous *Fagonia indica* for 24 hours. Wound closure was captured with an EVOS microscope at 0 hour and 24 hours timepoints at x10 magnification (A). (B) Wound closure was determined using ImageJ software and is displayed as percentage wound closure after 24 hours. Data denoted NS were not significant compared to an untreated control analysed by

unpaired students t test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

.

3.3.3 Mechanisms of action of *Fagonia indica* induced cytotoxicity in breast cancer

Vascular endothelial growth factor (VEGF), is an essential growth factor and a key player in the initiation of angiogenesis. Apatanib is a tyrosine kinase inhibitor which selectively inhibits VEGF receptor 2 (VEGFR2) (IC_{50} = 1 nM). Inhibition of VEGFR2 did not significantly reduce cell viability in untreated MDA-MB-231 cells (Figure 3.3.13A). Addition of Apatanib to *Fagonia indica* treated MDA-MB-231 cells also did not significantly reduce or increase cell viability, when compared to *Fagonia indica* treatment alone. In addition, mRNA expression of VEGFA in MDA-MB-231 cells was determined using qPCR (Figure 3.3.13B). In MDA-MB-231 cells expression of VEGFA was significantly reduced to 0.312-fold ($p<0.001$) of the control after treatment with 1 mg/mL *Fagonia indica* for 6 hours, at 24 hours this was reduced further to 0.198-fold ($p<0.001$). Inhibition of VEGFA in MCF-7 cells also had no significant effects on cell viability of both untreated or *Fagonia indica* treated cells (Figure 3.3.14A). There was a reduction in mRNA expression of VEGFA in MCF-7 cells to 0.213-fold ($p<0.05$) of the control, after 6 hours of treatment with *Fagonia indica* extract (Figure 3.3.14B). However, this effect was mitigated at 24 hours, where there were no significant changes in gene expression when compared to the untreated control.

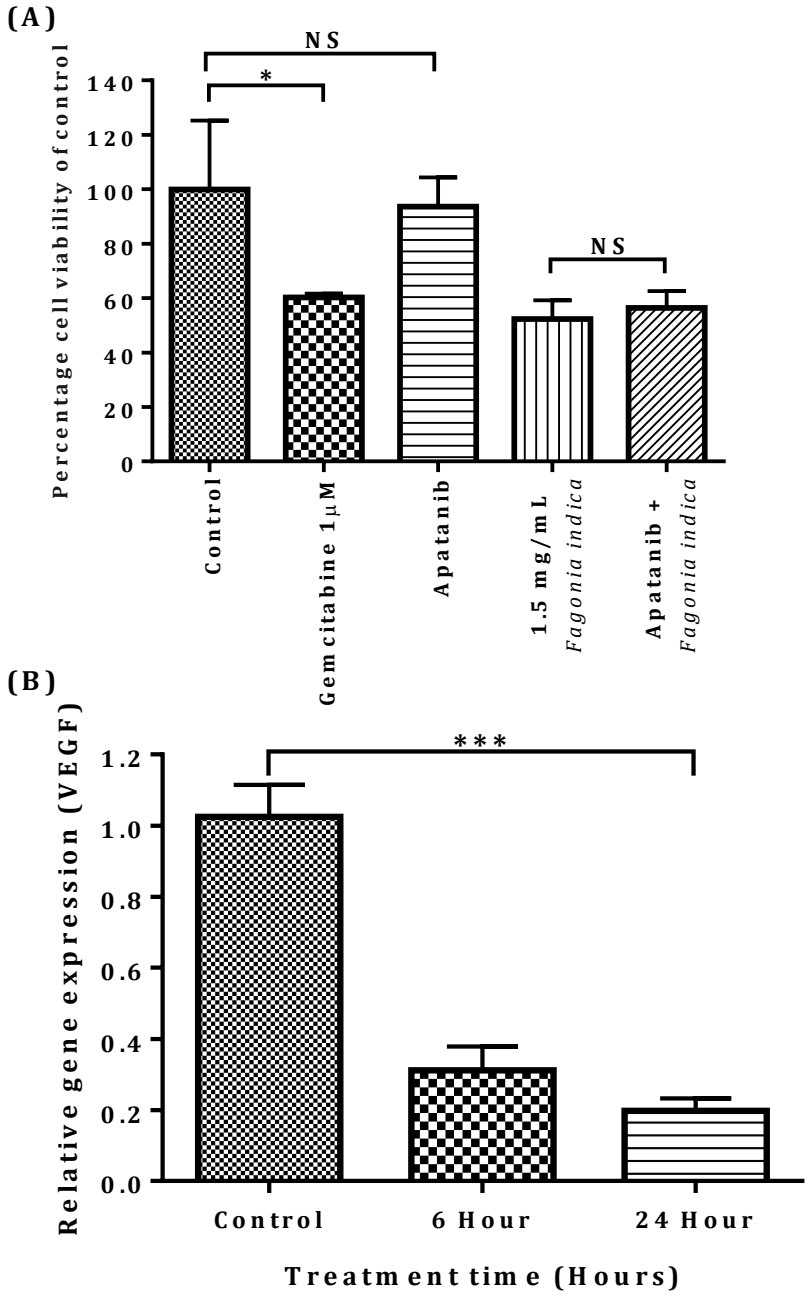

Figure 3.3.13: *Fagonia indica* extract treatment reduces expression of VEGF in MDA-MB-231 breast cancer cell line. (A) MDA-MB-231 cells were treated with 1.5 mg/mL aqueous *Fagonia indica* extract and/or 1 μM of VEGF inhibitor Apatanib. 1 μM of Gemcitabine was used as a positive control. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted * ($p<0.05$) were significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test (B) Gene expression of VEGF was determined on MDA-MB-231 breast cancer cell lines. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted *** ($p<0.001$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. Data denoted as NS were not significant when compared to an untreated control. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

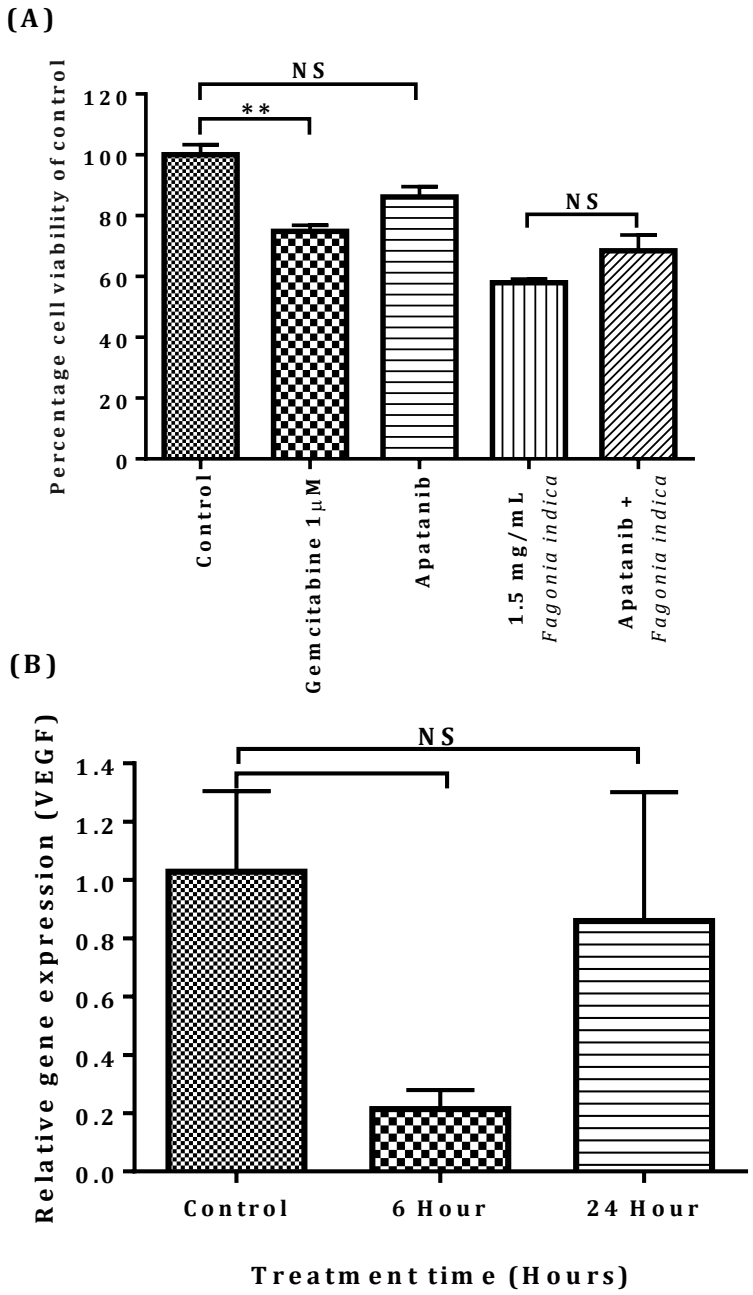

Figure 3.3.14: *Fagonia indica* extract treatment reduces expression of VEGF in MCF-7 breast cancer cell line. (A) MCF-7 cells were treated with 1.5 mg/mL aqueous *Fagonia indica* extract and/or 1 μM of VEGF inhibitor Apatanib. 1 μM of Gemcitabine was used as a positive control. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted * ($p<0.05$) were significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test (B) Gene expression of VEGF was determined on MCF-7 breast cancer cell lines. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted * ($p<0.01$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. Data denoted as NS were not significant when compared to an untreated control. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

The effect of VEGFR2 inhibition on *Fagonia indica* treated colony formation was also investigated. After treatment with apatanib and/or *Fagonia indica* extract for 24 hours, cells were seeded and colony formation was assessed after one week. Apatanib, *Fagonia indica* and combined treatment groups for both MDA-MB-231 and MCF-7 breast cancer cells formed significantly fewer colonies than the control group. In MDA-MB-231 cells, colony formation was significantly reduced by 46.4% ($p>0.001$) for cells treated with apatanib but only by 19.6% ($p>0.05$) in cells treated with a combination of apatanib and *Fagonia indica* extract (Figure 3.3.15A), compared to an untreated control. Overall, colony inhibition was greater in MDA-MB-231 cells treated with *Fagonia indica* alone ($p<0.0001$). In MCF-7 cells, colony formation was significantly reduced by 82.5% ($p<0.0001$) in cells treated with apatanib, but only 62.9% in ($p<0.0001$) those treated with both apatanib and *Fagonia indica* (Figure 3.3.15B). Overall, colony formation was not significantly different between single and combination treatments of apatanib and *Fagonia indica*.

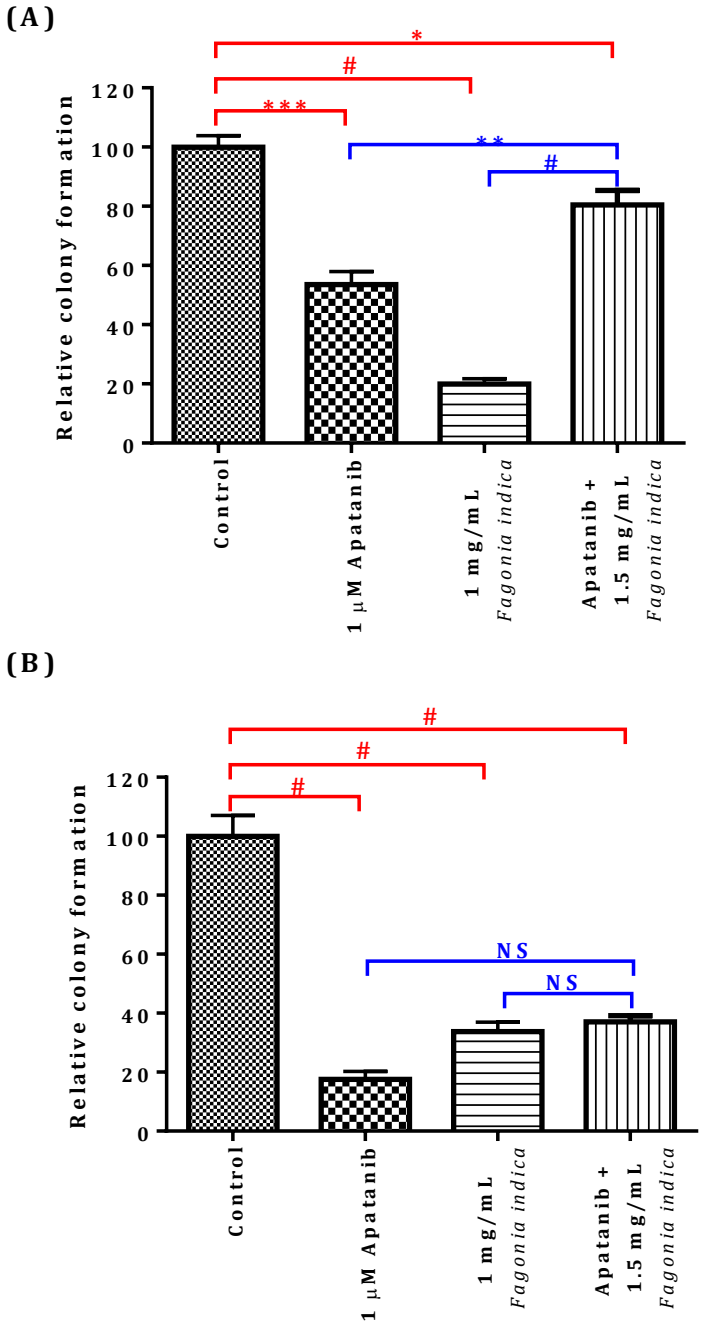

Figure 3.3.15: *Fagonia indica* and apatanib treatment inhibits proliferation from a single cell of MDA-MB-231 and MCF-7 breast cancer cell lines. (A) MDA-MB-231 cells and (B) MCF-7 cells were pre-treated with 1 µM VEGF inhibitor Apatanib, 1.5 mg/mL aqueous *Fagonia indica,* or a combination of the two for 24 hours and seeded at a density of 200 cells per well in a 24 well plate for 1 week. Colonies of 50> cells were stained using 0.01% crystal violet stain and colony formation was measured as a percentage of an untreated DMSO vehicle control. Data denoted * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and # ($p<0.0001$) were significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test. Data denoted as NS were not significant when compared to an untreated control. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

NF-κB is a major transcription factor which is able to upregulate genes related to immune function and inflammation. Caffeic acid phenethyl ester (CAPE) acts as a specific inhibitor of the nuclear transcription factor NF-κB. Inhibition of NF-κB did not significantly reduce cell viability in untreated or *Fagonia indica* treated MDA-MB-231 cells (Figure 3.3.16A). In addition, mRNA expression of NF-κB subunit p65 in MDA-MB-231 cells was determined using qPCR (Figure 3.3.16B). In MDA-MB-231 cells expression of p65 was significantly reduced to 0.147-fold ($p<0.01$) of the control after treatment with 1 mg/mL *Fagonia indica* for 6 hours and was reduced further to 0.055-fold ($p<0.001$) at 24 hours. In MCF-7 cells (Figure 3.3.17A), inhibition of NF-κB did not significantly reduce cell viability in untreated cells and had no effect on *Fagonia indica*-induced cytotoxicity. Treatment with *Fagonia indica* extract in MCF-7 cells also did not significantly impact expression of p65 (Figure 3.3.17B).

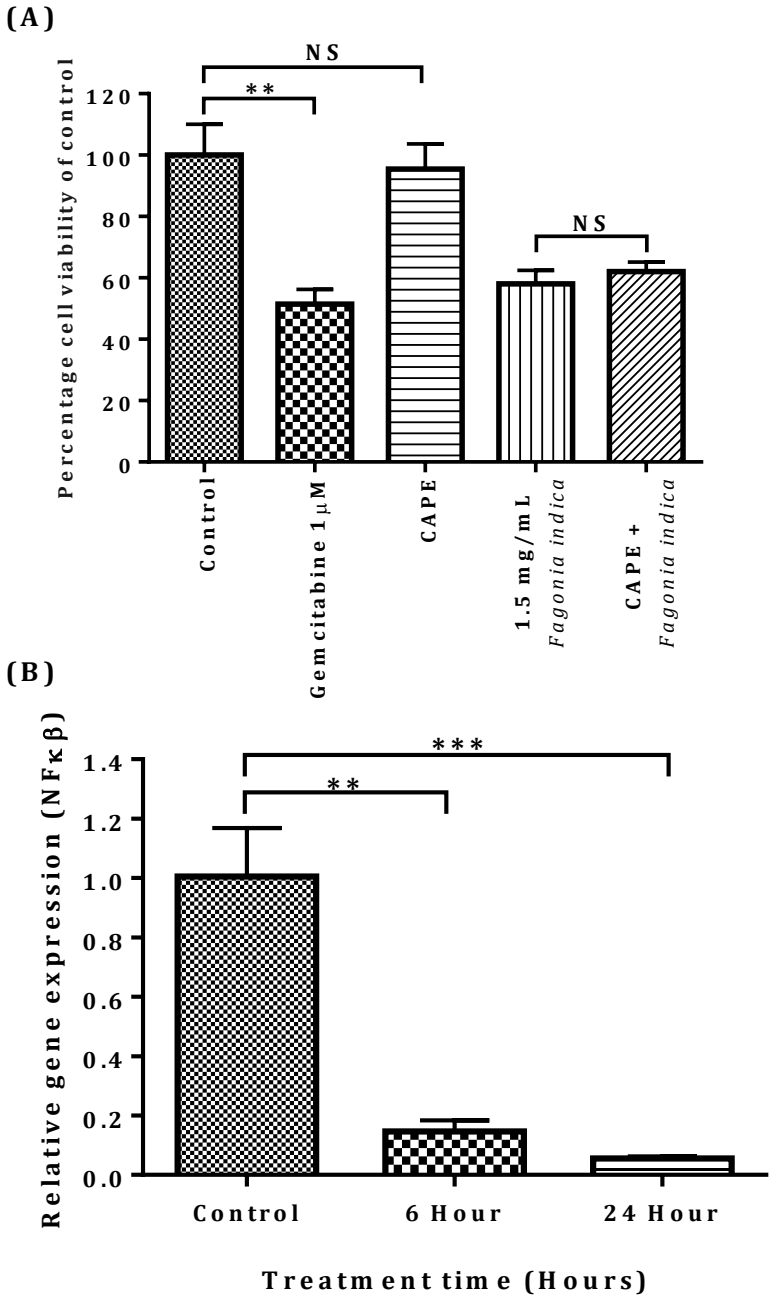

Figure 3.3.16: *Fagonia indica* extract treatment reduces expression of NF-κB in MDA-MB-231 breast cancer cell line. (A) MDA-MB-231 cells were treated with 1.5 mg/mL aqueous *Fagonia indica* extract and/or 1 μM of NF-κB inhibitor CAPE. 1 μM of Gemcitabine was used as a positive control. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted ** ($p<0.01$) were significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test (B) Gene expression of NF-κB was determined on MDA-MB-231 breast cancer cell lines. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted * ($p<0.01$) and ** ($p<0.001$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. Data denoted as NS were not

significant when compared to an untreated control. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

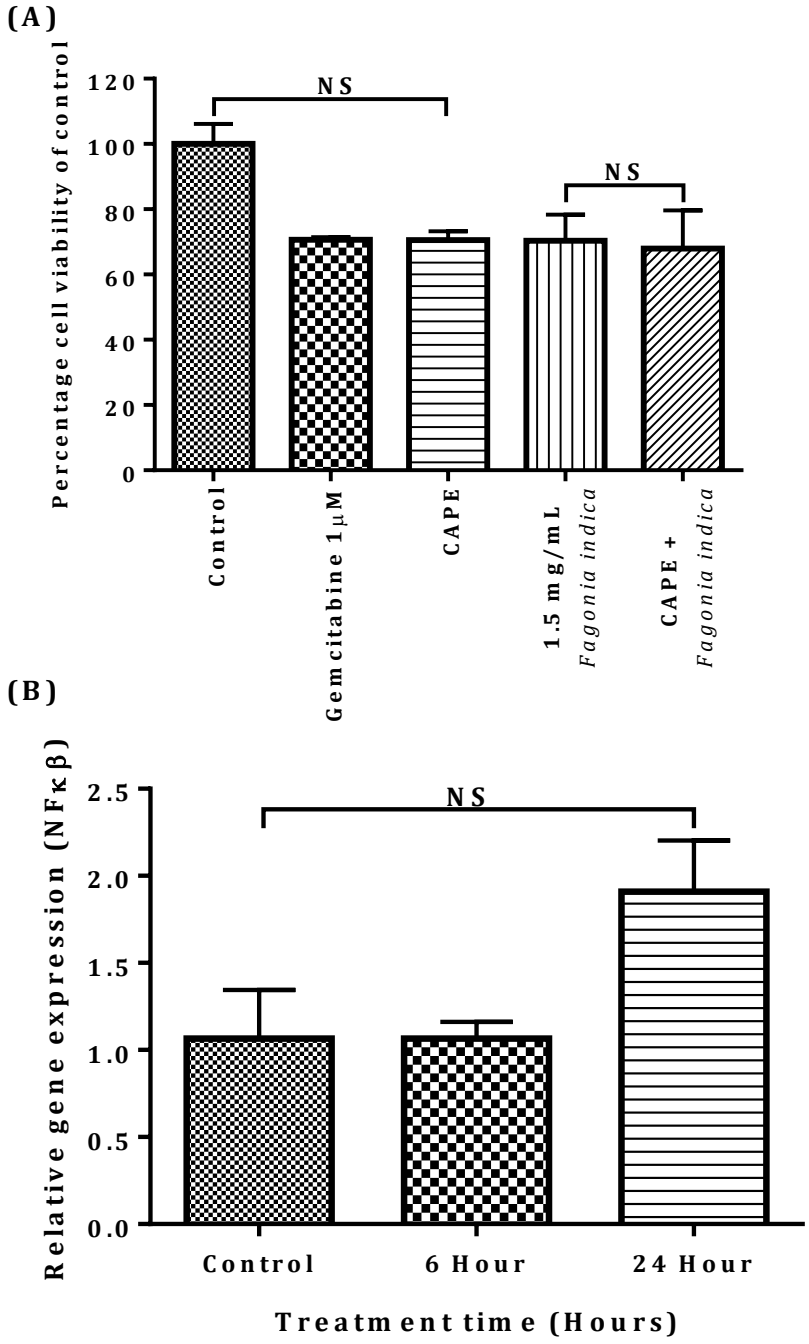

Figure 3.3.17: *Fagonia indica* extract treatment increases expression of NF-κB in MCF-7 breast cancer cell line. (A) MCF-7 cells were treated with 1.5 mg/mL aqueous *Fagonia indica* extract and/or 1 μM of NF-κB inhibitor CAPE. 1 μM of Gemcitabine was used as a positive control. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted as NS were not significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test (B) Gene expression of NF-κB was determined on MCF-7 breast cancer cell lines. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data

denoted as NS were not significant when compared to an untreated control using Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

Intracellular adhesion molecule-1 (ICAM1), is a cell surface glycoprotein and an adhesion molecule, with critical roles in inflammation and immune function. After treatment with *Fagonia indica* extract expression of ICAM-1 mRNA in MDA-MB-231 breast cancer cells was significantly reduced to 0.06-fold ($p<0.01$) of the control after 6 hours and this reduction was maintained at 24 hours (Figure 3.3.18A). Expression of ICAM-1 was similarly reduced in MCF-7 cells to 0.07- ($p<0.01$) and 0.24-fold ($p<0.05$) of the control after 6 and 24 hours of treatment with *Fagonia indica*, respectively (Figure 3.3.18B).

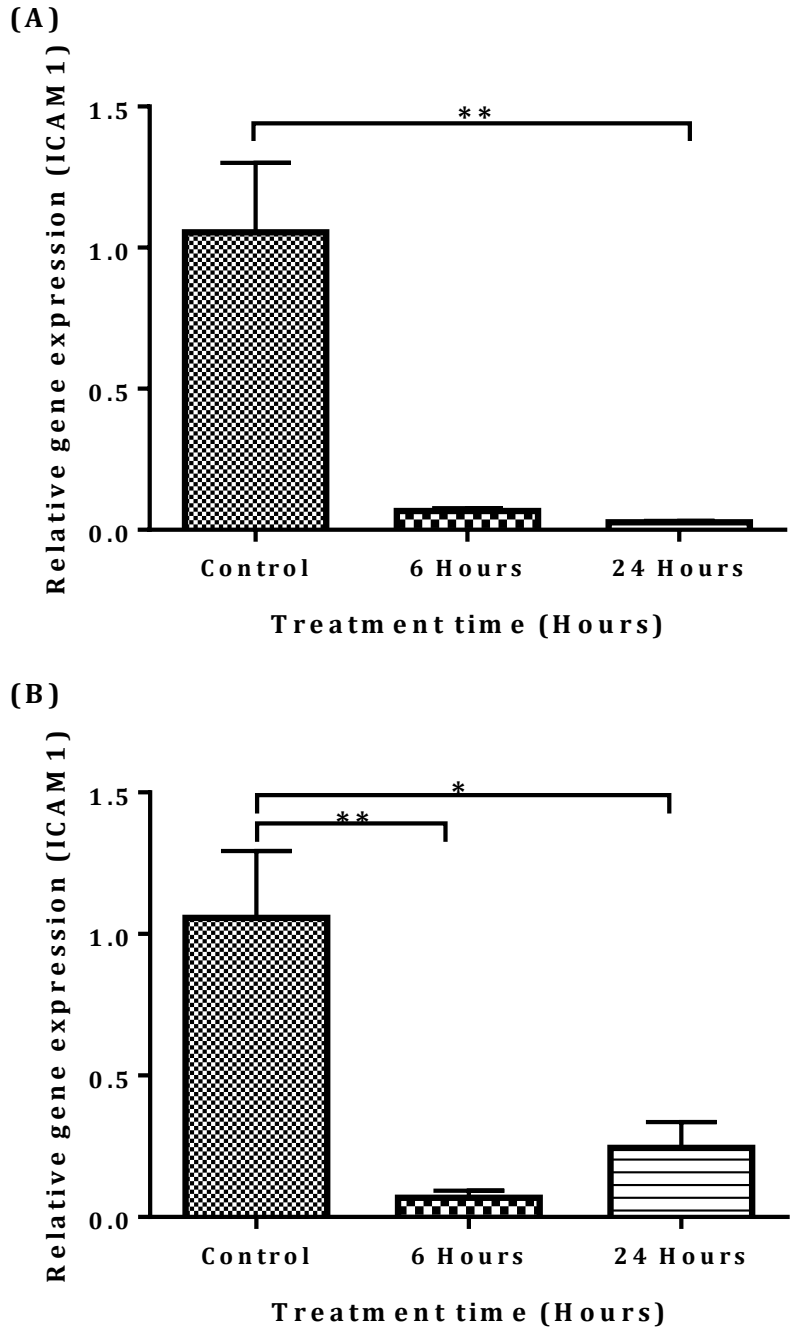

Figure 3.3.18: *Fagonia indica* extract treatment reduces expression of ICAM-1 in MDA-MB-231 and MCF-7 breast cancer cell lines. Gene expression of ICAM-1 was determined on (A) MDA-MB-231 and (B) MCF-7 breast cancer cell lines. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted * ($p<0.01$) and ** ($p<0.001$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

3.3.4 Effects of an aqueous *Fagonia indica* extract on chemotherapy-resistant MDA-MB-231 cells

Resistance to single chemotherapeutic agents occurs almost universally and drug tolerance in cancer is widely documented. It was therefore important to establish the effect of *Fagonia indica* on drug resistant breast cancer cell lines. Chemotherapy-resistant cell lines are developed by repeatedly exposing cells growing in culture medium to the relevant drug in increasing increments, the difference in response to the relevant chemotherapeutic agents between wild-type and drug resistant MDA-MB-231 cells can be found in Appendix Figure 7.1. An aqueous extract of *Fagonia indica* was prepared and used to determine the ability of a whole extract treatment to reduce breast cancer viability in chemotherapy-resistant cell lines. Using a MTT assay, treatment with *Fagonia indica* reduced cell viability in a time and dose dependent manner in gemcitabine resistant MDA-MB-231 cells (Figure 3.3.19). After 24 hours of treatment with 2.5 mg/mL *Fagonia indica*, cell viability was significantly reduced by 54.5% ($p<0.01$), which increased to 69.2% ($p<0.01$) after 72 hours.

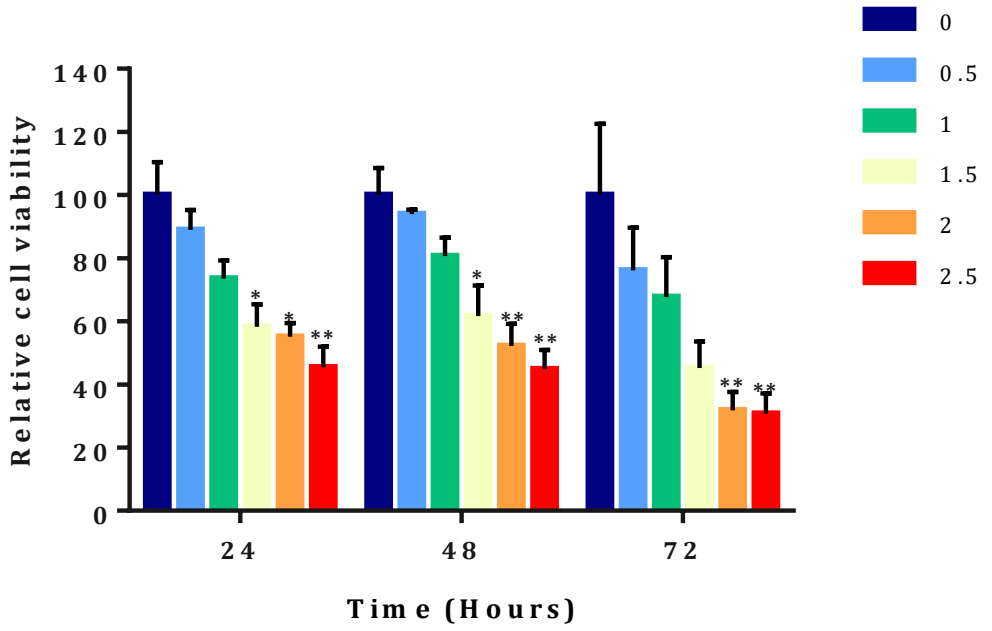

Figure 3.3.19: ***Fagonia indica*** **extract treatment reduces** ***in vitro*** **cell viability of gemcitabine resistant MDA-MB-231 breast cancer cell lines.** (A) Gemcitabine resistant MDA-MB-231 cells were treated with 0-2.5 mg/mL aqueous *Fagonia indica* extract for 24-72 hours. Cell viability was determined as a percentage of an untreated DMSO vehicle control using MTT assay. Data denoted * ($p<0.05$) and ** ($p<0.01$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

To determine whether *Fagonia indica* treatment is able to re-sensitise resistant cell lines to chemotherapy, gemcitabine resistant MDA-MB-231 cells were treated with 1 mg/mL of aqueous *Fagonia indica* extract for 24 hours, prior to treatment with the relevant chemotherapy for a further 48 hours. In gemcitabine-resistant MDA-MB-231 cells (Figure 3.3.20), there was no significant difference in cell viability of gemcitabine treated cell lines with or without the *Fagonia indica* pre-treatment.

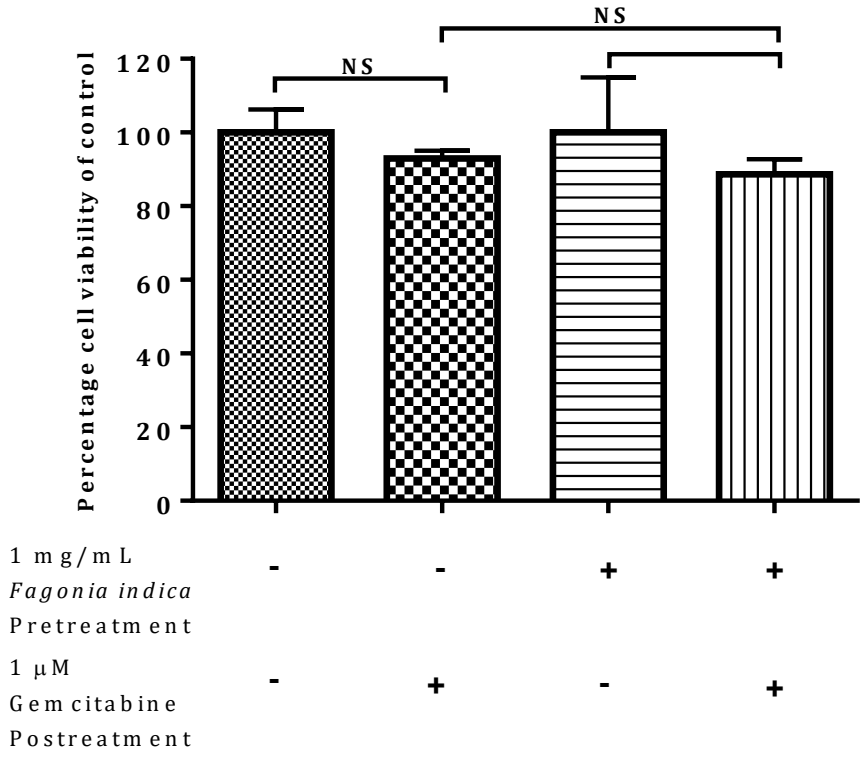

Figure 3.3.20: The effect of *Fagonia indica* extract pre-treatment on chemotherapy induced cytoxicity in resistant MDA-MB-231 breast cancer cell lines. Gemcitabine resistant MDA-MB-231 cells were pre-treated with 1 mg/mL aqueous *Fagonia indica* extract for 24 hours before treatment with Gemcitabine or Paclitaxel for 48 hours. Cell viability was determined as a percentage of relevant DMSO/ *Fagonia indica* vehicle control using MTT assay. Data denoted NS were not significant compared to the relevant control analysed by one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

The ATP-binding cassette (ABC) transporters, are a superfamily of membrane transporter proteins which are able to actively transport a wide-array of structurally diverse substrates out of the cell and are largely overexpressed in resistant cancer cell lines. In drug-resistant MDA-MB-231 cells, expression of ABC transporters ABCC4 and ABCG2, was determined using qPCR. Expression of ABCC4 was 4.9-fold ($p<0.05$) higher in gemcitabine resistant MDA-MB-231 cells compared to the wild-type cell line (Figure 3.3.21A). After treatment with *Fagonia indica* extract, expression of ABCC4 was significantly reduced in gemcitabine resistant MDA-MB-231 breast cancer cell lines by 0.21- ($p<0.05$) and 0.11-fold ($p<0.05$) of the control, after 6 and 24 hours respectively (Figure 3.3.21B). Additionally, expression of ABCG2 was significantly increased by 26.4-fold ($p<0.05$) in gemcitabine resistant MDA-MB-231 cells, compared to wild-type cell lines (Figure 3.3.22A). However, there were no significant changes in expression of ABCG2 in gemcitabine resistant cell lines after treatment with *Fagonia indica* between 6 and 24 hours (Figure 3.3.22B).

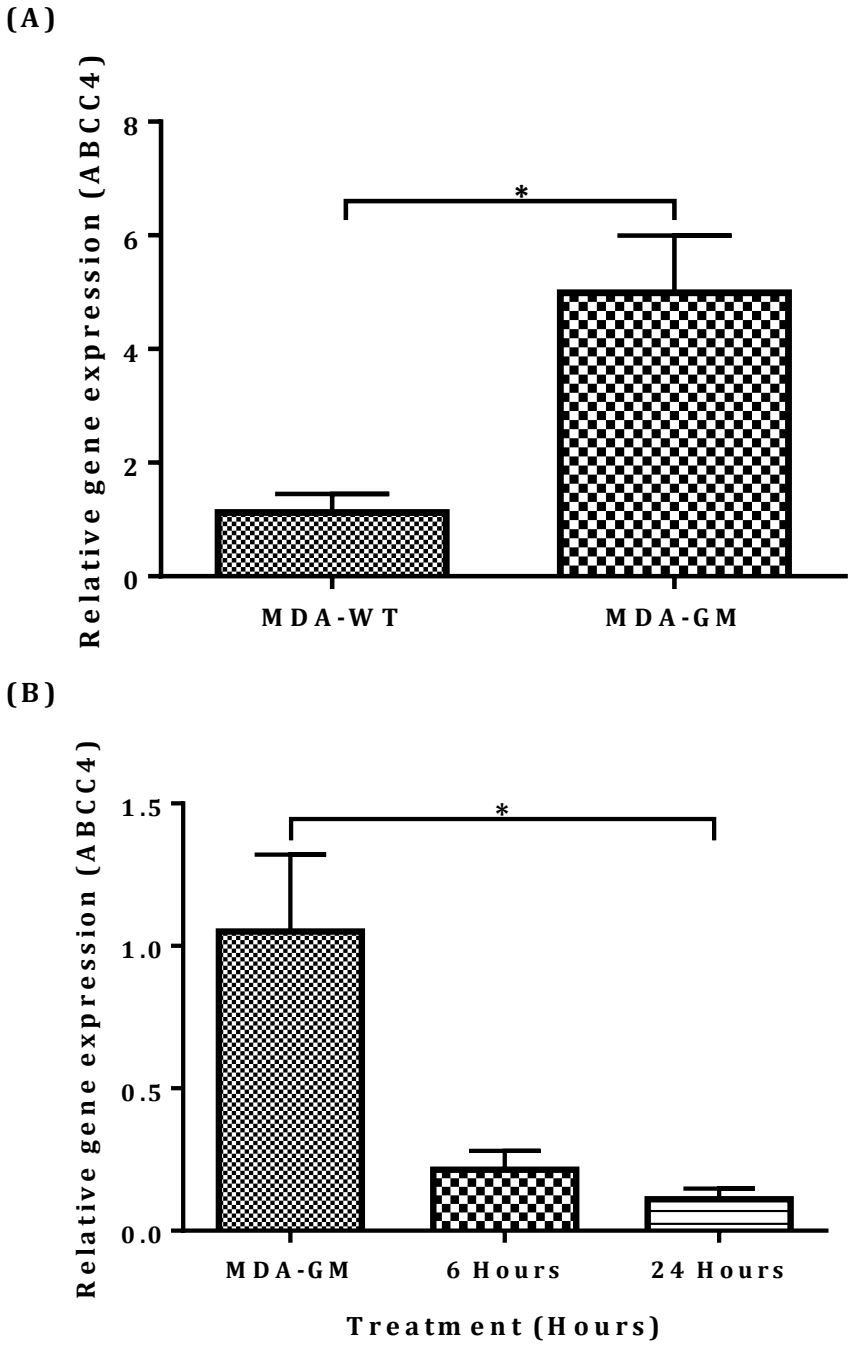

Figure 3.3.21: *Fagonia indica* extract treatment reduces expression of ABCC4 in Gemcitabine resistant MDA-MB-231 cells. Gene expression of ABCC4 was determined on (A) MDA-MB-231 wild-type (MDA-WT) vs Gemcitabine resistant cells (MDA-GM) and (B) *Fagonia indica* treated Gemcitabine-resistant MDA-MB-231 cells. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted * ($p<0.01$) were significant compared to the untreated control analysed by (A) unpaired student t-test and (B) one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

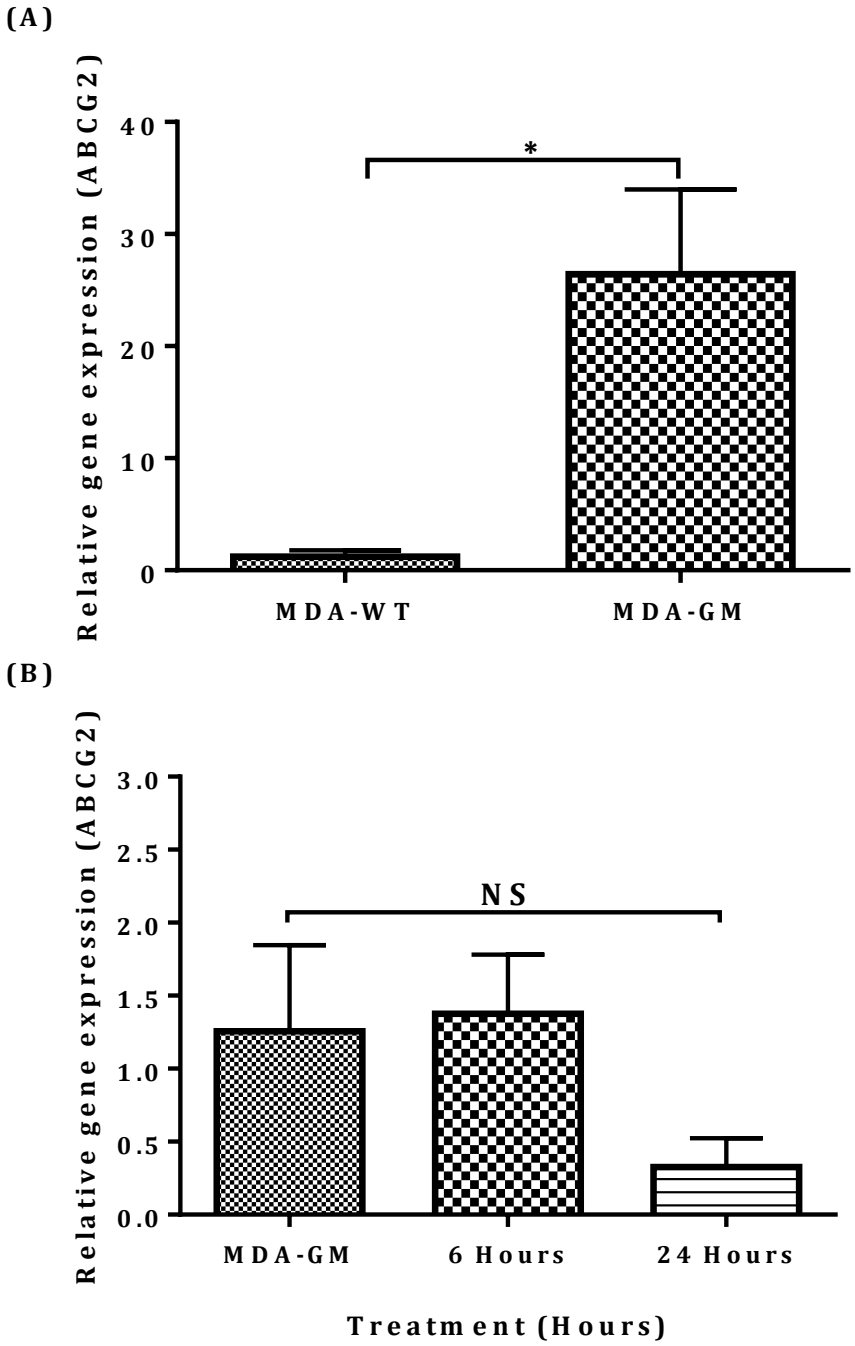

Figure 3.3.22: *Fagonia indica* extract treatment reduces expression of ABCG2 in Gemcitabine resistant MDA-MB-231 cells. Gene expression of ABCG2 was determined on (A) MDA-MB-231 wild-type (MDA-WT) vs Gemcitabine resistant cells (MDA-GM) and (B) *Fagonia indica* treated Gemcitabine-resistant MDA-MB-231 cells. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted * ($p<0.01$) were significant compared to the untreated control analysed by (A) unpaired student t-test and (B) one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM

MK-571 is an inhibitor of several multidrug resistant proteins; including ABCC1 and is used widely to demonstrate the role of ABC transporters in the efflux of drugs. Addition of MK571 to the culture medium did not significantly affect the cell viability of untreated gemcitabine resistant MDA-MB-231 cells (Figure 3.3.24). There were also no significant changes in cell viability between *Fagonia indica* treated cells and those treated with a combination of *Fagonia indica* and the MK571 inhibitor.

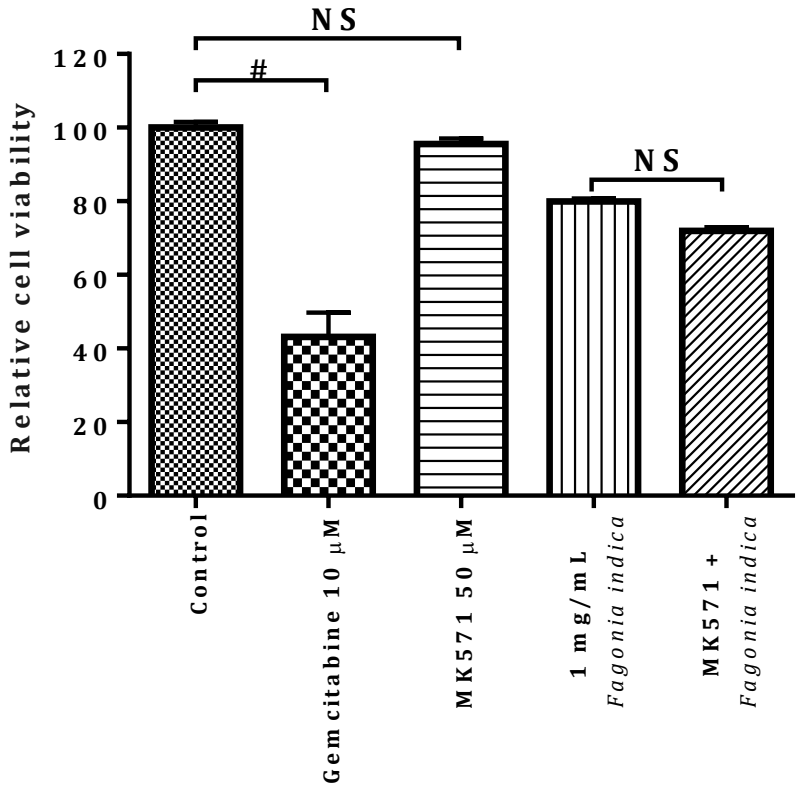

Figure 3.3.24: Effects of ABCC inhibition on extract induced MDA-MB-231 cellular cytotoxicity. MDA-MB-231 gemcitabine resistant cell lines were treated with 1 mg/mL of aqueous extract and/or 50 μM of MK571 for 48 hours. 10 μM Gemcitabine was used as a positive control. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted as # ($p<0.001$), was statistically significant compared to the untreated DMSO vehicle control, analysed using one-way ANOVA with Sidaks's multiple comparison test. Data denoted NS were not significant compared to the relevant control. All data points are representative of three independent experiments, performed in triplicate and are expressed as mean ± SEM.

Calcein-AM is a hydrophobic compound which diffuses across the cell membrane where it is subsequently hydrolysed by intracellular esterase's into a hydrophilic, highly fluorescent

compound which is retained within the cell. In the present study, Calcein-AM treatment was used to determine the differences in efflux of ABCC transporters between wild-type and gemcitabine resistant MDA-MB-231 breast cancer cells (Figure 3.3.25A). There were no significant changes in intracellular Calcien fluorescence between wild type and gemcitabine resistant cell lines after 30 minutes of Calcein-AM treatment. Secondly, to determine the effect of *Fagonia indica* treatment on efflux of ABCC transporters, wild-type and gemcitabine resistant MDA-MB-231 cell lines were treated with a combination of Calcein-AM and *Fagonia indica* extract for up to 30 minutes (Figure 3.3.25). In gemcitabine resistant MDA-MB-231 cell lines, Calcein fluorescence was significantly reduced in *Fagonia indica* treated cell lines between 28-30 minutes of efflux activity ($p<0.05$) (Figure 3.3.25B). This effect was not seen in the wild-type MDA-MB-231 cell lines, whereby there were no significant changes in Calcein fluorescence between *Fagonia indica* treated cells and the untreated control between 0-30 minutes (Figure 3.3.25C).

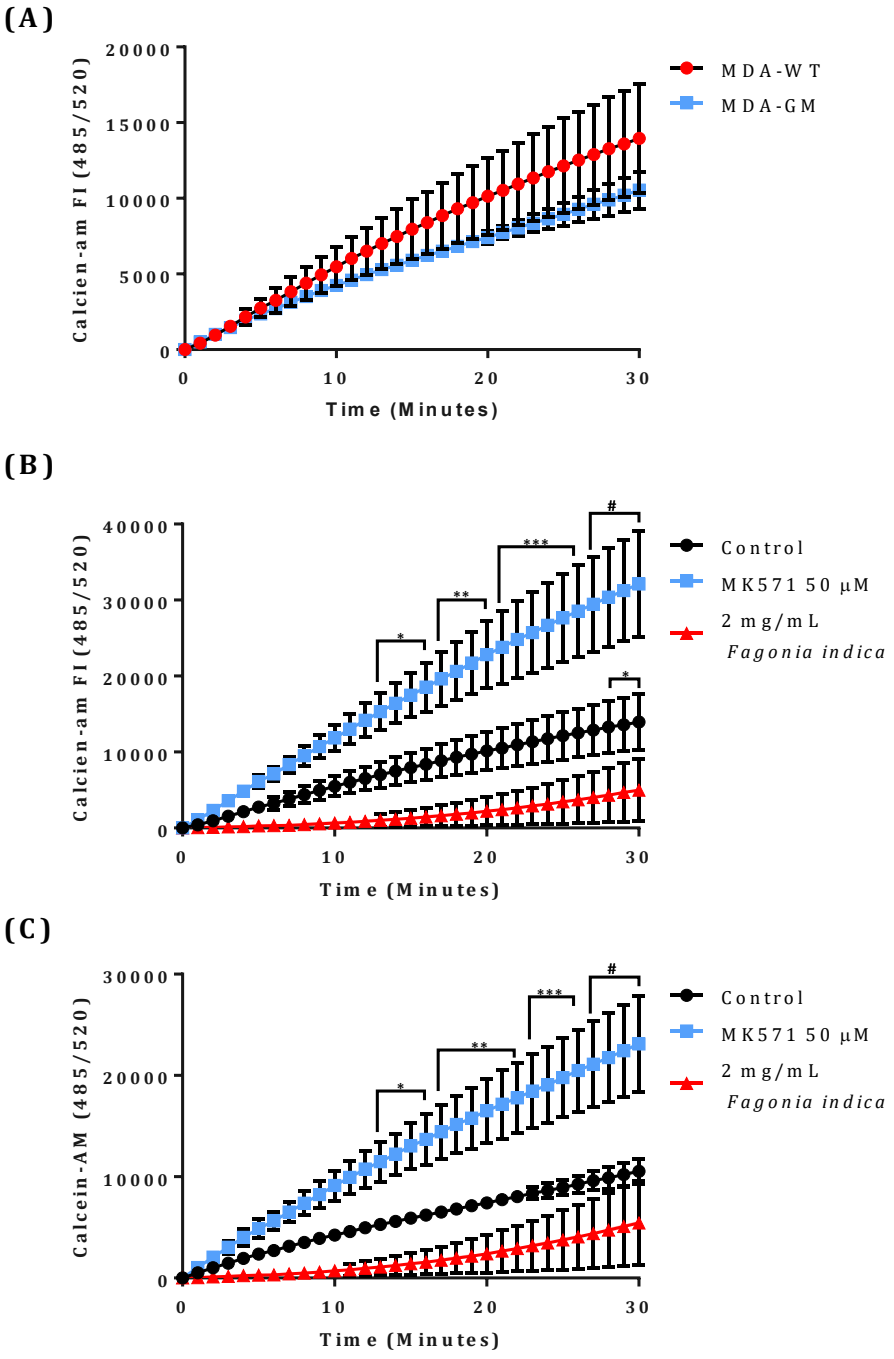

Figure 3.3.25: Fluorometric measurement of ABCC1/B1 transporter protein activity in MDA-MB-231 wt and gemcitabine resistant breast cancer cell lines. Mean cleaved calcein fluorescence was detected at 1-minute intervals for 30 minutes after treatment with 5 mM Calcein-AM in (A) wild-type vs gemcitabine resistant MDA-MB-231 cells, (B) gemcitabine resistant MDA-MB-231 treated with 2 mg/mL *Fagonia indica* or untreated control and (C) wild type MDA-MB-231 cells treated with 2 mg/mL *Fagonia indica* or untreated control. 50 µM MK571 inhibitor was used as a positive control. Data denoted as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and # ($p<0.0001$), was statistically significant compared to the untreated control, analysed using two-way ANOVA with Tukey's multiple comparison test. All data points are representative of two independent experiments, performed in triplicate and are expressed as mean ± SD.

3.4 Discussion

Fagonia indica is a medically significant plant, used as a traditional medicine for a myriad of disease states (Rahman *et al*., 1984). In typical indigenous cultures, an aqueous remedy of *Fagonia indica* is used as a herbal therapy for early-stage breast cancers (Waheed *et al*., 2012). In the present study an aqueous extract of *Fagonia indica* caused a time- and dose-dependent decrease in cell viability in two phenotypically diverse breast cancer cell lines. There was a maximum reduction in cell viability at 72 hrs of 48% in MCF-7 cells and 41% in MDA-MB-231 cells. Lam *et al* (2012), previously demonstrated the cytotoxic potential of an aqueous extract of *Fagonia indica* against p53 positive and negative breast cancer cell lines. They showed that treatment with 2 mg/mL of aqueous *Fagonia indica* caused a greater reduction in both MCF-7 and MDA-MB-231 cells at 75% and 67%, respectively. The increased sensitivity to *Fagonia indica* in MFC-7 cells in this study was attributed to p53 status and p53 associated apoptosis. In the present study, although sensitivity to *Fagonia indica* was similarly higher for MCF-7 cells, the overall sensitivity was greatly reduced. Concentration of 'bioactive' compounds in isolated plant extracts is likely to differ, partly due to culturing conditions in the plant whereby increased melatonin, light quality and access to white light has been identified as a prominent factor in the biosynthesis of phytochemicals and secondary metabolites in *Fagonia indica* (Khan *et al*., 2019).

Interestingly, cytotoxicity of *Fagonia indica* was increased for MDA-MB-231 cells when measured using the NR assay. It has been previously reported that using different methods of measuring cytotoxicity can yield different results due to the chemical and cytotoxic processes employed in the assays (Bacanli *et al.*, 2017). The NR assay is based upon the ability of viable cells to maintain pH gradients via the production of ATP, the dye carries a net charge of zero as it crosses the cell membrane, becoming charged in the lysosome where it concentrates (Repetto *et al.*, 2008). In contrast, the MTT assay uses a tetrazolium salt which is converted into a blue formazan product by dehydrogenases in the electron transport chain (Mosmann, 1983). The NR and MTT assays have proven to be the most sensitive and accurate assays in

detecting cytotoxic events (Fotakis & Timbrell, 2006); however, studies have indicated that major differences in cytotoxicity values are evident between assays (Weyermann, Lochmann, & Zimmer, 2005). It is therefore, important to note that the value of cytotoxicity as demonstrated in the above assays may differ slightly.

Another key indication of the cytotoxic potential of an aqueous extract of *Fagonia indica*, is demonstrated by the loss in intracellular ATP concentrations. Intracellular ATP concentration has been used as an indicator of the physiological state of human cells, as cells with deficiency in ATP lack the ability to perform vital biological activities (Bajerski *et al*., 2018). Metabolic plasticity is an emerging feature of cancer and unlike healthy cells, they can up-regulate glucose transporters and use aerobic glycolysis to rapidly upregulate ATP production to facilitate tumorigenesis (Jia *et al*., 2019). In the current study, *Fagonia indica* extract was able to significantly reduce intracellular ATP in both MCF-7 and MDA-MB-231 cell lines, by over 50%. Although this is the first time that *Fagonia indica* treatment has been linked to metabolic dysregulation of breast cancer cells, studies examining other plant species have demonstrated an ability of a dry plant extract to diminish intracellular ATP levels, in correlation with a decline in cellular proliferation in both 2D and 3D breast cancer cell cultures (Hernández *et al*., 2017).

The caspase family was identified as a potential target of *Fagonia indica* induced cytotoxicity, due to the identification of p53 and FOXO3a activation in *Fagonia indica* induced cell death (Lam *et al*., 2012). Both p53 and FOXO3a activation have seen linked with subsequent activation of caspases and the induction of apoptosis. P53 is critically involved in cell cycle progression and DNA repair mechanisms, it has also been shown to activate caspase-8, 9 and 3 leading to programmed cell death (Schuler *et al*., 2000). Nuclear FOXO3a translocation via Akt activation, has also been identified as an activator of caspase-8 and apoptosis (Skurk *et al*., 2004). In the current study, mRNA expression of caspase-8 was determined in both p53 positive MCF-7 and p53 negative MDA-MB-231 breast cancer cells. After 6 hours of treatment with a low-dose concentration of *Fagonia indica*, expression of caspase-8 had

increased in both cell lines, with a 2-fold upregulation in MCF-7 cells. Studies of head and neck cancers found that p53 acts as an upstream regulator of caspase-8 and drug induced apoptosis (J. Liu *et al.*, 2011). Caspase inhibitor Z-VAD-FMK did not significantly affect *Fagonia indica* induced cell viability in either cell line, suggesting a diminished role of caspases in extract induced cell death. However, it is worth noting that pan-caspase inhibitors have been the subject of some criticism due to a lack of binding specificity and occurrence of non-specific effects (Martinet *et al*., 2006). The current data indicates a role of caspase-8 up-regulation and the death receptor pathway as a potential mechanism of *Fagonia indica* induced cytotoxicity.

Akt is a major protein kinase with a central function in signalling pathways related to proliferation and cell survival, as such it is often over-activated in various cancers. In contrast, p38 and other MAPK kinases are activated by environmental stresses and lead to the induction of apoptosis and senescence (Sheng *et al*., 2020). As stress activated p38 is common upon treatment with cytotoxic agents, selective inhibitor SB203580 was used in the current study to assess the importance of p38-MAPK in *Fagonia indica* induced apoptosis (Deschesnes *et al.*, 2001). Inhibition of p38-MAPK did not significantly alter extract-induced apoptosis in MCF-7 breast cancer cells and actually increased cell death in MDA-MB-231 cells. Chen *et al* (2012), found that treatment of MCF-7 cells with Cryptoanshinone; a naturally derived compound from *Salvia miltiorrhiza*, led to p38 activation and apoptosis in absence of caspase induction. In the present study caspase induction occurred independently of p38 activation. As MAPK pathways are linked with Akt; a negative regulator of apoptosis, activation of Akt with SC79 also displayed negligible effects on both cell lines (Weir *et al*., 2007).

In vitro colony formation assays are a quantitative technique used to assess the ability of a single cell to develop into a large colony via clonal expansion (Rajendran & Jain, 2018). In the present study, treatment with *Fagonia indica* extract caused a significant reduction in colony formation in both MCF-7 and MDA-MB-231 breast cancer cell lines. Lam *et al* (2012), found that treatment with *Fagonia indica* induces cell cycle arrest in MCF-7 cells at G1-phase,

inhibiting proliferation and initiating apoptotic mechanisms. Treatment with *Fagonia indica* also significantly reduced wound healing in both breast cancer cell lines as demonstrated by a scratch wound assay, indicating the ability of the extract to inhibit cell migration and invasion. Mishra & Kaur (2013) found that an ethanolic extract of *Tinospora corfiolia* showed anti-migratory and anti-invasive potential via a reduction in wound healing, in relation to reduced expression of plasticity markers such as NCAM , PSA-NCAM and ICAM-1 adhesion molecules. ICAM-1 is a transmembrane glycoprotein expressed on the surface of multiple cell types and its upregulation has been associated with increased cancer metastases (Cheng & Liang, 2015). Strell *et al* (2010), found that up-regulation of ICAM-1 in MDA-MB-468 breast cancer cells coincided with migratory activity and cell-cell interactions with neutrophils. This cell interaction resulted in an ICAM-1 clustering-dependent migration of the tumour cells. In the present study, ICAM-1 mRNA expression was significantly reduced in both MCF-7 and MDA-MB-231 breast cancer cell lines after 6 and 24hr treatment with *Fagonia indica* extract. This reduced expression of ICAM-1 is important in further understanding the anti-migratory mechanisms of *Fagonia indica*.

Vascular endothelial growth factor (VEGF) is a major hallmark of cancer, largely in relation to its ability to drive vascular growth and angiogenesis (Sia *et al*., 2014). Further research has also indicated that VEGF signalling promotes cell proliferation in cancer via signalling pathways and indirect activation of target proteins such as ERK (Lin *et al*., 2017). In the present study, treatment with *Fagonia indica* caused a reduction in mRNA expression of VEGF-A in both MDA-MB-231 and MCF-7 cells after 6 hours. Although this is the first time that VEGF has been identified as a target of *Fagonia indica* treatment, Sartippour *et al* (2002) described the ability of a green tea extract to inhibit secretion of VEGF and to reduce expression at the transcriptional level in MDA-MB-231 breast cancer cells. The current study also demonstrates a functional role of VEGF signalling in *Fagonia indica*, inhibition of VEGF caused an increase in colony formation when compared to *Fagonia indica* treatment alone. Wen *et al* (2008), used a whole grape seed extract to inhibit VEGF signalling in endothelial

cells and MDA-MB-231 injected mouse models. The extract was shown to directly inhibit VEGFR2 kinase activity, however in the current study it is unknown whether VEGF activity is directly inhibited at the receptor by *Fagonia indica*, or if expression of VEGF is an indirect effect of treatment.

Nuclear factor – κB (NF-κB) represents a family of inducible transcription factors, which together regulate a vast array of genes involved in processes from proliferation and apoptosis to immune and inflammatory responses (T. Liu *et al.*, 2017). There are currently more than 100 known targets of NF-κB, including adhesion molecule ICAM-1 and angiogenic factor VEGF (Smith, Lyu, & Cai, 2014). A study on mRNA expression levels of NF-κB target genes in invasive breast cancer found a direct link between the transcription factor and up-regulation of tumour-promoting genes including ICAM-1 (Lerebours *et al*., 2008). As mRNA levels of ICAM-1 in the current study were greatly reduced by *Fagonia indica* treatment, a link between NF-κB expression and ICAM-1 upon treatment was investigated. Interestingly in the current study, expression of NF-κB subunit P65 was significantly reduced after 6 and 24 hours of *Fagonia indica* treatment in MDA-MB-231 breast cancer cells but remained relatively unchanged in MCF-7 cells under the same conditions. Biswas *et al* (2004) found that nuclear NF-κB was largely detected in the stroma of ER-negative breast tumours and NF-κB signalling in ER-negative cell line SKBr3 was essential for modulating cellular proliferation and avoiding apoptosis. As MDA-MB-231 breast cancer cells are triple negative in terms of ER, PR and HER2 status, they require different approaches to maintain a malignant state. Further studies have indicated the importance of NF-κB signalling in MDA-MB-231 cells and targeting this pathway is a promising line of therapy for TNBC (Poma *et al*., 2017), this could be relating to the changes in gene expression in the current study. Despite the effects in mRNA expression, the inhibition of NF-κB did not significantly alter the effect of *Fagonia indica* treatment on cell viability of breast cancer cell lines.

Notably, MDA-MB-231 breast cancer cell lines did not respond as significantly in terms of anti-inflammatory and antiangiogenic effects after treatment with *Fagonia indica*. MDA-MB-

231 is a triple negative breast cancer cell line (TNBC), which means it does not express ER, PR, nor does it display HER-2 amplification. Triple negative breast cancers make up approximately 10-15% of all breast cancer cases and are typically correlated with poorer outcomes and lack of validated molecular targets (Chavez, Garimella, & Lipkowitz, 2010). AUF-1 is an RNA binding protein which is able to both stabilise and destabilise mRNA transcripts, related to several key cancer genes (AlAhmari *et al*., 2020). Studies have shown that AUF-1 binds and destabilises TGF-β and IL-6 mRNA reducing their overall turnover, leading to subsequent down-regulation of p53 and p21 in breast cancer (Hendrayani, Al-Khalaf, & Aboussekhra, 2014). In the current study, AUF-1 mRNA expression in MDA-MB-231 was significantly down-regulated after treatment with *Fagonia indica* extract. This could highlight a new mechanism for *Fagonia indica* in MDA-MB-231 cell lines, as previous research has shown that extract treatment regulates expression of IL-6 (Azam *et al*., 2018).

Despite the advancements in the treatment and management of cancers, resistance to the classical chemotherapeutic agents and novel targeted agents remains a significant problem (Wang, Zhang, & Chen, 2019). The ATP binding cassette (ABC) transporters are membrane proteins which facilitate the transport of a wide array of structurally diverse substrates, including common chemotherapeutic agents (Al-Eitan *et al*., 2019). Hansen *et al* (2016) used whole exome sequencing to identify successive mutations in the acquisition of resistance to docetaxel in MCF-7 and MDA-MB-231 breast cancer cells. Of these mutations, several occurred in ABC transporter genes including ABCG2 and ABCC4. In the current study, mRNA expression of ABCC4 and ABCG2 was greatly increased in MDA-MB-231 gemcitabine resistant cells in comparison to a wild-type control. Other studies have demonstrated a similar upregulation of ABC transporters; including ABCG2, in cancers treated with gemcitabine (Sun *et al*., 2016). Treatment with 1 mg/mL *Fagonia indica* reduced the overexpression of ABCC4 after 6 hours and of ABCG2 after 24 hours, in gemcitabine resistant MDA-MB-231.

Despite the diminished ability of gemcitabine to induce apoptosis in these resistant MDA-MB-231 clones, *Fagonia indica* was able to reduce cell viability to a similar degree of the wild-type cells. This suggests that the mechanisms of apoptosis are different from those of gemcitabine and that *Fagonia indica* treatment is able to overcome modes of resistance. Gemcitabine acts a pro-drug which is phosphorylated by deoxycytidine kinase to become active and inhibit processes essential for DNA synthesis (Plunkett *et al*., 1995). As *Fagonia indica* extract is likely a mix of a number of bioactive compounds, the biological mechanisms of the extract are difficult to elucidate precisely. In regards to overcoming acquired resistance mechanisms, other studies have demonstrated the potential of compounds such as Metformin, which alone down-regulated ABC transporter P-gp and showed synergistic activity with doxorubicin against resistant MCF-7 breast cancer cells (Ying Li *et al*., 2018). As *Fagonia indica* treatment was able to reduce the expression of ABCG2 and ABCC4 mRNA, the current study investigated the ability of *Fagonia indica* treatment to re-sensitise resistant cell lines to gemcitabine. Pre-treating gemcitabine resistant MDA-MB-231 cells with *Fagonia indica* extract did not significantly change the effect of gemcitabine on the cell viability of drug resistant MDA-MB-231 cell lines. This could suggest that although *Fagonia indica* is able to reduce the gene expression of ABC transporters in resistant breast cancer cell lines, this was not sufficient to reverse the effects of acquired resistance in these cell lines. It was also noted that inhibition of ABCC1 transporters with the inhibitor MK571 did not significantly increase *Fagonia indica* induced apoptosis against gemcitabine resistant cell lines. This could be due in part to an accumulation of several acquired mechanisms of resistance, which could include decreased drug influx or increased DNA repair mechanisms (Mansoori *et al*., 2017).

To determine the extent of *Fagonia indica* treatment on gemcitabine resistant MDA-MB-231 breast cancer cells, the present study investigated the potential of an aqueous extract to directly inhibit or impact the activity of ABCC membrane transporters. Calcein acetoxymethyl ester (Calcein AM) is a molecule frequently used to evaluate transporter-mediated chemoresistance. Calcein-AM is hydrophobic and can easily cross the cell membrane. Upon uptake it is cleaved

by intracellular esterase to form a hydrophilic fluorescent free Calcein molecule which can only be exported by transporter proteins (Szerémy *et al*., 2011). Cells which are overexpressing multidrug transporters should only accumulate low amounts of fluorescent free Calcein due to increased rates of active efflux. There has been growing interest in dietary phytochemicals, as studies have indicated that some plant phytochemicals can act as substrates, inhibitors and modulators of ABC transporting proteins (Yan Li *et al*., 2010). In the current study, *Fagonia indica* extract significantly reduced Calcein fluorescence in both wild-type and chemotherapy resistant MDA-MB-231 cell lines. This would suggest that the response to treatment with *Fagonia indica* extract is a rapid increase in transporter-mediated efflux. As efflux is increased this would strongly indicate that the extract does not act as an inhibitor to drug-related transport in these cell lines. There were also no significant differences between wildtype and parental cell lines.

It is worth noting a possible link between the reduction in intracellular ATP concentration and the significant reduction in mRNA expression of ABCC4 and ABCG2 transporters after *Fagonia indica* treatment. In eukaryotic cells, ABC transporters mediate transport of substrates against a chemical gradient, in a process which requires ATP hydrolysis as its primary driving force (Wilkens, 2015). As the activity of these membrane transporters is exclusively dependent on the availability and accessibility of ATP, decreased mitochondrial functioning can result in impaired ABC transporter activity and subsequently reduced efflux (Pahnke *et al*., 2013). In the present study, treatment with *Fagonia indica* extract resulted in a significant loss in intracellular ATP in both MCF-7 and MDA-MB-231 wild-type cells, this disruption in mitochondrial pathways and ATP production could contribute to the reduced expression and functioning of ATP-reliant transporters (Scheffler *et al*., 2012). Nakano *et al* (2011), targeted glycolysis; the main mode of ATP production in cancerous cells, to explore the effect on ABC transporter function and drug sensitivity. In this study, inhibition of glycolysis by 3-bromopryuvtae reduced the activity of ABC transporters restoring the retention of chemotherapeutic agents daunorubicin and mitoxantrone. Although pre-treatment

with *Fagonia indica* extract did not significantly restore sensitivity of MDA-MB-231 cells to gemcitabine, it could still be valuable to determine the effect of extract treatment in the inhibition of glycolysis in relation to ATP production.

3.5 Conclusion

This chapter has demonstrated the ability of an aqueous extract of *Fagonia indica* to induce cell death in two phenotypically distinct breast cancer cell lines; MCF-7 and MDA-MB-231. Following on from previous research; which proposed p53 activation and subsequent transcription of effector molecules p21 and BAX as a mechanism of apoptotic action for *Fagonia indica*, this chapter confirmed the presence of apoptotic markers via YP1 staining, which could be related to increased caspase activation. Investigation into the role of p38/ Akt signalling pathways in the induction of cell death in MCF-7 and MDA-MB-231 was inconclusive. Further research would utilise flow cytometry to identify cell surface markers of apoptosis and necrosis, to help establish a clear pathway of apoptosis for *Fagonia indica* treatment.

Despite the ability of *Fagonia indica* to reduce cell viability being well established, the mechanisms of action have remained elusive. This chapter eluded; for the first time, potential targets of aqueous *Fagonia indica* extract against breast cancer cells. These included several genes associated with the hallmarks of cancer; inflammation, migration and angiogenesis, including NF-κB, ICAM-1 and VEGF. Inhibition of VEGF-A receptor also alleviated some of the effects of *Fagonia indica* extract, including the ability to induce cell death and inhibit proliferation. An interaction between *Fagonia indica* extract and VEGF signalling could be further explored by targeting additional downstream signalling targets of VEGF. It would also be valuable in future experiments to determine the effect of *Fagonia indica* on the protein expression of VEGF, or to determine the effect on secretion of VEGF into culture medium using an ELISA assay.

This chapter also introduced the potential of *Fagonia indica* extract to induce cell death in chemotherapy resistant breast cancers. Aqueous extract of *Fagonia indica* was able to reduce cell viability in resistant cell lines to the same degree or more so than in the wild-type clones. One proposed mechanism of action in resistant cell lines was the inhibition or targeting of ABC transporters. The chapter demonstrated the ability of *Fagonia indica* to reduce the expression of various ABC transporters, however a functional assay was able to show that this was not likely due to direct inhibition of the transporters themselves. It was also shown that reduced mRNA expression of ABC transporters did not significantly re-sensitise resistant cell lines to chemotherapy. Further work is required to elucidate the mechanisms of action in chemotherapy resistant breast cancers and to determine to what degree the extract is able to reverse acquired resistance in breast cancer.

4 Chapter 4: Colon cancer

4.1 Introduction

The colon is the second most frequent site of diagnosed cancer cases in Europe and as such is a common cause of cancer-related mortality (Ferlay *et al*., 2018). Incidence rates of colon cancer correlate with increasing levels of Westernisation (Lauby-Secretan *et* al., 2018). Increased risks of colon cancer include diets high in processed red meat, increased BMI and obesity, smoking, abnormal bowel movement and aspirin use (Drew *et al*., 2017; Lewin *et al*., 2006; McCleary *et al*., 2010; Shaukat *et al.*, 2017). Colon cancer screening programmes have increased early detection of high-risk adenomas and have shown to reduce overall mortality from colon cancer (Bretthauer *et al*., 2016). Current therapeutic regimes for colon cancer include a surgical resection and combination chemotherapy, including 5-fluorouracil (5-FU) and oxaliplatin (Xie, Chen, & Fang, 2020). Treatment in combination with lifestyle changes such as maintaining a healthy body weight, increased physical exercise and implementing vegetables and whole gains into the diet, has been shown to increase survival of colon cancer patients (Van Blarigan *et al*., 2018; Van Vulpen *et al*., 2016). Despite improvements in diagnosis, colon cancer remains a significant health burden in westernised countries and those undergoing nutritional transition, such as Middle Eastern and African countries (Belahsen, 2014; El kinany *et al*., 2018).

Plants, plant-based extracts and secondary metabolites are frequently utilised in the treatment of diseases, including a potential role in the prevention and treatment of cancers (Aiello *et al*., 2019). In previous studies, compounds isolated from plant extracts have been used in clinical studies as potential adjuvants to chemotherapy in colon cancer (Chen *et al*., 2014; Kummar *et al*., 2011). *Fagonia indica* is a good candidate for treating colon cancer in traditional communities due to the composition and availability of bioactive compounds (Khan *et al*., 2020). Previous studies have demonstrated the cytotoxic ability of alcoholic extracts of *Fagonia indica* against colon cancer cell lines. An ethyl acetate extract and its fractions produced by Shehab *et al* (2015), displayed marked antitumour activity against HCT colon

cancer cell lines. An isolated saponin glycoside from an ethanolic fraction of *Fagonia indica*, reduced cell viability in Caco-2 colon cancer cells after 24 hours (Waheed *et al*., 2012). Despite this, there was been no research demonstrating the effect of an aqueous extract against colon cancer, nor has there been any research regarding the mechanism of action of a *Fagonia indica* extract in colon cancer.

The p38 MAPK pathway is typically activated in response to environmental stresses and double strand DNA damage (Wood *et al*., 2009). P38 plays a vital role in stress-induced responses by initiating cell cycle arrest and apoptosis via the phosphorylation of p53 and the induction of p53-dependent G2/M checkpoints (Thornton & Rincon, 2009; Cuenda *et al*., 2007). P38 MAPK pathways also; in part, contribute to the cellular response to chemotherapy in colon cancer, therefore it is important to understand how this pathway is activated post treatment (Sui *et al*., 2014). The role of *Fagonia indica* extract and the induction of apoptotic pathways in colon cancer has not been explored in previous studies and the cytotoxic capacity of *Fagonia indica* in this cancer type is relatively unknown.

VEGF-A plays a central role in endothelial cell proliferation, invasion, migration and survival of primary tumors (Nishida *et al*., 2006). Overexpression of VEGF-A has been identified as a hallmark in colon cancer and is correlated with an increased risk of invasion and metastasis (Bhattacharya *et al*., 2017). VEGF has also been shown to directly regulate the expression of adhesion factor ICAM-1 via the phosphatidylinositol 3 OH-kinase (PI3K)/AKT pathway (Radisavljevic, *et al*., 2000). Aberrant expression of both VEGF and ICAM-1 has been used to predict clinical outcome of patients with metastatic colon cancer. Yeh (2019) identified a significant positive correlation between protein expression of both VEGF and ICAM-1 in biopsied colon cancer tissue. The relationship was significantly higher in tumors of increased size or with metastasis (Dymicka-Piekarska *et al*., 2012). Increased expression of VEGF has also been associated with increased inflammation. Scaldaferri *et al* (2009) demonstrated the capacity of VEGF to induce a pro-inflammatory phenotype via the induction of ICAM-1 and VCAM-1 in inflammatory bowel disease (IBD). The mechanism behind the induction of

VEGF as a pro-inflammatory cytokine has been; in part, attributed to stimulation from the nuclear factor NF-kB signaling pathway. VEGF activation stimulated activity of NF-kB, resulting in an increase in protein levels of ICAM-1 and VCAM-1. Additionally, regulation of angiogenic factors such as VEGF by NF-kB stimulation have also been attributed to the overall progression of colon cancer (Soly *et al*, 2009).

Drug resistance to current chemotherapies for colon cancers occurs universally, with resistance developing as a selection pressure via DNA mutation and metabolic alterations (Hu *et al*., 2016). The ability of transmembrane ABC proteins to mediate transport of chemotherapeutic compounds and their metabolites has been linked to aberrant expression of multiple ABC transporters in resistant colon cancers (Meng *et al*., 2018). Studies have shown that exposure of colon cancers to low-dose chemotherapeutic agents for 10 days was sufficient to upregulate ABCG2 expression (Calcagno *et al*., 2008; Xie *et al*., 2014). Gradilone *et al* (2008) found that combination therapies for colon cancer actually conferred resistance to 5-FU and oxaliplatin via the upregulation of ABC transporter -4. Inhibition of ABCC4 was able to enhance the accumulation of intracellular drug concentration and re-sensitise tumours to existing chemotherapies, including colon cancer treatment 5-FU (Chen *et al*., 2017; Drenberg *et al*., 2016). There has been no research demonstrating the effect of *Fagonia indica* extracts or derivatives against chemotherapy resistant colon cancer *in vitro*.

The primary aims of this chapter were; for the first time, to establish the cytotoxic capabilities of a whole plant aqueous extract of *Fagonia indica* in colon cancer *in vitro* and to demonstrate whether treatment disrupts the ability of the cancer cells to proliferate and migrate. This chapter also aimed to uncover some of the potential mechanisms of action of a *Fagonia indica* extract. There was a particular focus on members of the Akt and p38 pathways, VEGF, NFκB and ICAM-1; to link not only to apoptotic pathways but also other key aspects of tumorigenesis. This chapter also explored the ability of *Fagonia indica* to induce cell death in chemotherapy resistant/ multidrug resistant colon cancer and highlights the relationship between *Fagonia indica* treatment and the expression of multi-drug resistance proteins.

4.2 Materials and methods

4.2.1 Extract preparation

An aqueous extract of *Fagonia indica* was prepared as described in section 2.2.

4.2.2 MTT assay

The effect of an aqueous extract of *Fagonia indica* on H630-WT, RKO-WT, H630-GM, H630-5FU and RKO-TDX cell viability was determined using the MTT assay as described in section 2.5.1. In brief, cells were seeded at $1x10^4$ prior to treatment with 0-2.5 mg/mL of aqueous *Fagonia indica* extract for up to 72 hours, prior to analysis using MTT. The effect of pathway inhibitors and activators on extract treatment was determined using varying concentrations of selective inhibitors; Z-VAD-FMK, SC79, SB203580, CAPE, Apatanib and MK571for 48 hours, alongside treatment with 1.5 mg/mL of *Fagonia indica* prior to MTT analysis (as described in further detail in section 2.X). The ability to restore sensitivity of chemotherapeutic agents; gemcitabine, tomudex and 5-fluourouracil, was determined by pre-treating resistant cell lines for 24 hours with 1 mg/mL *Fagonia indica* prior to treatment with tomudex or gemcitabine, prior to MTT analysis.

4.2.3 NR assay

The effect of an aqueous extract of *Fagonia indica* on H630 and RKO cell viability was determined using the NR assay as described in section 2.5.2. In brief, cells were seeded at $1x10^4$ prior to treatment with 0-2.5 mg/mL of aqueous *Fagonia indica* extract for up to 72 hours, prior to analysis using NR.

4.2.4 YO-PRO-1 staining

The effect of an aqueous extract of *Fagonia indica* extract inducing apoptosis in H630 and RKO colon cancer cells was determined using YO-PRO-1 fluorescent microscopy, as described in section 2.4.

4.2.5 ATP assay

The level of intracellular ATP produced in H630 and RKO colon cancer cells was detected using the luminescent ATP assay as detailed in section 2.6.

4.2.6 RNA extraction, cDNA production and QPCR

The effects of aqueous *Fagonia indica* extract treatment on VEGF, Caspase-8, ICAM-1, NFkB, ABCC4 and ABCG2 mRNA expression in H630 and RKO colon cancer cells was determined by real-time qPCR as outlined in sections 2.7.1, 2.7.2 and 2.7.3. A total of 500 ng of extracted RNA per treatment was reversed transcribed into cDNA, prior to qPCR analysis. YHWAZ and Actin were both used as housekeeping genes.

4.2.7 Colony formation assay

The effect of *Fagonia indica* on H630 and RKO colony formation was detected using the colony formation assay as described in section 2.9. To determine the effect of VEGF signalling in *Fagonia indica*, H630 and RKO colon cancer cells were pre-treated alongside 1 μM of apatanib for 24 hours prior to seeding the colony formation assay.

4.2.8 Scratch wound assay

The effect of *Fagonia indica* on wound healing and migration was determined on H630 and RKO colon cancer cells after 24 hours as described in section 2.8.

4.2.9 Calcein-AM assay

To determine whether an aqueous extract of *Fagonia indica* could inhibit the activity of ABCC1, the level of cellular efflux between H630-WT/ H630-GM and RKO-WT/ RKO-TDX cells was measured using the calcein-AM fluorescent microplate assay, as detailed in section 2.10.

4.3 Results

4.3.1 An aqueous extract of *Fagonia indica* induces apoptosis and inhibits proliferation of colon cancer cells H630 and RKO.

An aqueous extract of *Fagonia indica* was prepared and tested using the MTT assay to determine the ability to reduce colon cancer viability in two colon cancer cell lines with homozygous p53 expression. Treatment with *Fagonia indica* extract reduced cell viability of H630 and RKO colon cancer cell lines in a time- and concentration-dependent manner (Figure 4.3.1). After 24 hours of treatment with 2.5 mg/mL *Fagonia indica* extract, cell viability of H630 cells was significantly reduced by 29.3% ($p<0.001$), increasing to 50.7% ($p<0.0001$) after 72 hours, compared to an untreated control (Figure 4.3.1A). In RKO cells there were no significant changes in cell viability after 24 hours, however after 72 hours cell viability was significantly reduced by 30.7%, compared to an untreated control ($p<0.001$) (Figure 4.3.1B).

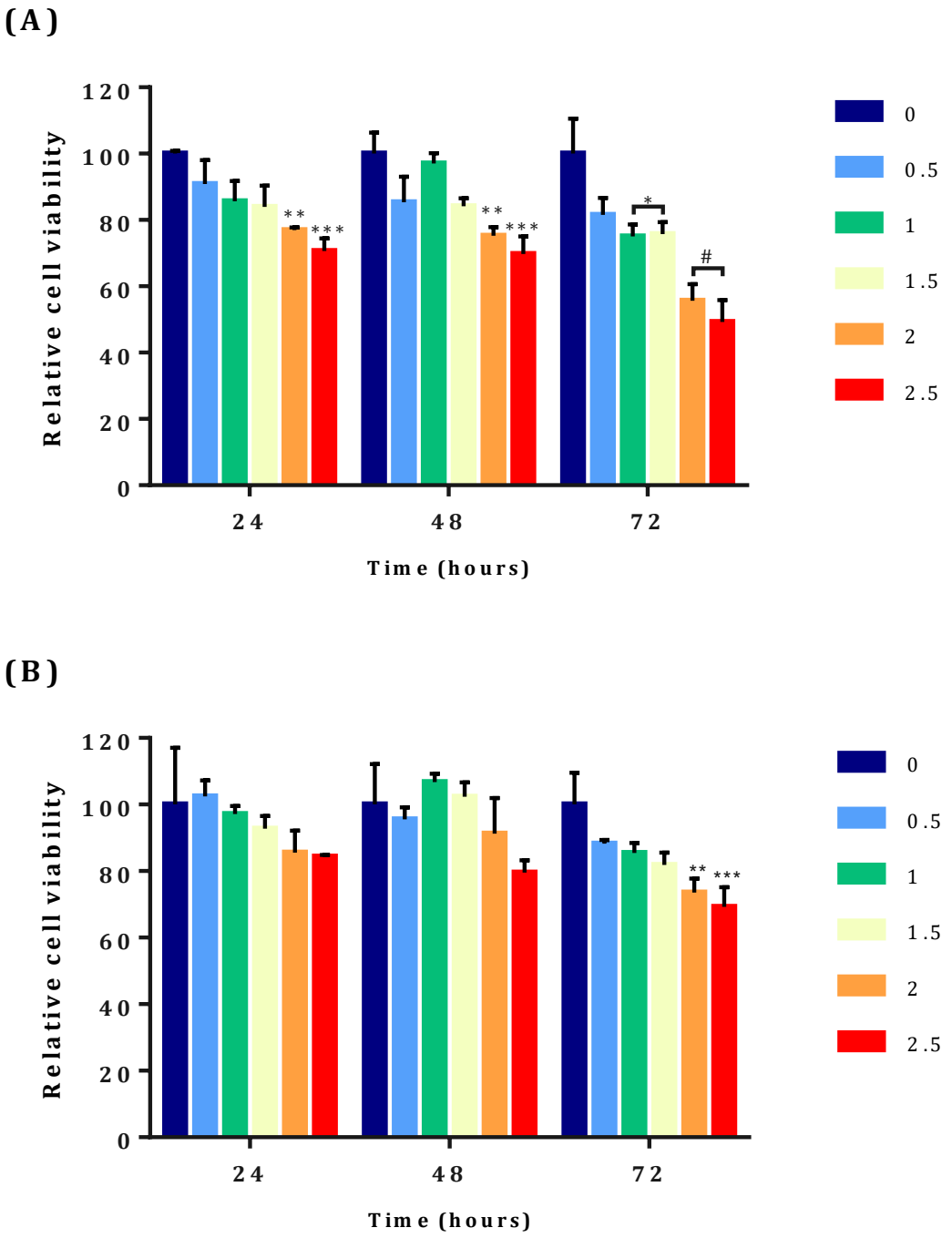

Figure 4.3.1: *Fagonia indica* extract treatment reduces *in vitro* cell viability of H630 and RKO colon cancer cell lines. (A) H630 and (B) RKO cells were treated with 0-2.5 mg/mL aqueous *Fagonia indica* extract for 24-72 hours. Cell viability was determined as a percentage of an untreated DMSO vehicle control using MTT assay. Data denoted * ($p<0.05$), ** ($p<0.01$),*** ($p<0.001$) and # ($p<0.0001$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM

Extract induced cytotoxicity was also determined using the NR assay (Figure 4.3.2). In H630 cells, cell viability was significantly reduced after treatment with 2.5 mg/mL *Fagonia indica* extract by 19.8% ($p<0.01$) at 24 hours, and 55.9% ($p<0.0001$) after 72 hours (Figure 4.3.2A). RKO cells were more sensitive to *Fagonia indica* extract under NR assay, with loss in cell viability of 51.3% ($p<0.0001$) after 24 hours of treatment with 2.5 mg/mL of extract, increasing to 65.8% at 72 hours ($p<0.0001$) (Figure 4.3.2B).

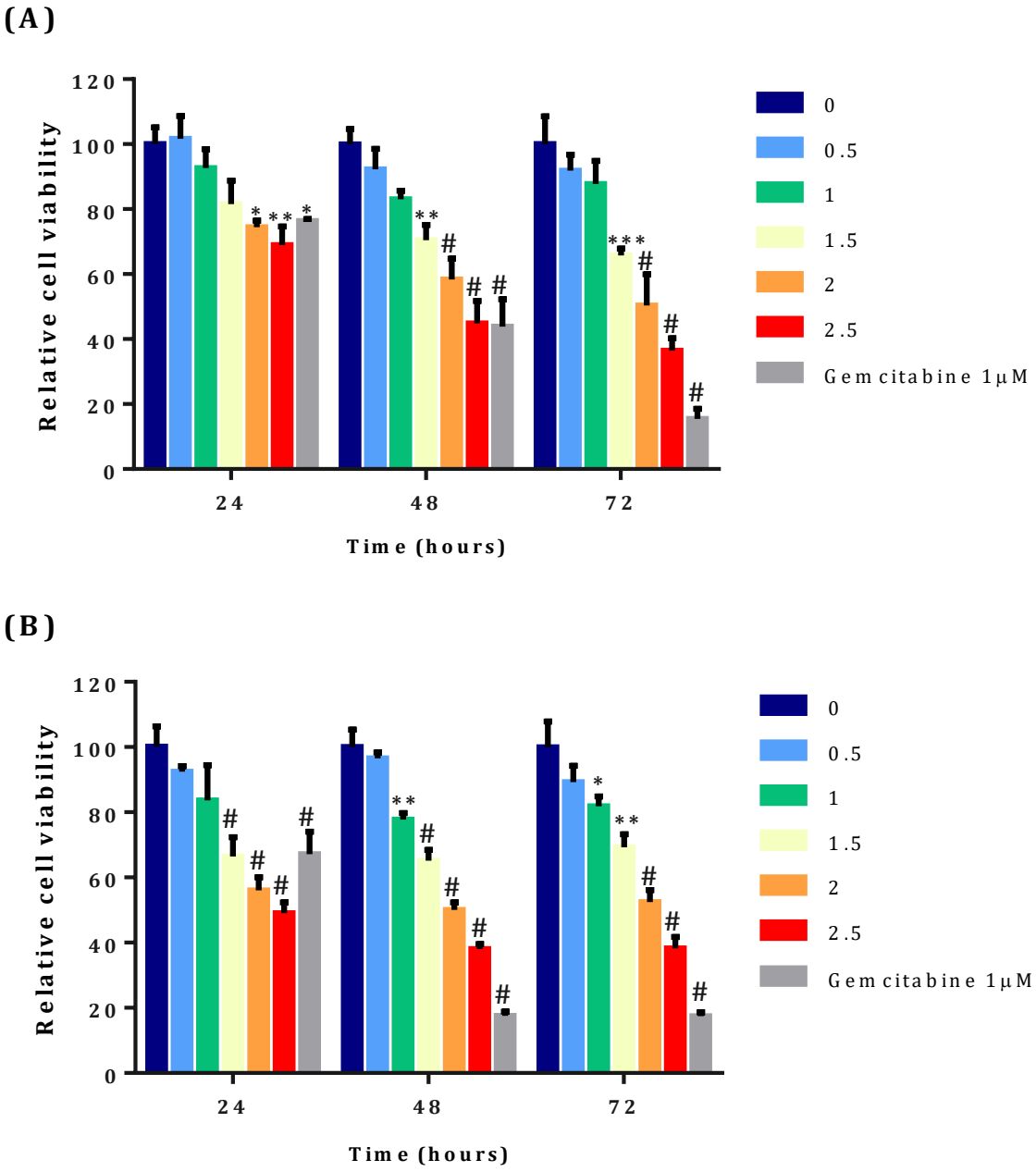

Figure 4.3.2: *Fagonia indica* extract treatment reduces *in vitro* cell viability of H630 and RKO colon cancer cell lines. (A) H630 and (B) RKO cells were treated with 0-2.5 mg/mL aqueous *Fagonia indica* extract or 1 µM of Gemcitabine for 24-72 hours. Cell viability was determined as a percentage of an untreated DMSO vehicle control using the NR assay. Data denoted * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and # ($p<0.0001$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

Aberrant metabolism is a key feature of tumorigenesis with high intracellular ATP levels and increased glycolysis being synonymous with tumour development. Intracellular ATP levels were measured in *in vitro* colon cancer cell lines (Figure 4.3.3). Treatment with 1 mg/mL *Fagonia indica* extract over 24 hours induced a significant reduction in both H630 (Figure 4.3.3A) and RKO (Figure 4.3.3B) intracellular ATP levels by 68.3% ($p<0.01$) and 70.3% ($p<0.01$) respectively, compared to an untreated control.

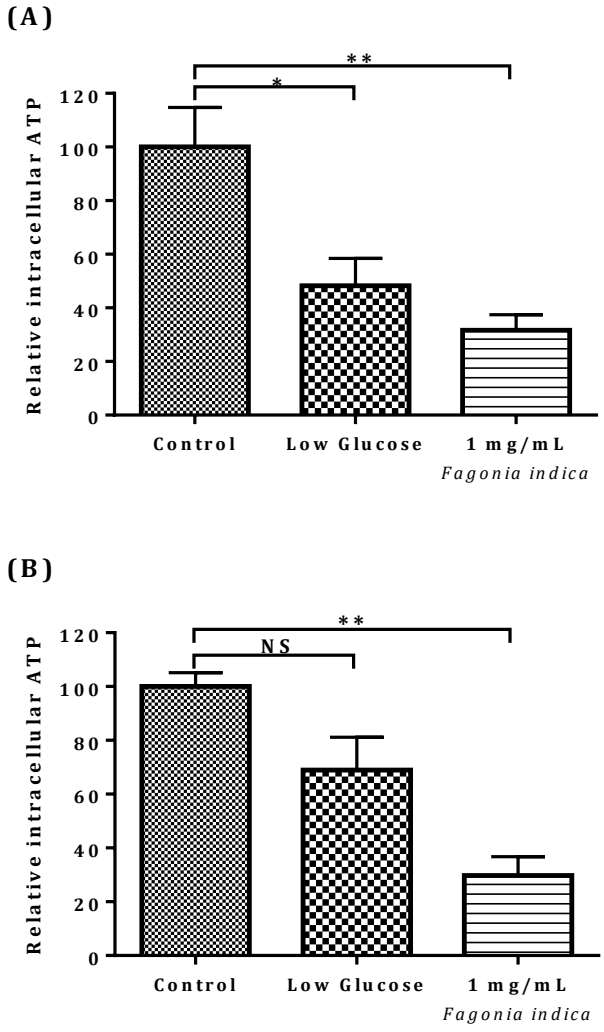

Figure 4.3.3: *Fagonia indica* extract treatment reduces in vitro intracellular ATP concentrations in H630 and RKO colon cancer cell lines. (A) H630 and (B) RKO cells were treated with 1 mg/mL aqueous Fagonia indica extract or low glucose supplemented DMEM for 24 hours prior to analysis of intracellular ATP with a luminescent ATP assay. ATP concentration was determined as a percentage of an untreated control. Data denoted * ($p<0.05$) and ** ($p<0.01$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. Data denoted NS was not statistically significant compared to the untreated control. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

The caspases are a family of distinct protease enzymes with a key role in initiating programmed cell death. Inhibition of pan-caspases with z-VAD-FMK, can aid in the determination between distinct sects of cell death, including apoptotic and necrotic cell death. Inhibition of caspases, did not significantly affect cell viability in the untreated control group, nor in *Fagonia indica* treated H630 (Figure 4.3.4A) and RKO colon cancer cells (Figure 4.3.4B).

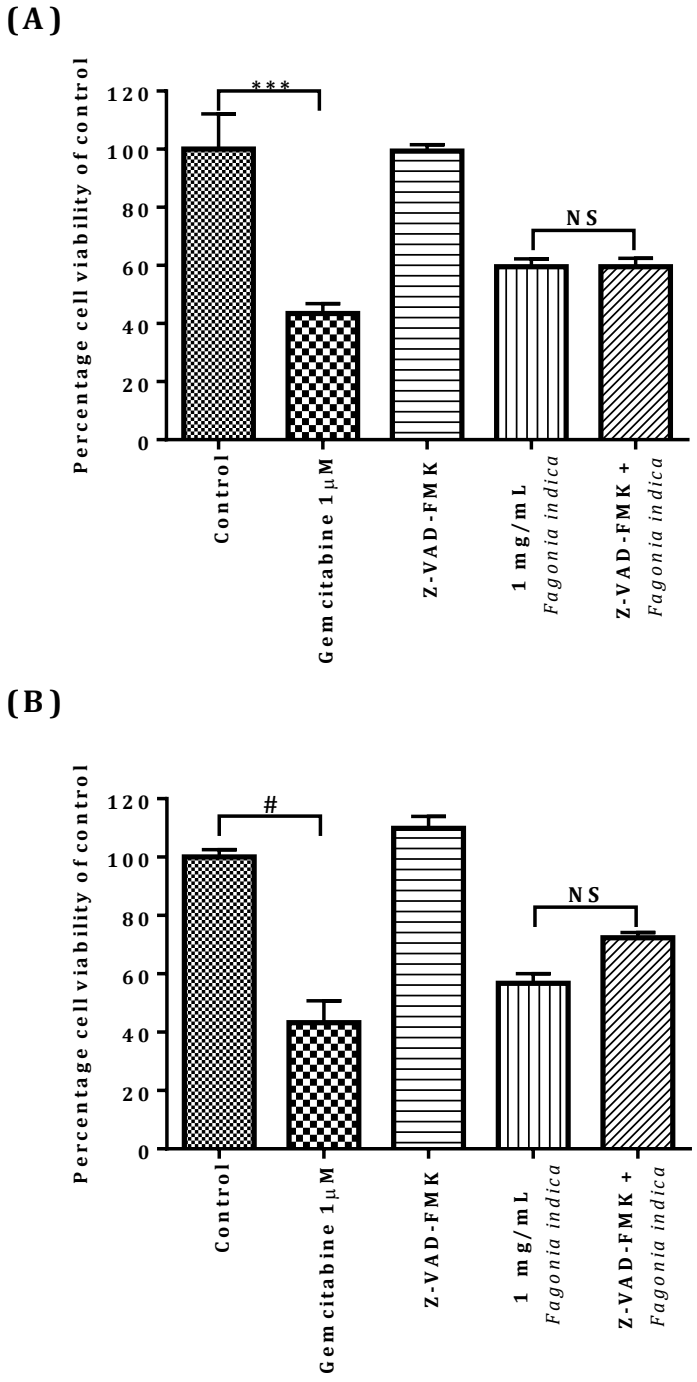

Figure 4.3.4: The effect of pan-caspase inhibition on *Fagonia indica* extract treatment in H630 and RKO colon cancer cell lines (A) H630 cells and (B) RKO cells were treated with 1 mg/mL of aqueous extract and/or 50 μM of pan-caspase inhibitor Z-VAD-FMK. 1 μM of Gemcitabine was used as a positive control. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted *** ($p<0.005$) and # ($p<0.001$) were significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. All data points are representative of three independent experiments, performed in triplicate and are expressed as mean ± SEM.

P38 mitogen activated protein kinase (MAPK) initiates the activation of caspases via extrinsic and intrinsic pathways for apoptosis. SB203580 is a pharmacological inhibitor of p38 MAP kinase (IC_{50} = 34 nM) and was used to inhibit activation of p38 transduction pathways in H630 and RK0 cell lines (Figure 4.3.5). Inhibition of p38 MAPK did not affect cell viability in untreated H630 cells, but significantly increased *Fagonia indica* treated cell viability in H630 cells by 35% ($p<0.01$) (Figure 4.3.5A). When activated, p38 MAPK can indirectly inhibit oncogenic activation of Akt. Akt signalling pathways promote cellular survival and growth in response to extracellular signals. In H630 cells, activation of Akt with SC79 did not significantly affect cell viability in untreated cells (Figure 4.3.5C), but alleviated the reduction in cell viability of *Fagonia indica* treated cells by 31.6% ($p<0.0001$). However, in RKO cells treatment with SB20 inhibitor and SC79 activator, did not significantly affect untreated nor *Fagonia indica* treated cells (Figure 4.3.5B/ 4.3.5D).

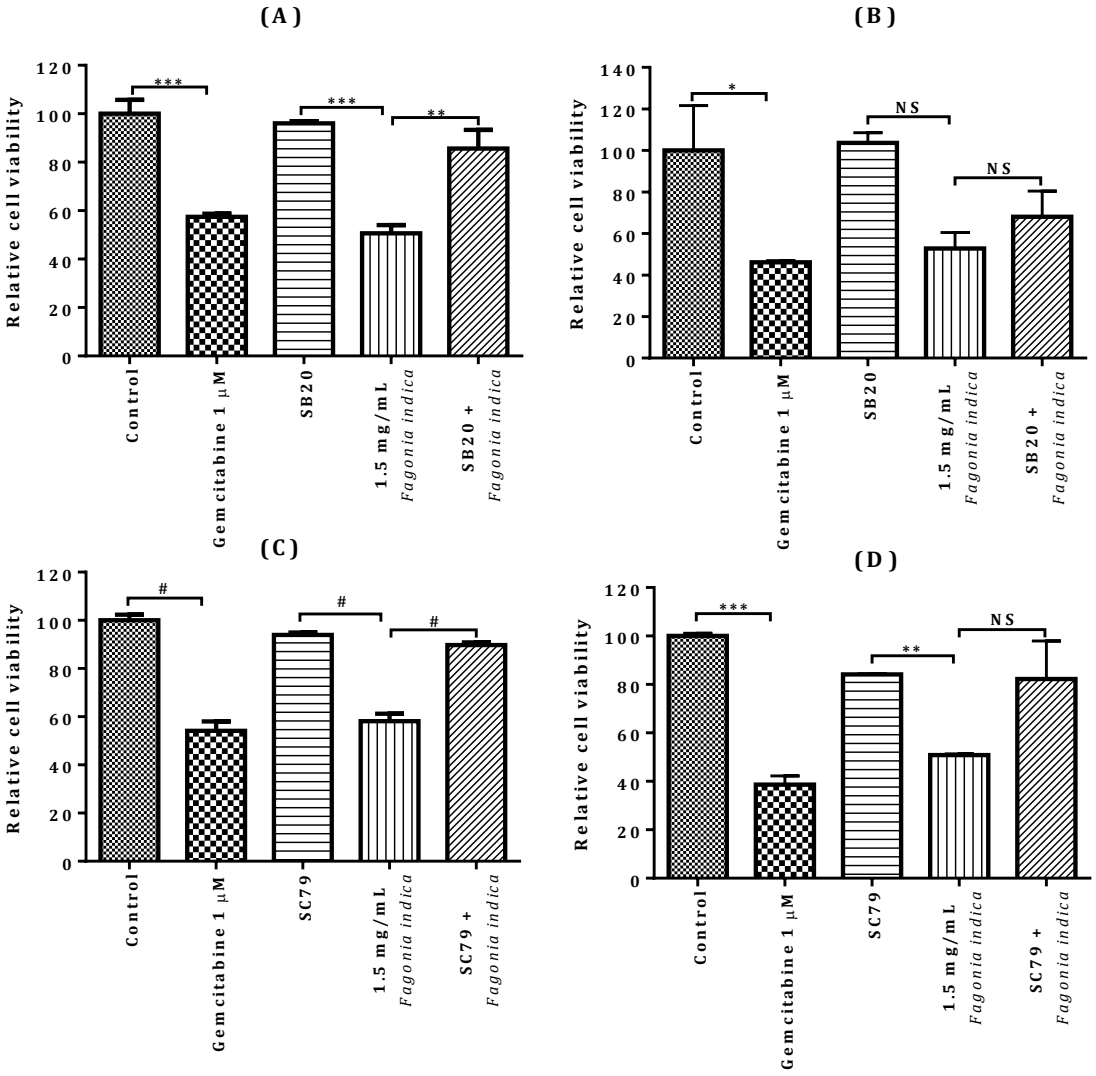

Figure 4.3.5: Effects of p38 MAP kinase inhibition and AKT activation on extract induced H630 and RKO colon cancer cytotoxicity. (A) H630 and (B) RKO cells were treated with 1.5 mg/mL of aqueous extract and/or 1 μM of SB203580 (SB20) p38 MAP kinase inhibitor for 48 hours. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. (C) and (D) MCF7 cells were treated with 1 mg/mL of aqueous extract and 1 μM of SC79 Akt activator for 48 hours. 1 μM Gemcitabine was used as a positive control. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted as * ($p<0.05$) , ** ($p<0.01$), *** ($p<0.001$) and # ($p<0.0001$), was statistically significant compared to the untreated DMSO vehicle control, analysed using one-way ANOVA with Sidaks's multiple comparison test. All data points are representative of three independent experiments, performed in triplicate and are expressed as mean ± SEM.

YO-PRO-1 (YP1) is used as a nuclear marker of early-stage apoptosis. YP1 is only able to enter cells during apoptosis due to jeopardized membrane structures, which would typically keep a molecule of this size from penetrating an intact barrier. YP1 was used to demonstrate the initiation of apoptosis in human colon cancer cells H630 and RKO, after treatment with *Fagonia indica* extract. After 24 hours, there was a higher degree of YP1 staining in *Fagonia indica* treated H630 and RKO cell lines compared to an untreated control (Figure 4.3.6). Notably, there was also an observed reduction in adherence, which could coincide with cell viability.

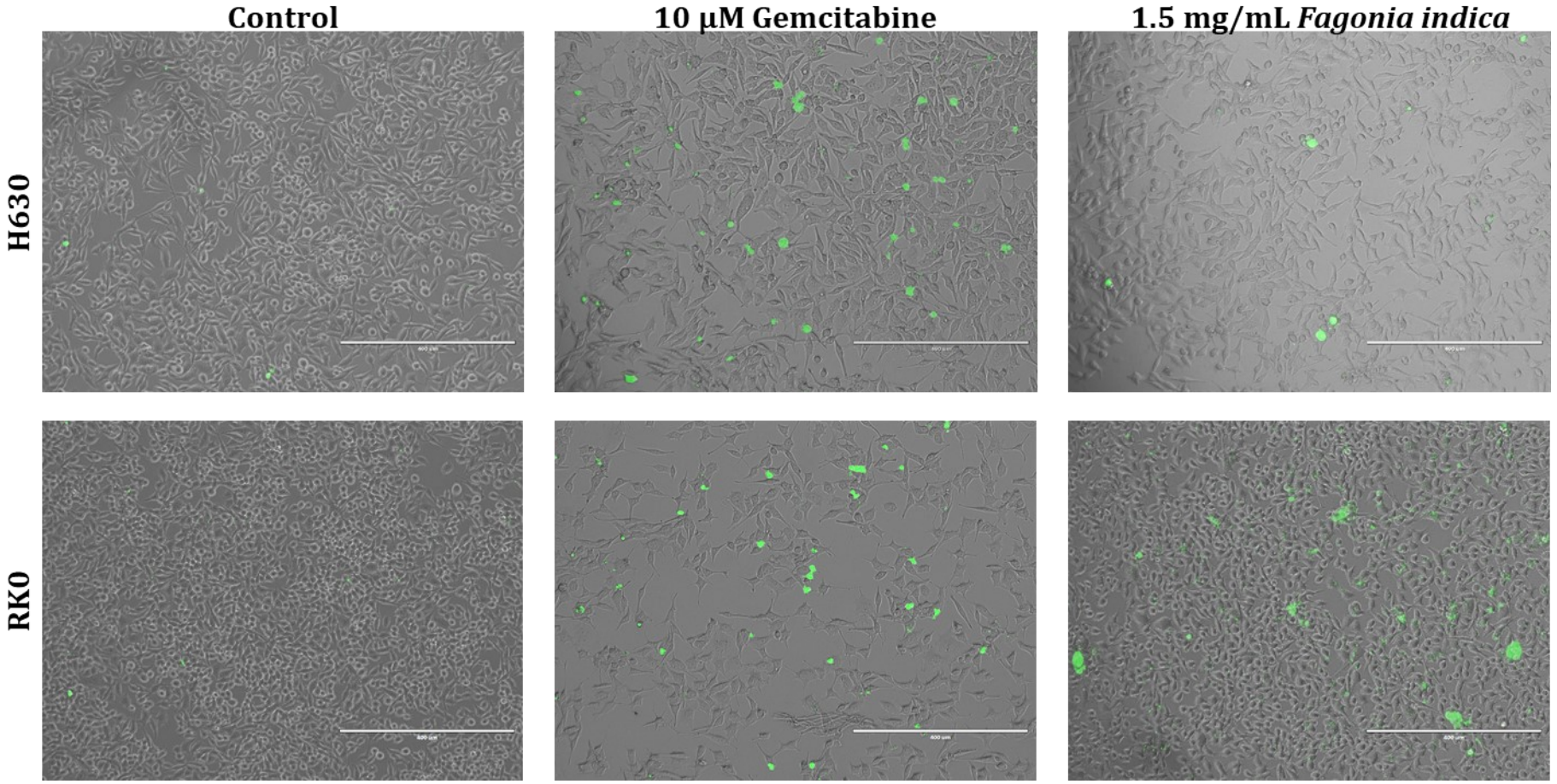

Figure 4.3.6: YO-PRO-1 staining of apoptotic H630 and RKO colon cancer cells. H630 (top) and RKO (bottom) cells were treated with 1.5 mg/mL aqueous *Fagonia indica* extract for 24 hours prior to staining with 1 µM yo-pro-1. 10 µM Gemcitabine was used as a positive control. Images were captured on an EVOS fluorescent microscope at 10X magnification. All images are representative of N=3. Scale bar = 400 µM.

4.3.2 An aqueous extract of *Fagonia indica* inhibits cologenic formation, wound healing and migration of colon cancer lines H630 and RKO.

The effect of *Fagonia indica* on proliferation from a single cell, colony formation and migration in both cell lines was also investigated. The capacity of a single cell to proliferate after pre-treatment with *Fagonia indica* extract for 24 hours was determined using a colony formation assay. *Fagonia indica* extract treated H630 and RKO cells formed significantly fewer colonies than the untreated control group after one week of colony formation (Figure 4.3.7/4.3.8). In H630 cells, colony formation was significantly reduced by 29.6% ($p<0.001$) for cells seeded at 200 cells/ per well, compared to an untreated control (Figure 4.3.7B). Similarly, colony formation in RKO cells was also significantly reduced by 29.5% ($p<0.01$), compared to an untreated control (Figure 4.3.8B).

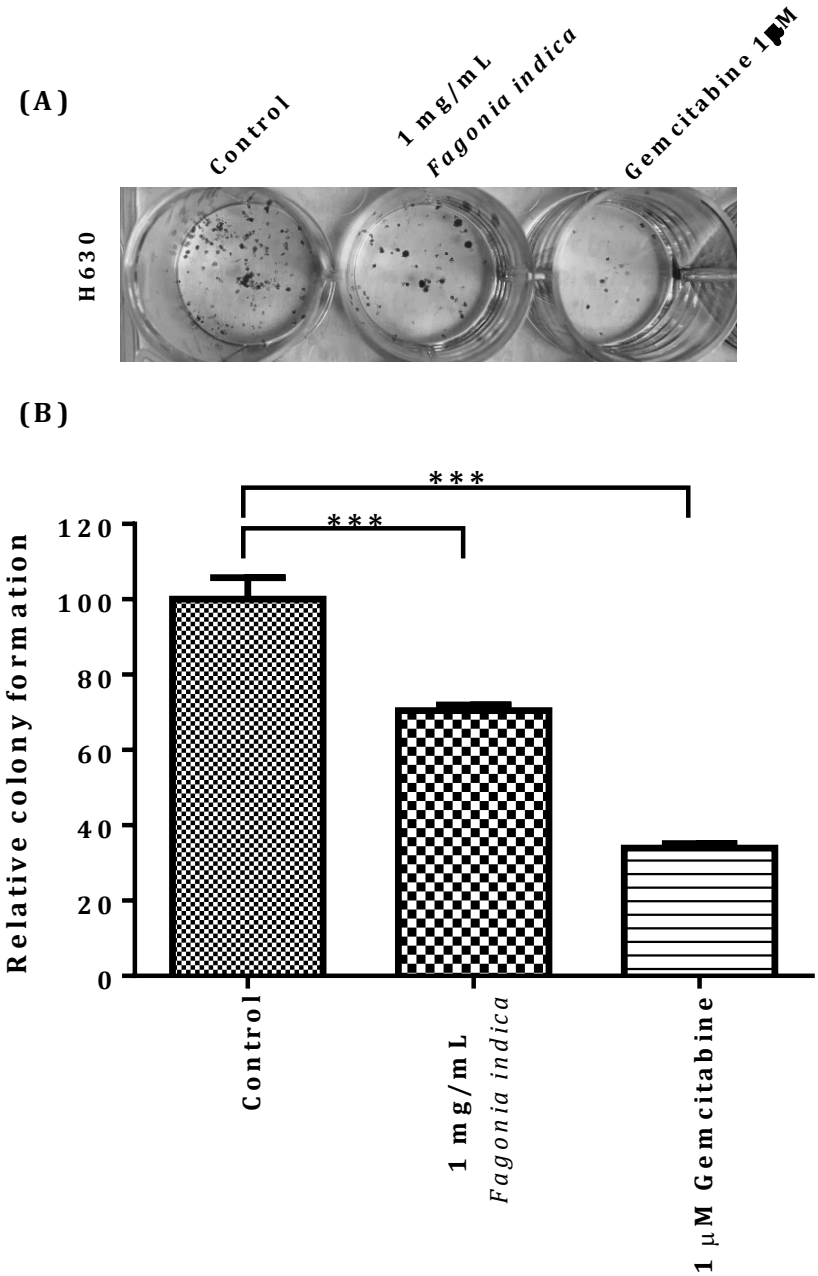

Figure 4.3.7: *Fagonia indica* treatment inhibits proliferation from a single cell of H630 colon cancer cell lines. H630 cells were pre-treated with 1.5 mg/mL aqueous *Fagonia indica* or 1 μM Gemcitabine for 24 hours and seeded at a density of 200 cells per well in a 24 well plate for 1 week. Colonies of 50> cells were stained using 0.01% crystal violet stain and counted using an EVOS microscope (A). (B) Colony formation was measured as a percentage of an untreated DMSO vehicle control. Data denoted ***($p<0.001$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

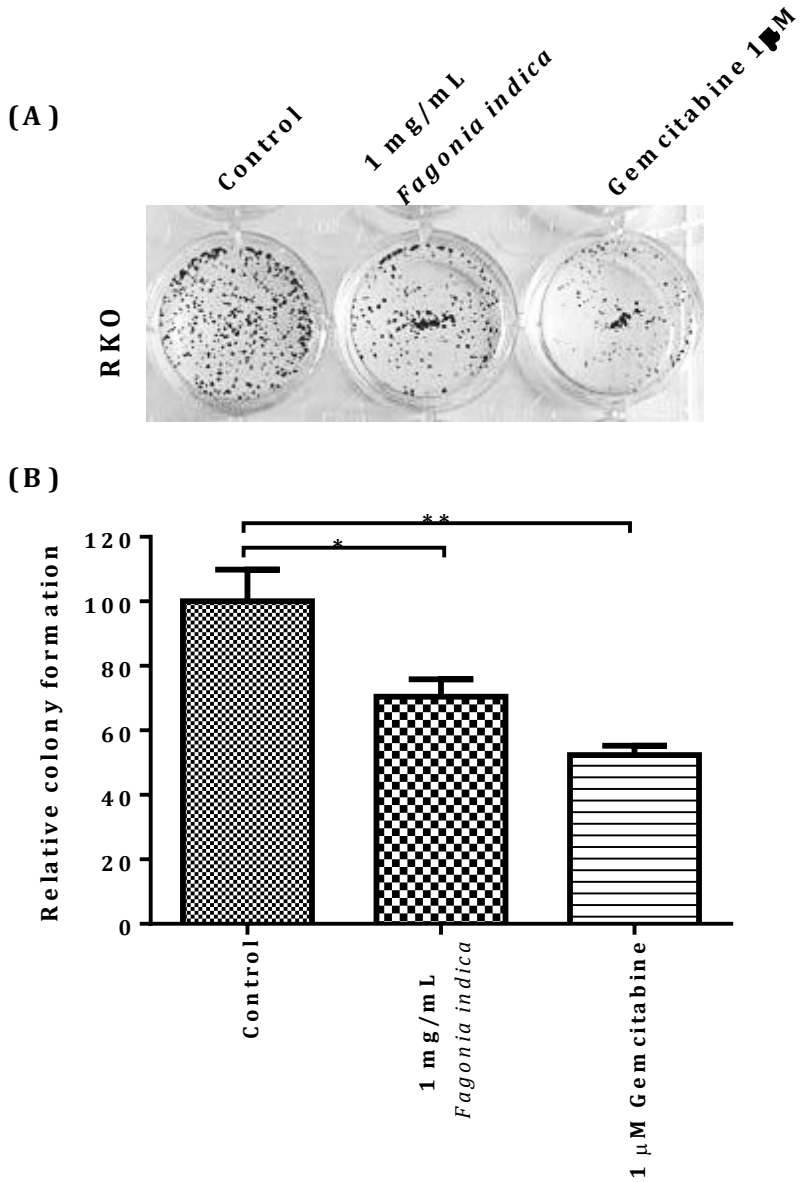

Figure 4.3.8: *Fagonia indica* treatment inhibits proliferation from a single cell of RKO colon cancer cell lines. RKO cells were pre-treated with 1 mg/mL aqueous *Fagonia indica* or 1 µM Gemcitabine for 24 hours and seeded at a density of 400 cells per well in a 24 well plate for 1 week. Colonies of 50> cells were stained using 0.01% crystal violet stain and counted using an EVOS microscope (A). (B) Colony formation was measured as a percentage of an untreated DMSO vehicle control. Data denoted * ($p<0.05$),** ($p<0.01$) and ***($p<0.001$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

In addition, a scratch wound assay was used to determine whether *Fagonia indica* extract inhibited the migration of H630 and RKO colon cancer cells. After 24 hours of treatment with *Fagonia indica*, wound healing was not significantly affected in either H630 (Figure 4.3.9) or RKO colon cancer cell lines (Figure 4.3.10), when compared to an untreated control group.

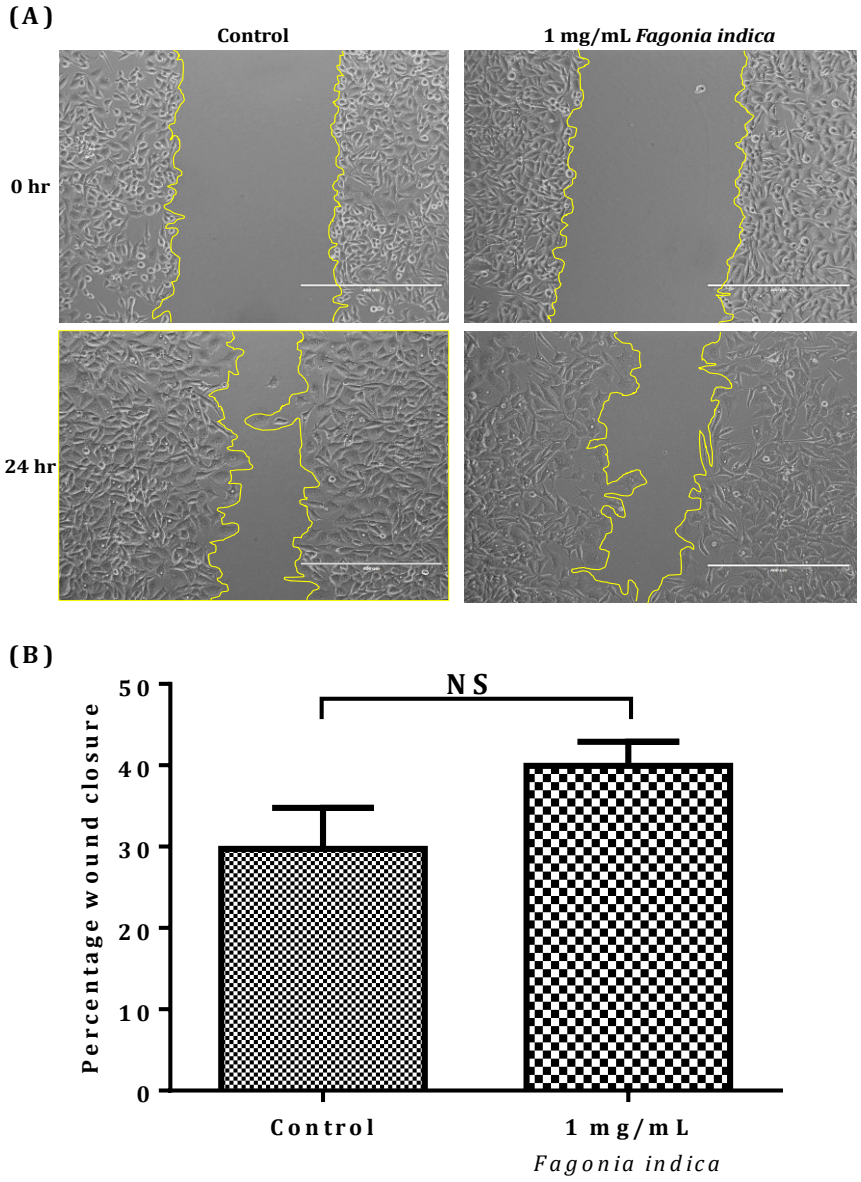

Figure 4.3.9: *Fagonia indica* treatment inhibits migration of H630 colon cancer cell lines. H630 cell monolayers were scratched with a 20 µL pipette tip and treated with 1 mg/mL of aqueous *Fagonia indica* for 24 hours. Wound closure was captured with an EVOS microscope at 0 hour and 24 hours timepoints at x10 magnification (A). (B) Wound closure was determined using ImageJ software and is displayed as percentage wound closure after 24 hours. Data denoted NS were not significant compared

to an untreated control analysed by unpaired student's t test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

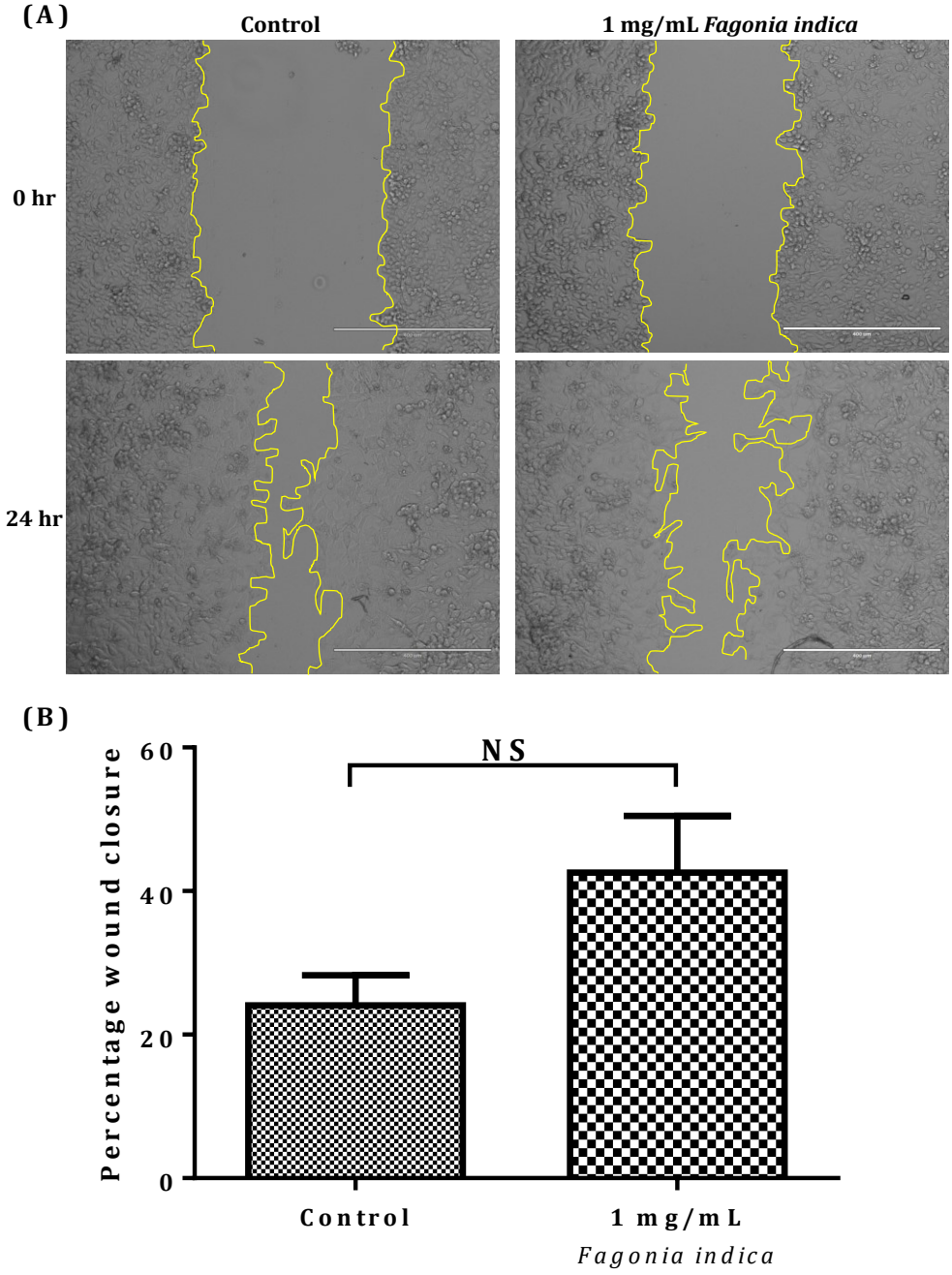

Figure 4.3.10: *Fagonia indica* treatment inhibits migration of RKO colon cancer cell lines. RKO cell monolayers were scratched with a 20 µL pipette tip and treated with 1 mg/mL of aqueous *Fagonia indica* for 24 hours. Wound closure was captured with an EVOS microscope at 0 hour and 24 hours timepoints at x10 magnification (A). (B) Wound closure was determined using ImageJ software and is displayed as percentage wound closure after 24 hours. Data denoted NS were not significant compared to an untreated control analysed by unpaired student's t test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

4.3.3 Mechanisms of action of *Fagonia indica* induced cytotoxicity in colon cancer

Vascular endothelial growth factor (VEGF), is an essential growth factor and a key player in the initiation of angiogenesis. Apatanib is a tyrosine kinase inhibitor which selectively inhibits VEGF receptor 2 (VEGFR2) (IC_{50} = 1 nM). Inhibition of VEGFR2 did not significantly reduce cell viability in untreated H630 cells (Figure 4.3.11A). Addition of Apatanib to *Fagonia indica* treated H630 cells significantly reduced cytotoxicity by 8.1% ($p<0.05$), compared to *Fagonia indica* treatment alone. In addition, mRNA expression of VEGFA in H630 cells was determined using qPCR (Figure 4.3.11B). In H630 cells expression of VEGFA was significantly reduced to 0.26-fold ($p<0.05$) of the control after treatment with 1 mg/mL *Fagonia indica* for 6 hours, at 24 hours there was no significant change in mRNA expression. The effect of VEGFA inhibition was more profound in RKO cells (Figure 4.3.12A), with an increase in cell viability of 19.6% ($p<0.01$) when combined with *Fagonia indica* treatment, compared to *Fagonia indica* treatment alone. However, there were no significant changes in VEGFA mRNA expression in *Fagonia indica* treated RKO cells between 6-24 hours (Figure 4.3.12B). Inhibiting VEGFR2 with Apatanib also alleviated the significant effect of *Fagonia indica* extract on colony formation in H630 cells (Figure 4.3.13).

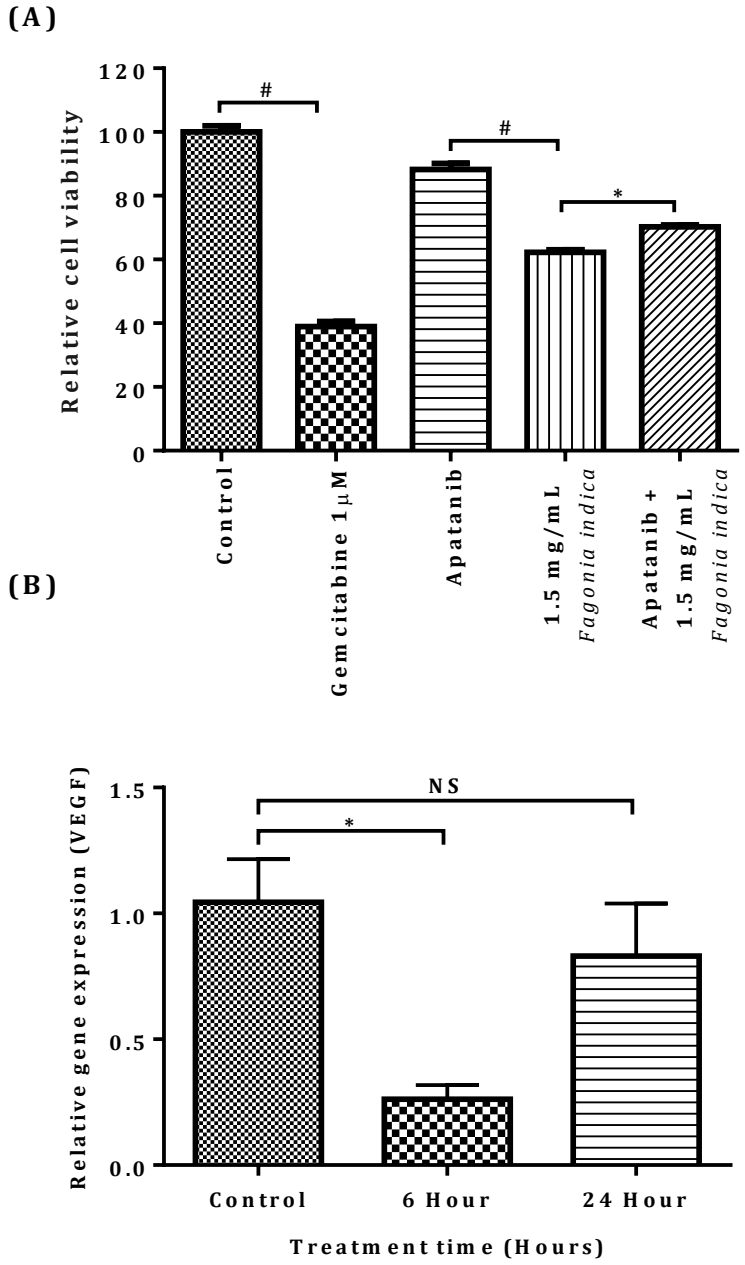

Figure 4.3.11: *Fagonia indica* extract treatment reduces expression of VEGF in H630 colon cancer cell line. (A) H630 cells were treated with 1.5 mg/mL aqueous *Fagonia indica* extract and/or 1 µM of VEGF inhibitor Apatanib. 1 µM of Gemcitabine was used as a positive control. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted * ($p<0.05$) and # ($p<0.0001$) were significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test (B) Gene expression of VEGF was determined on H630 colon cancer cell lines. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted * ($p<0.05$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. Data denoted as NS were not significant when compared to an untreated control. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

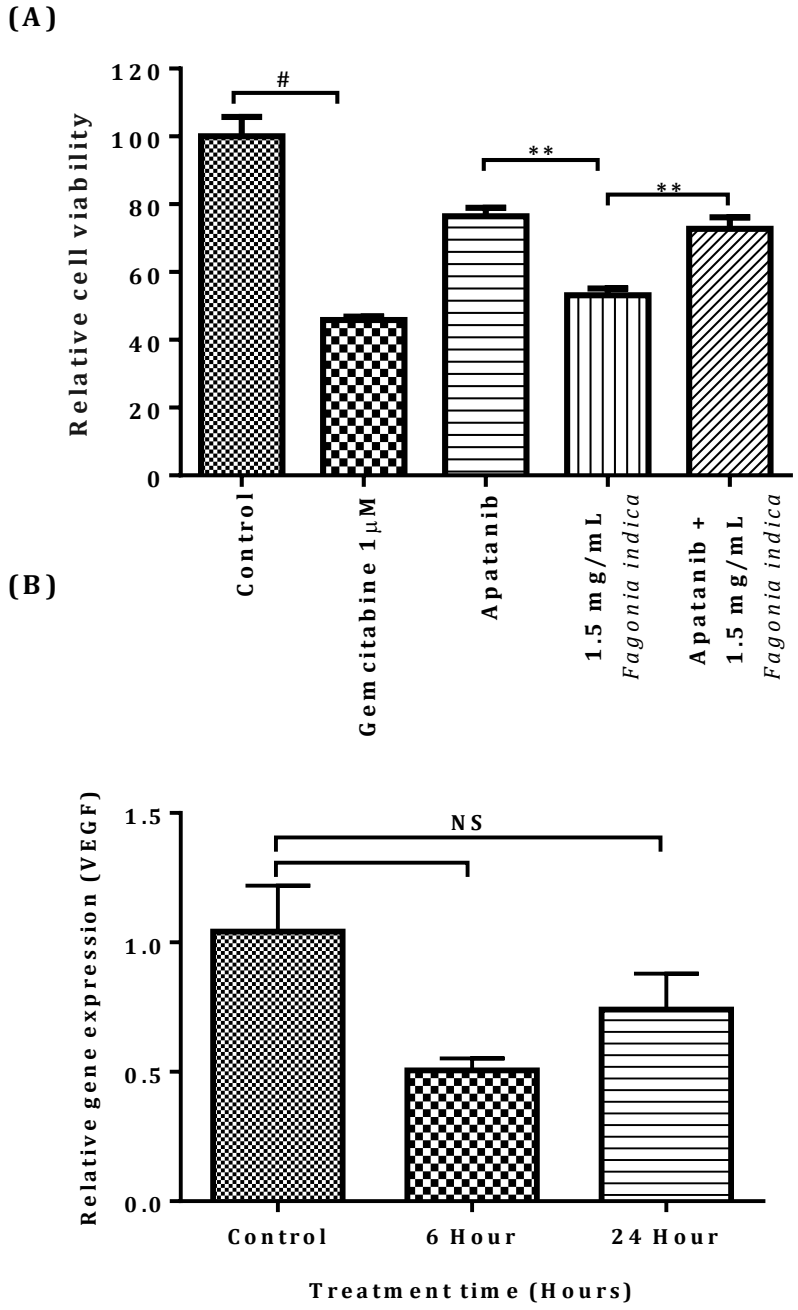

Figure 4.3.12: *Fagonia indica* extract treatment reduces expression of VEGF in RKO colon cancer cell line. (A) RKO cells were treated with 1.5 mg/mL aqueous *Fagonia indica* extract and/or 1 µM of VEGF inhibitor Apatanib. 1 µM of Gemcitabine was used as a positive control. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted ** ($p<0.01$) and # ($p<0.0001$) were significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test (B) Gene expression of VEGF was determined on RKO colon cancer cell lines. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted as NS were not significant when compared to an untreated control. by one-way ANOVA with

Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

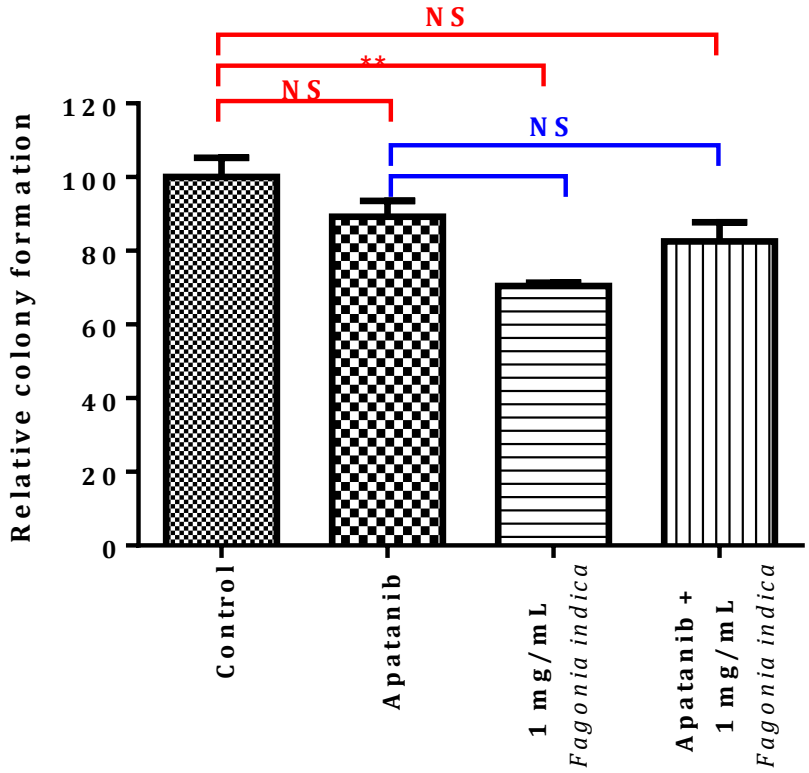

Figure 4.3.13: *Fagonia indica* treatment and VEGF inhibition inhibits proliferation from a single cell of H630 colon cancer cell lines. H630 cells were pre-treated with 1 µM VEGF inhibitor Apatanib and 1 mg/mL aqueous *Fagonia indica* for 24 hours and seeded at a density of 200 cells per well in a 24 well plate for 1 week. Colonies of 50> cells were stained using 0.01% crystal violet stain and colony formation was measured as a percentage of an untreated DMSO vehicle control. Data denoted ** ($p<0.01$) was significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. Data denoted NS was considered to be not significant. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

NF-κB is a major transcription factor, upregulating genes related to immune function and inflammation. Caffeic acid phenethyl ester (CAPE) acts as a specific inhibitor of the nuclear transcription factor NF-κB and was used to determine the effect of NF-κB inhibition on *Fagonia indica* treatment. Inhibition of NF-κB with CAPE did not significantly reduce cell viability in untreated H630 (Figure 4.3.14A). Addition of CAPE to *Fagonia indica* treated H630 cells did not significantly affect cytotoxity, compared to *Fagonia indica* treatment alone. In addition, mRNA expression of NF-κB subunit p65 in H630 cells was determined using qPCR (Figure 4.3.14B). In H630 cells expression of p65 was significantly reduced to 0.06-($p<0.001$) and 0.32-fold ($p<0.01$) of the untreated control after treatment with 1 mg/mL *Fagonia indica* for 6 hours and 24 hours, respectively. Similarly, in RKO cells inhibition of NF-κB did not significantly affect cell viability of untreated or *Fagonia indica* treated cells (Figure 4.3.15A). However, treatment with *Fagonia indica* extract let to a significant reduction in p65 expression in RKO cells to 0.47 ($p<0.01$) and 0.16-fold ($p<0.05$) of the untreated control at 6 and 24 hours, respectively (Figure 4.3.15B).

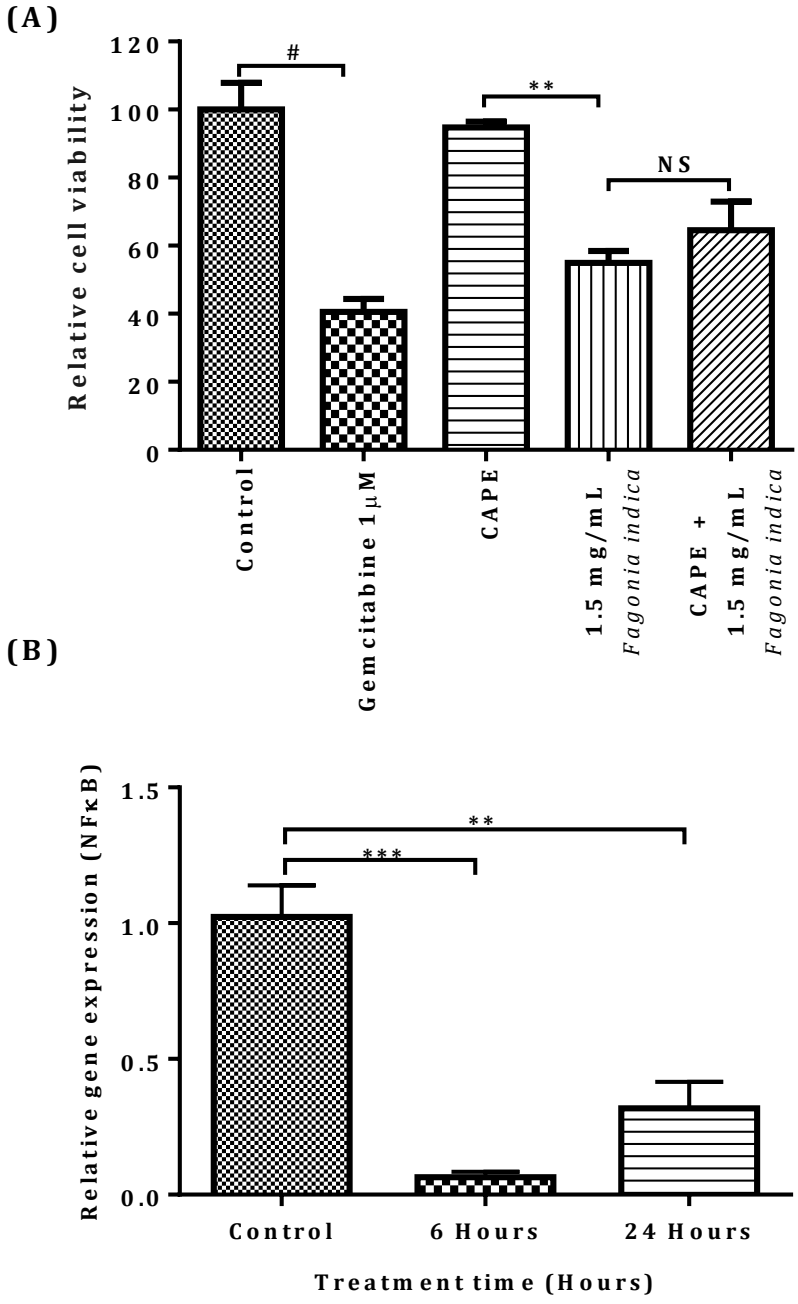

Figure 4.3.14: *Fagonia indica* extract treatment reduces expression of NF-κB in H630 colon cancer cell line. (A) H630 cells were treated with 1 mg/mL aqueous *Fagonia indica* extract and/or 1 µM of NFκβ inhibitor CAPE. 1 µM of Gemcitabine was used as a positive control. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted ** ($p<0.01$) were significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test (B) Gene expression of NF-κB was determined on H630 colon cancer cell lines. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted * ($p<0.01$) and ** ($p<0.001$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. Data denoted as NS were not significant when

compared to an untreated control. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

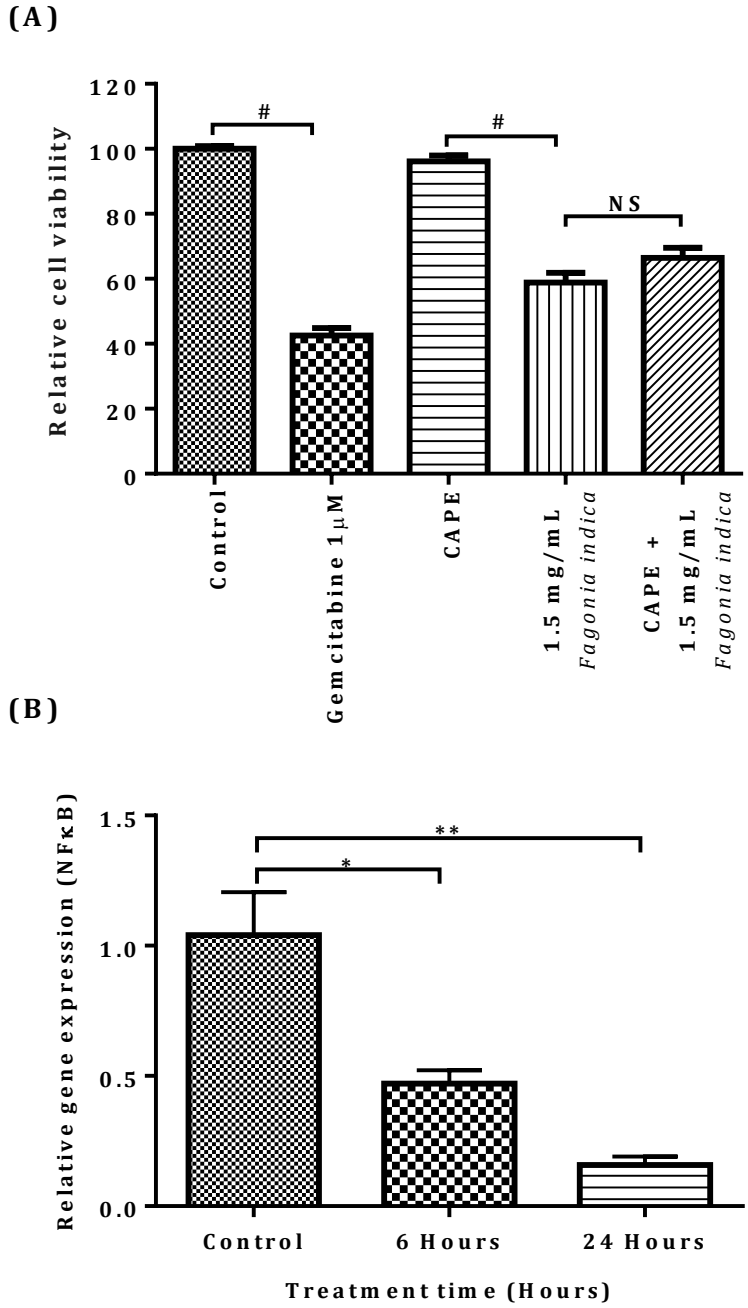

Figure 4.3.15: *Fagonia indica* extract treatment reduces expression of NF-κB in RKO colon cancer cell line. (A) RKO cells were treated with 1 mg/mL aqueous *Fagonia indica* extract and/or 1 μM of NFκB inhibitor CAPE. 1 μM of Gemcitabine was used as a positive control. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted ** ($p<0.01$) were significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test (B) Gene expression of NFκβ was determined on RKO colon cancer cell lines. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted * ($p<0.01$) and ** ($p<0.001$) were significant compared to the untreated control analysed by one-way

ANOVA with Dunnett's multiple comparison test. Data denoted as NS were not significant when compared to an untreated control. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

Intracellular adhesion molecule-1 (ICAM1), is a cell surface glycoprotein and an adhesion molecule, with demonstrated roles in inflammation and immune function. Expression of ICAM-1 mRNA was tested in *Fagonia indica* treated H630 and RKO cell lines using qPCR. After treatment with 1 mg/mL *Fagonia indica* extract for 6 hours, expression of ICAM-1 in H630 cells was significantly reduced to 0.18-fold ($p<0.05$) of the untreated control (Figure 4.3.16A). This reduced expression was not noted at 24 hours, where expression recovered to that of the untreated control. Expression of ICAM-1 was similarly reduced in RKO cells to 0.42- fold ($p<0.01$) of the untreated control at 6 hours, however in RKO cell lines expression was further reduced at 24 hours to 0.19-fold ($p<0.001$) of the untreated control (Figure 4.3.16B).

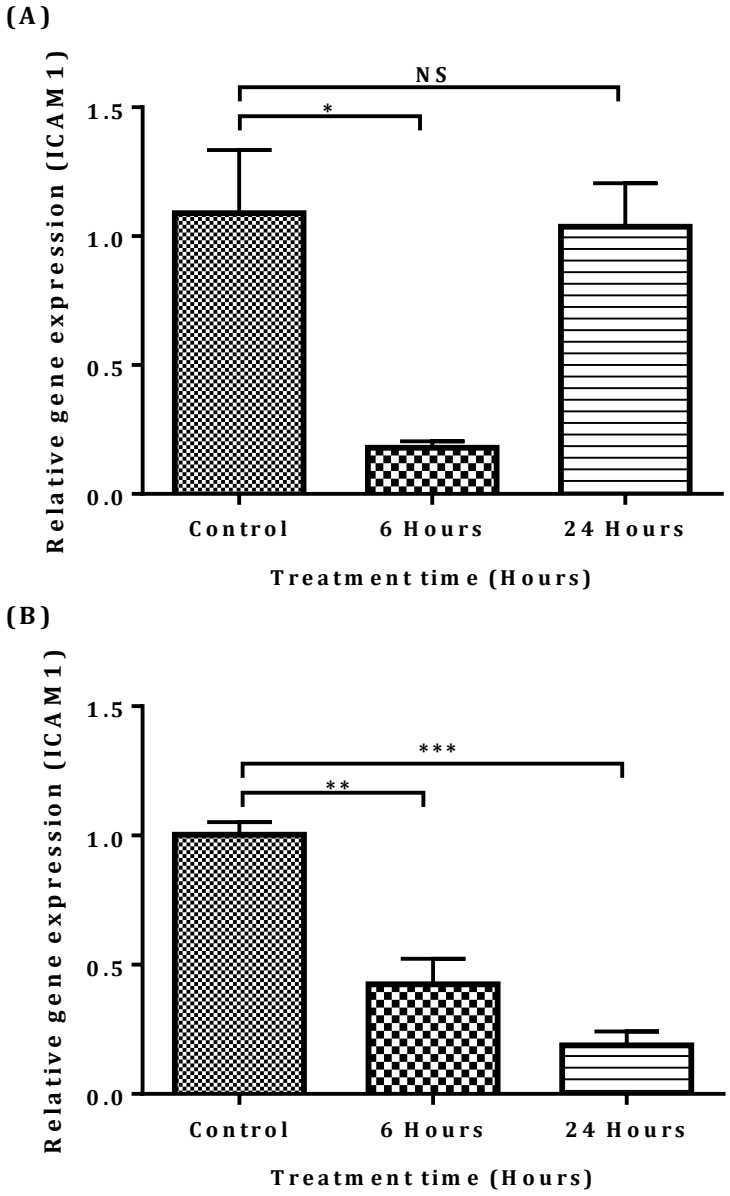

Figure 4.3.16: *Fagonia indica* extract treatment reduces expression of ICAM-1 H630 and RKO colon cancer cell lines. Gene expression of ICAM1 was determined on (A) H630 and (B) RKO colon cancer cell lines. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted * ($p<0.01$) and ** ($p<0.001$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

4.3.4 Effects of an aqueous *Fagonia indica* extract on chemotherapy-resistant colon cancer cells

Resistance to single chemotherapeutic agents occurs almost universally and drug tolerance in cancer is widely documented. It was therefore important to establish the effect of *Fagonia indica* on drug resistant colon cancer cell lines. Chemotherapy-resistant cell lines are developed by repeatedly exposing cells growing in culture medium to the relevant drug in increasing increments, the difference in response to the relevant chemotherapeutic agents between wild-type and drug resistant H630 and RKO cells can be found in Appendix Figure 7.2 and 7.3. Treatment with F*agonia indica* reduced cell viability in a time and concentration dependent manner in gemcitabine (H630-GM) and 5-fluorouracil resistant H630 cells (H630-5FU) (Figure 4.3.17). After 24 hours of treatment with 2.5 mg/mL *Fagonia indica*, H630-GM cell viability was significantly reduced by 29.4% ($p<0.0001$), increasing to 63.2% ($p<0.0001$) after 72 hours, compared to an untreated control (Figure 4.3.17A). Treatment with *Fagonia indica* extract also significantly reduced cell viability of 5-flourouracil resistant H630 cells (H630-5FU), however this did not appear to be in time-dependent manner (Figure 4.3.17B). After 24 hours of treatment with 2.5 mg/mL of *Fagonia indica* extract, cell viability of H630-5FU cells was significantly reduced by 29.7% ($p<0.0001$), which only slightly increased at 72 hours to 34.3% ($p<0.01$), compared to a untreated control. *Fagonia indica* extract was also able to reduce cell viability in a time and concentration-dependent manner in tomudex resistant RKO cell lines (RKO-TDX) (Figure 4.3.18). Treatment with 2.5 mg/mL of *Fagonia indica* significantly reduced cell viability by 35.2% ($p<0.0001$) after 24 hours and 57.7% ($p<0.0001$) after 72 hours respectively, compared to an untreated control.

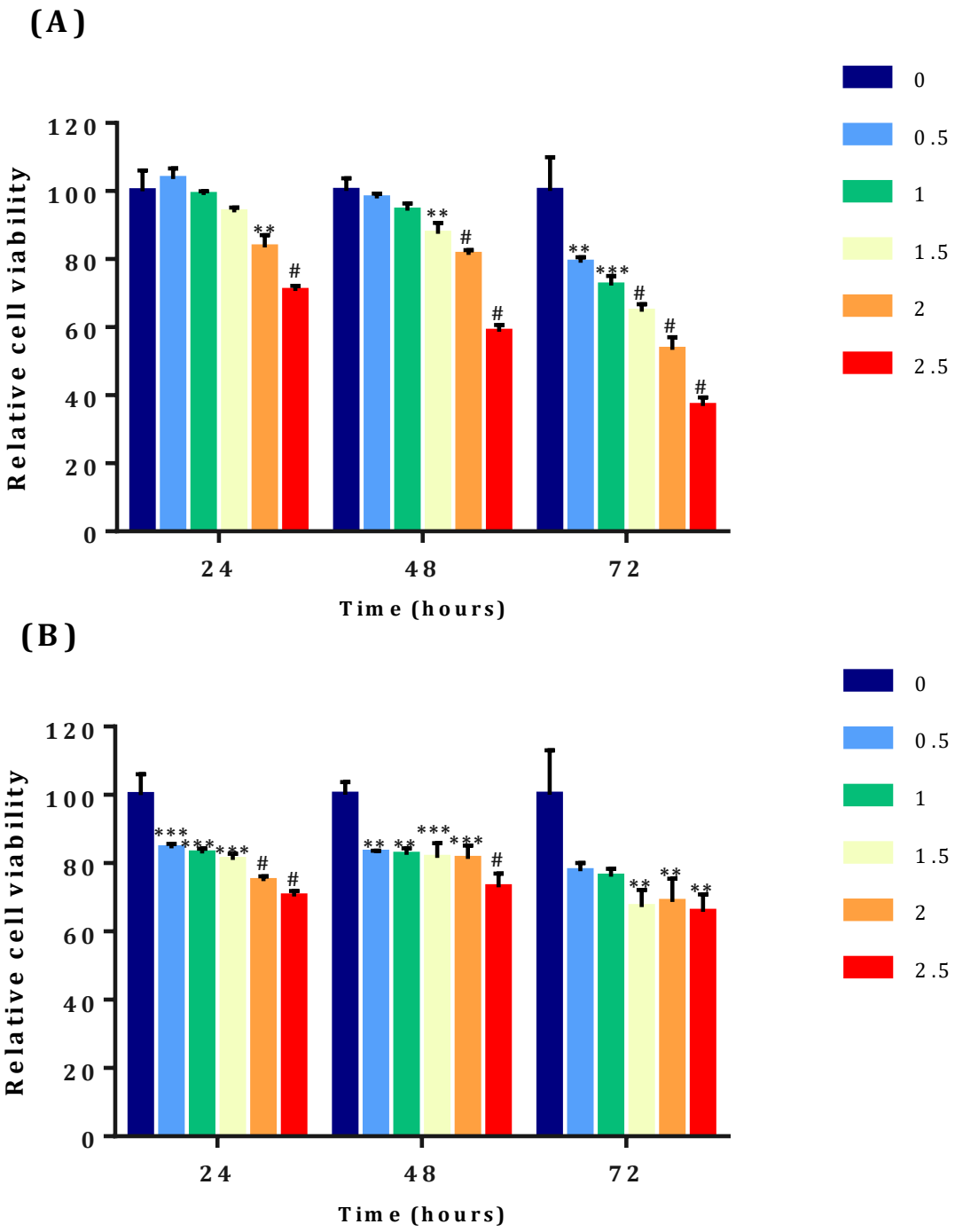

Figure 4.3.17: *Fagonia indica* extract treatment reduces *in vitro* cell viability of gemcitabine and 5-fluorouracil resistant colon cancer cell lines. (A) Gemcitabine resistant H630 (B) 5-fluorouracil resistant cells were treated with 0-2.5 mg/mL aqueous *Fagonia indica* extract for 24-72 hours. Cell viability was determined as a percentage of an untreated DMSO vehicle control using MTT assay. Data denoted * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and # ($p<0.0001$) were significant compared to the untreated control analysed by two-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

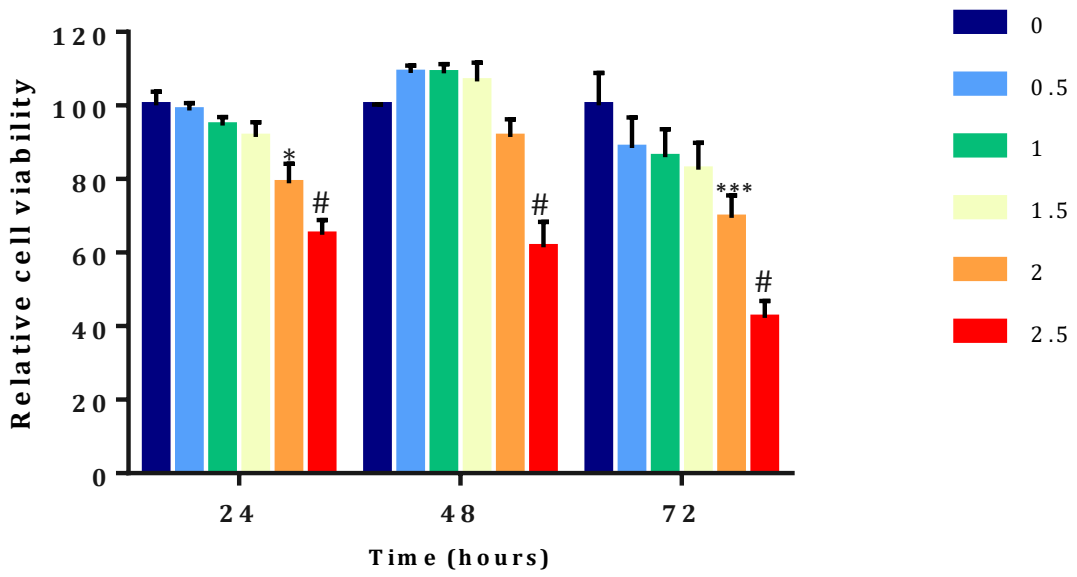

Figure 4.3.18: *Fagonia indica* extract treatment reduces *in vitro* cell viability of tomudex resistant colon cancer cell lines. (A) Tomudex resistant RKO cells were treated with 0-2.5 mg/mL aqueous *Fagonia indica* extract for 24-72 hours. Cell viability was determined as a percentage of an untreated DMSO vehicle control using MTT assay. Data denoted * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and # ($p<0.0001$) were significant compared to the untreated control analysed by two-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

As *Fagonia indica* extract was shown to significantly reduce cell viability in chemotherapy resistant H630 and RKO colon cancer cells, it was prudent to determine the potential of *Fagonia indica* to act as an adjuvant therapy alongside chemotherapeutic agents; gemcitabine and 5-fluourouracil. In H630-WT cells, gemcitabine treatment was able to significantly reduce cell viability by 41.6% ($p<0.01$) at 48 hours and 70.2% ($p<0.0001$) at 72 hours, compared to the untreated control (Figure 4.3.19A). In contrast, in H630-GM cells were not significantly affected by gemcitabine treatment after 24-72 hours (Figure 4.3.19B). In H630-WT cells there were also significant reductions in cell viability for cells treated with a combination of gemcitabine and *Fagonia indica* at 48 and 72 hours, compared to *Fagonia indica* treatment alone. However, there were no significant differences in cell viability between cells treated with gemcitabine compared to those with treated with the combination therapy between 24-72 hours. In contrast, in H630-GM cells there were significant reductions in cell viability at 48 hours for cells treated with a combination therapy of gemcitabine and *Fagonia indica*, compared with both gemcitabine and *Fagonia indica* treatments alone (34.8% ($p<0.0001$) and 21.4% ($p<0.01$), respectively).

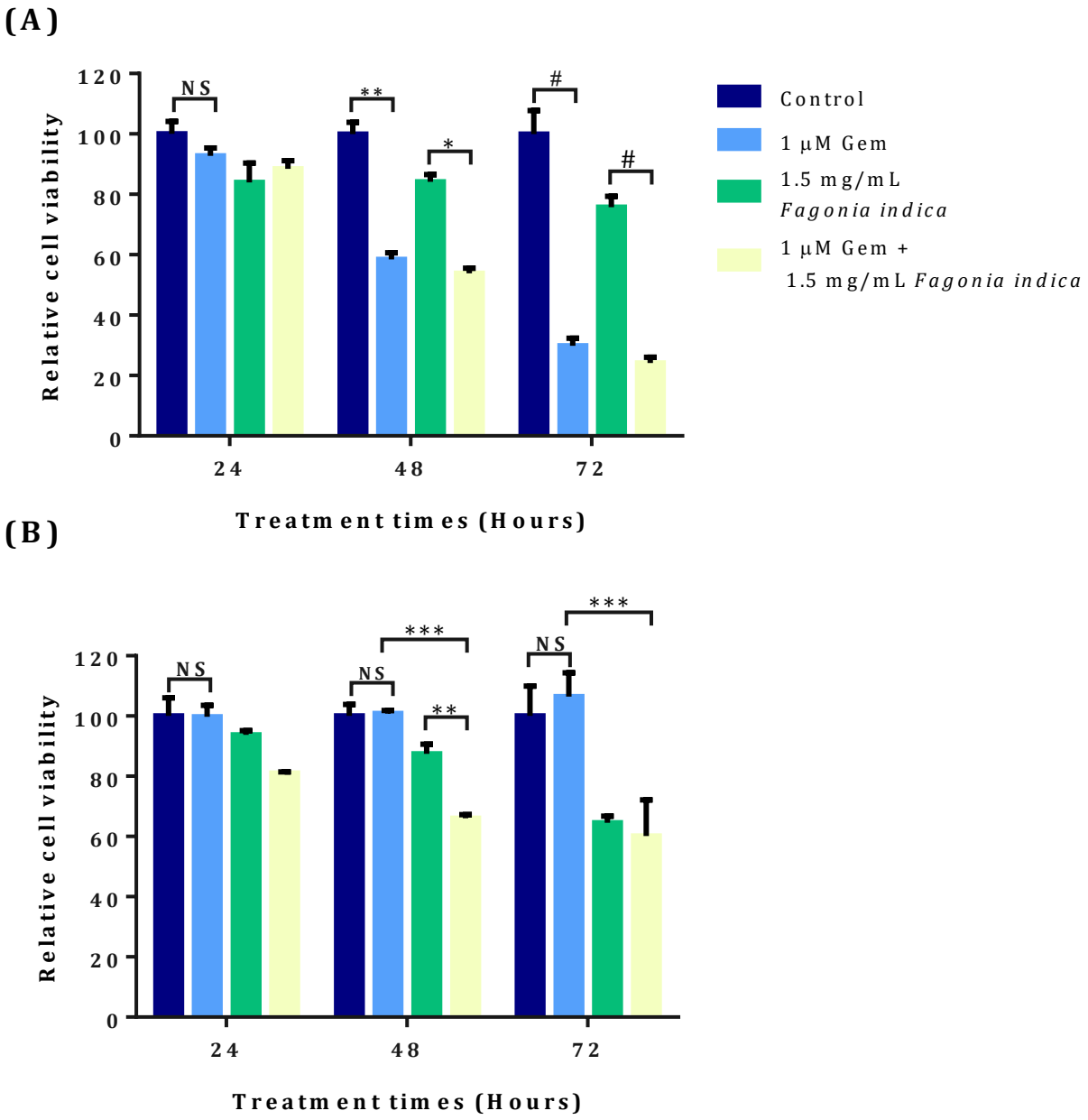

Figure 4.3.19: *Fagonia indica* extract and gemcitabine combination against *in vitro* wild-type and chemotherapy resistant H630 cells. (A) H630-WT and (B) H630-GM cells were treated with 1 µM gemcitabine and/or 1.5 mg/mL aqueous *Fagonia indica* extract for 24-72 hours. Cell viability was determined as a percentage of an untreated DMSO vehicle control using MTT assay. Data denoted * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and # ($p<0.0001$) were significant compared to the untreated control analysed by two-way ANOVA with Tukey's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

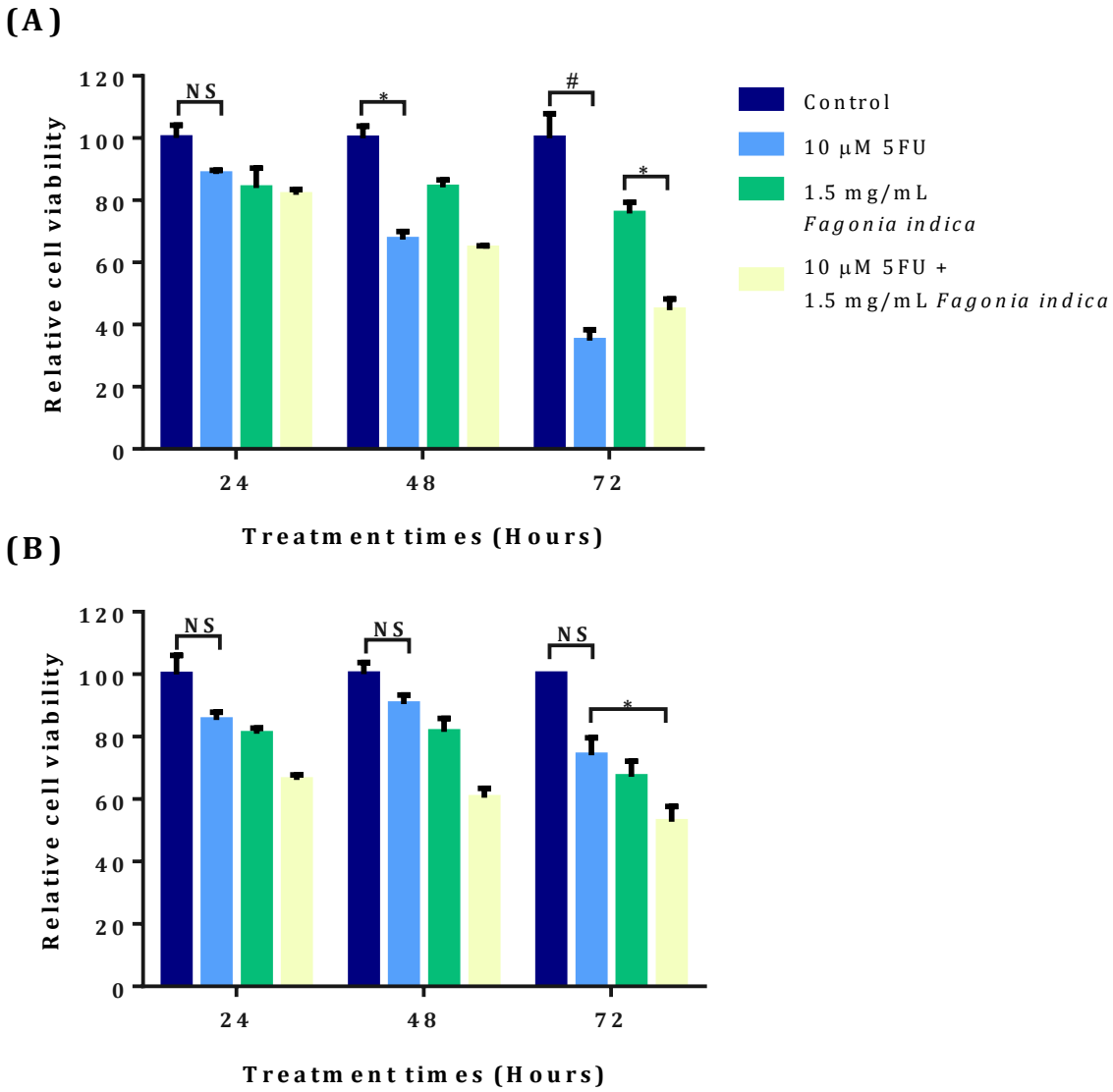

Figure 4.3.20: *Fagonia indica* extract and 5-Fluorouracil combination against *in vitro* wild-type and chemotherapy resistant H630 cells. (A) H630-WT and (B) H630-5FU cells were treated with 10 µM 5-fluorouracil and/or 1.5 mg/mL aqueous *Fagonia indica* extract for 24-72 hours. Cell viability was determined as a percentage of an untreated DMSO vehicle control using MTT assay. Data denoted * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and # ($p<0.0001$) were significant compared to the untreated control analysed by two-way ANOVA with Tukey's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

To determine whether *Fagonia indica* treatment is able to re-sensitise resistant cell lines to treatment with chemotherapeutic agents, H630 and RKO resistant cell lines were pre-treated with 1 mg/mL of aqueous *Fagonia indica* extract for 24 hours, prior to treatment with the relevant chemotherapeutic compound for 48 hours. In H630-GM cells which had no prior treatment with *Fagonia indica*, the cells which underwent subsequent treatment with gemcitabine for 48 hours had a significant increase in cell viability of 24.2% ($p<0.05$), when compared to a gemcitabine untreated control (Figure 4.3.22A). However, for H630-GM cells which were pre-treated with *Fagonia indica* extract for 24 hours, there was no significant difference between gemcitabine treated and untreated H630-GM cells. There was a significant reduction in cell viability of 33.7% ($p<0.01$) between H630-GM cells treated with gemcitabine post pre-treatment with *Fagonia indica*, compared to H630-GM cells treated only with gemcitabine. A similar pattern was noted in RKO-TDX cells with no prior treatment with *Fagonia indica*, subsequent treatment with tomudex for 48 hours caused a significant increase in cell viability of 22.3% ($p<0.05$) compared to a tomudex untreated control (Figure 4.3.22B). Again, this effect was lost between tomudex treated and untreated RKO-TDX cells after *Fagonia indica* pre-treatment. There was also a significant reduction in cell viability of 27.9% ($p<0.05$) between RKO-TDX cells treated with tomudex post pre-treatment with *Fagonia indica*, compared to RKO-TDX cells treated only with tomudex.

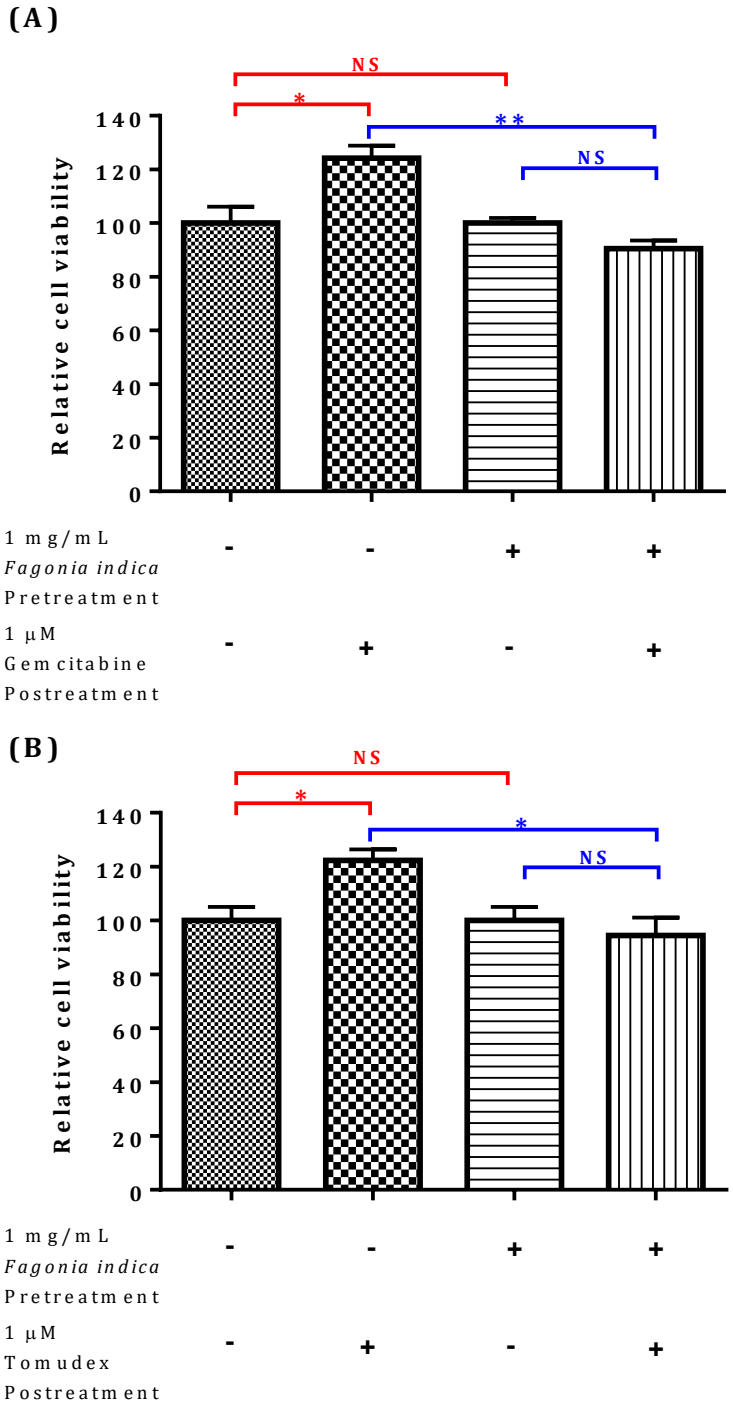

Figure 4.3.22: The effect of *Fagonia indica* extract pre-treatment on chemotherapy induced cytoxicity in resistant H630 and RKO colon cancer cell lines. (A) Gemcitabine resistant H630 and (B) Tomudex resistant RKO cells were pre-treated with 1 mg/mL aqueous *Fagonia indica* extract for 24 hours before treatment with Gemcitabine or Tomudex for 48 hours. Cell viability was determined as a percentage of relevant DMSO/ *Fagonia indica* vehicle control using MTT assay. Data denoted * ($p<0.05$) and ** ($p<0.001$) were significant and those denoted NS were not significant compared to the relevant control analysed by one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

The ATP-binding cassette (ABC) transporters, are a superfamily of membrane transporter proteins which are able to actively transport a wide-array of structurally diverse substrates and are frequently overexpressed in multi-drug resistant cancers. In chemotherapy resistant H630 and RKO cells, expression of ABC transporters; ABCG2 and ABCC4, was determined using qPCR. Expression of ABCG2 was not significantly different between H630-WT and H630-GM cells (Figure 4.3.23A). However, after treatment with *Fagonia indica* extract, mRNA expression of ABCG2 in H630-GM cell lines was significantly decreased at 6 and 24 hours to 0.32 ($p<0.01$) and 0.5-fold ($p<0.05$) of the untreated control, respectively (Figure 4.3.23B). In H630-5FU cells, expression of AGCG2 was significantly increased by 5.7-fold ($p<0.01$) of the H630-WT cell lines (Figure 4.3.24A). Similarly, *Fagonia indica* treatment significantly reduced ABCG2 expression in H630-5FU cells by 0.24 ($p<0.05$) and 0.31-fold ($p<0.05$) of an untreated control, after 6 and 24 hours respectively (Figure 4.3.24B). Interestingly, mRNA expression of ABCG2 was not significantly different between RKO-WT and RKO-TDX resistant cell lines (Figure 4.3.25A). After treatment with *Fagonia indica* extract for 24 hours, expression of ABCG2 increased significantly in RKO-TDX cells by 3.3-fold ($p<0.05$) of the untreated control (Figure 4.3.25B).

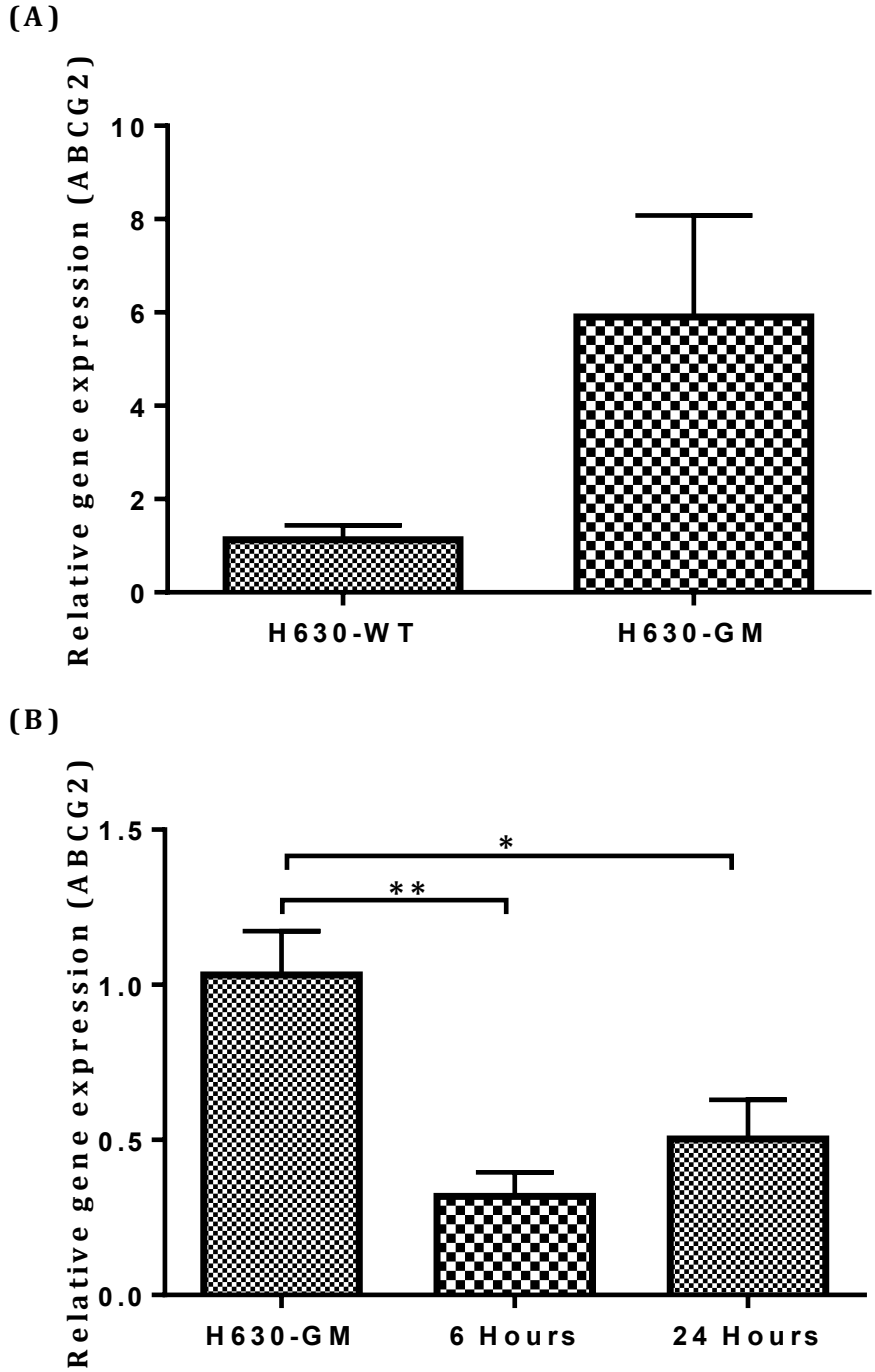

Figure 4.3.23: *Fagonia indica* extract treatment reduces expression of ABCG2 in gemcitabine resistant H630 cells. Gene expression of ABCG2 was determined on (A) H630-WT and H630-GM cell lines and (B) *Fagonia indica* treated H630-GM cells, at 6 and 24 hours. Pellets were collected and mRNA extracted immediately, or after of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted * ($p<0.05$) and ** ($p<0.01$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

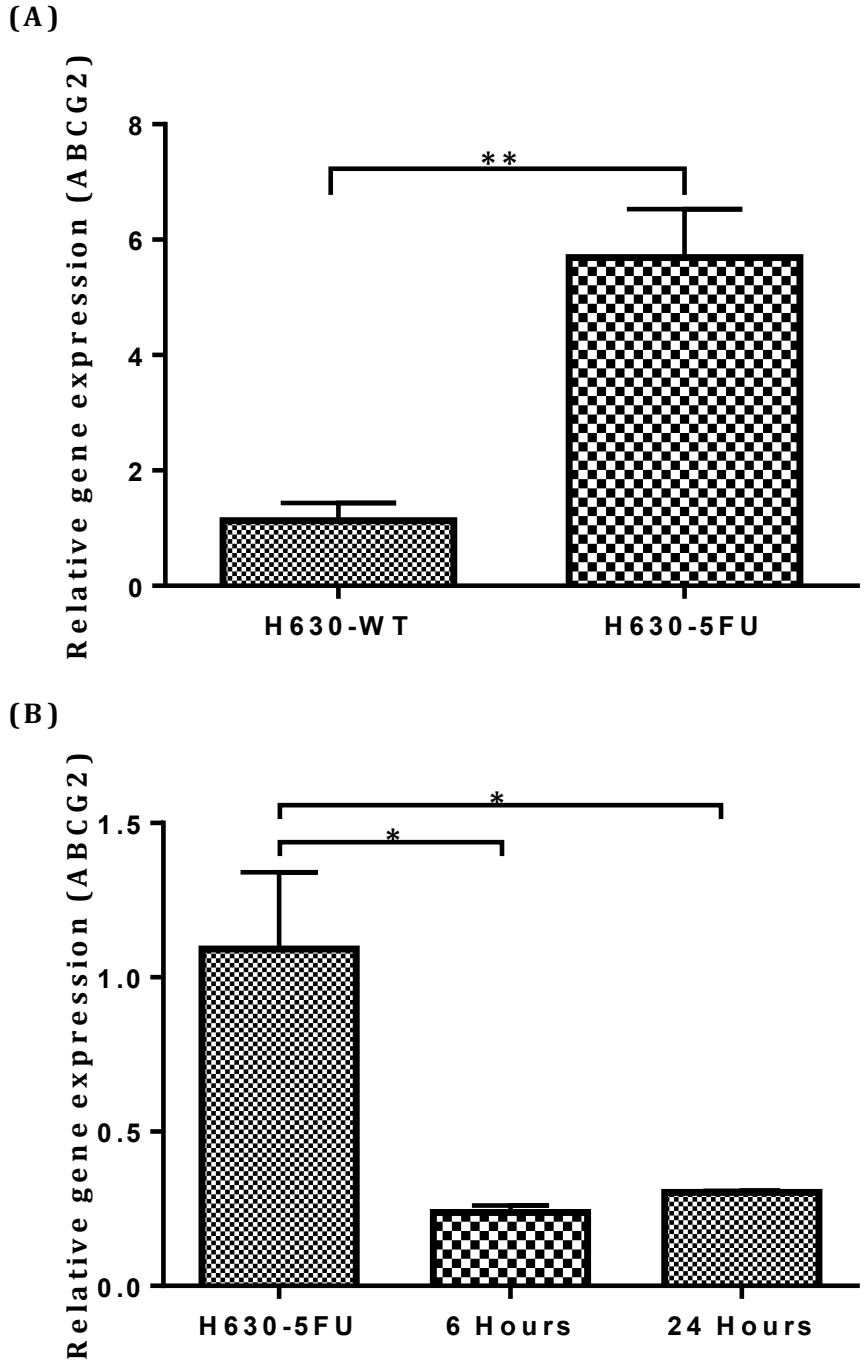

Figure 4.3.24: *Fagonia indica* extract treatment reduces expression of ABCG2 in 5-Fluorouracil resistant H630 cells. Gene expression of ABCG2 was determined on (A) H630-WT and H630-5FU and (B) *Fagonia indica* treated H630-5FU cells. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted * ($p<0.05$) and ** ($p<0.01$) were significant compared to the untreated control analysed by one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

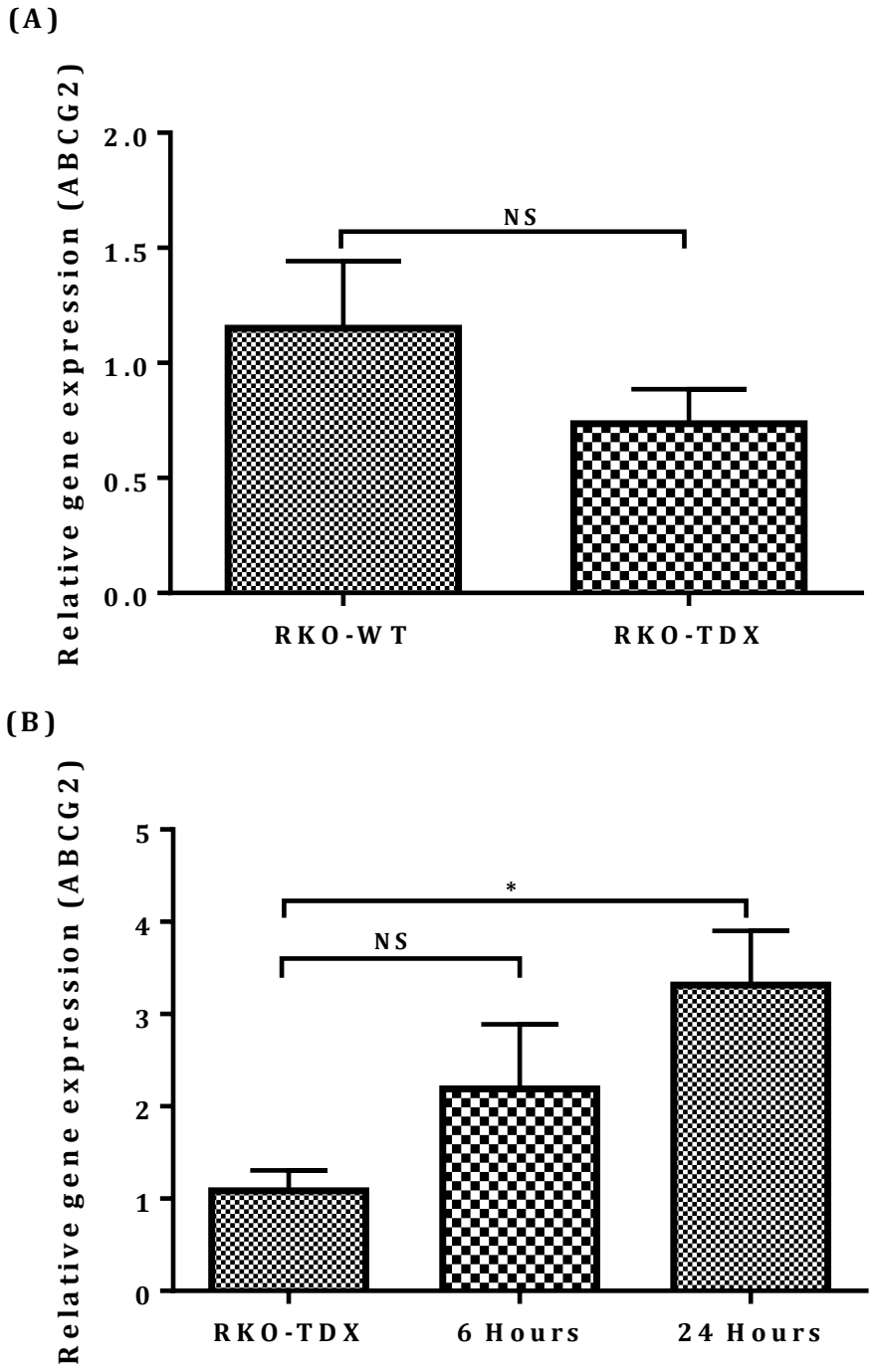

Figure 4.3.25: *Fagonia indica* extract treatment reduces expression of ABCG2 in Tomudex resistant RKO cells. Gene expression of ABCG2 was determined on (A) RKO wild-type vs TDX resistant cells and (B) *Fagonia indica* treated TDX-resistant RKO cells. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted * ($p<0.05$) and ** ($p<0.01$) were significant compared to the untreated control analysed by (A) unpaired student t test and (B) one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM

Expression of ABCC4 mRNA was also determined using qPCR. There was a significant increase in ABCC4 expression in H630-GM cells of 7.7-fold ($p<0.05$), compared to H630-WT cells. (Figure 4.3.26A) After treatment with *Fagonia indica,* expression of ABCC4 in H630-GM cells was significantly reduced to 0.12-fold ($p<0.001$) at 6 hours and 0.05-fold ($p<0.001$) at 24 hours, of an untreated control (Figure 4.3.26B). Similarly, expression of ABCC4 was significantly increased in H630-5FU cell lines by 12.4-fold ($p<0.01$) of the H630-WT cells (Figure 4.3.27A). Again, treatment with *Fagonia indica* extract significantly reduced expression of ABCC4 in H630-5FU cells by 0.18-fold at 6 hours and 0.13-fold at 24 hours, compared to an untreated control (Figure 4.3.27B). Finally, expression of ABCC4 was also significantly increased in RKO-TDX cells by 3.9-fold ($p<0.01$) compared to RKO-WT cell lines (Figure 4.3.28A). However, there were no significant changes in ABCC4 expression upon treatment with *Fagonia indica* in RKO-TDX cell lines (Figure 4.3.28B).

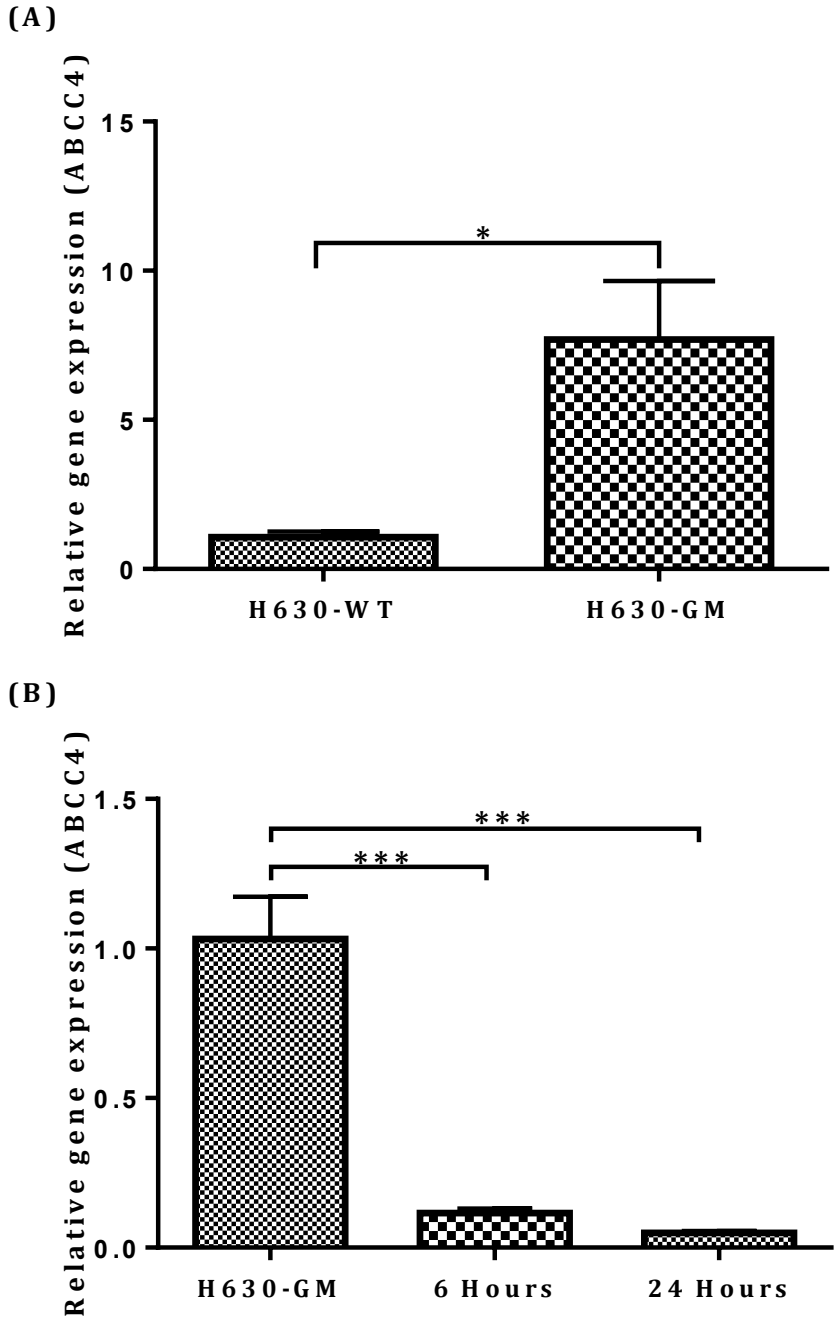

Figure 4.3.26: *Fagonia indica* extract treatment reduces expression of ABCC4 in gemcitabine resistant H630 cells. Gene expression of ABCC4 was determined on (A) H630-WT and H630-GM cells and (B) *Fagonia indica* treated H630-GM cells. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1 mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted * ($p<0.05$) and ** ($p<0.01$) were significant compared to the untreated control analysed by (A) unpaired student t-test and (B) one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

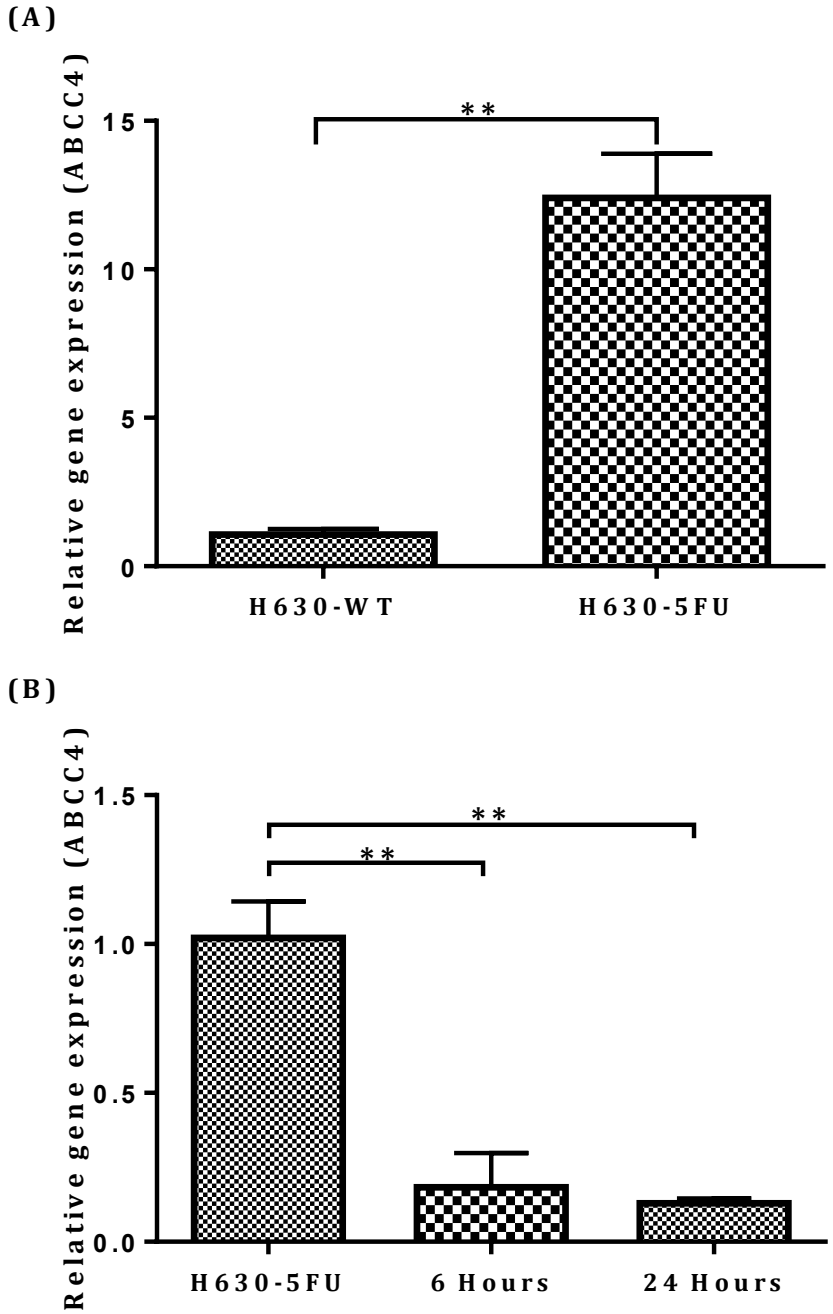

Figure 4.3.27: *Fagonia indica* extract treatment reduces expression of ABCC4 in 5-Fluorouracil resistant H630 cells. Gene expression of ABCC4 was determined on (A) H630-WT and H630-5FU cells and (B) *Fagonia indica* treated H630-5FU cells. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted * ($p<0.05$) and ** ($p<0.01$) were significant compared to the untreated control analysed by (A) unpaired student t-test and (B) one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

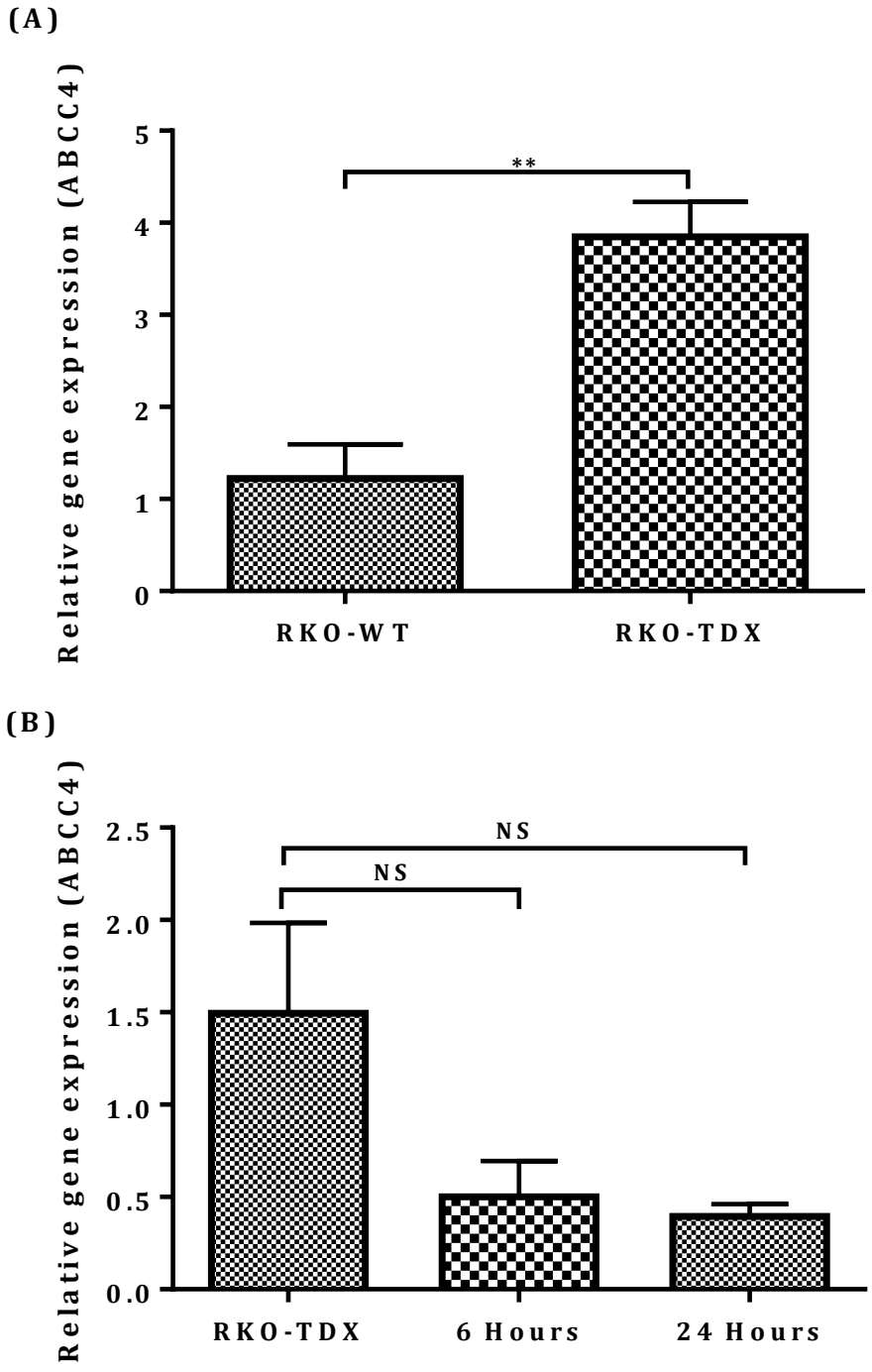

Figure 4.3.28: *Fagonia indica* extract reduces mRNA expression of ABCC4 in Tomudex resistant RKO colon cancer cells. Gene expression of ABCC4 was determined on (A) RKO wild-type vs TDX resistant cells and (B) *Fagonia indica* treated TDX-resistant RKO cells. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL aqueous extract. Values were normalised to housekeeping genes actin and YHWAZ. Data was analysed using the inverse delta Ct method and displayed as relative to an untreated control. Data denoted * ($p<0.05$) and ** ($p<0.01$) were significant compared to the untreated control analysed by (A) unpaired student t-test and (B) one-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

MK571 is an inhibitor of several ABCC multidrug resistance proteins, including ABCC1 and ABCC4, and is used widely to study the effects of ABCC transporters in models of disease. Treatment with MK571 did not significantly reduce cell viability in either H630-GM or RKO-TDX cell lines (Figure 4.3.30). Additionally, treatment with MK571 also did not significant affect *Fagonia indica* induced cytotoxicity in either H630-GM or RKO-TDX resistant cell lines (Figure 4.3.30).

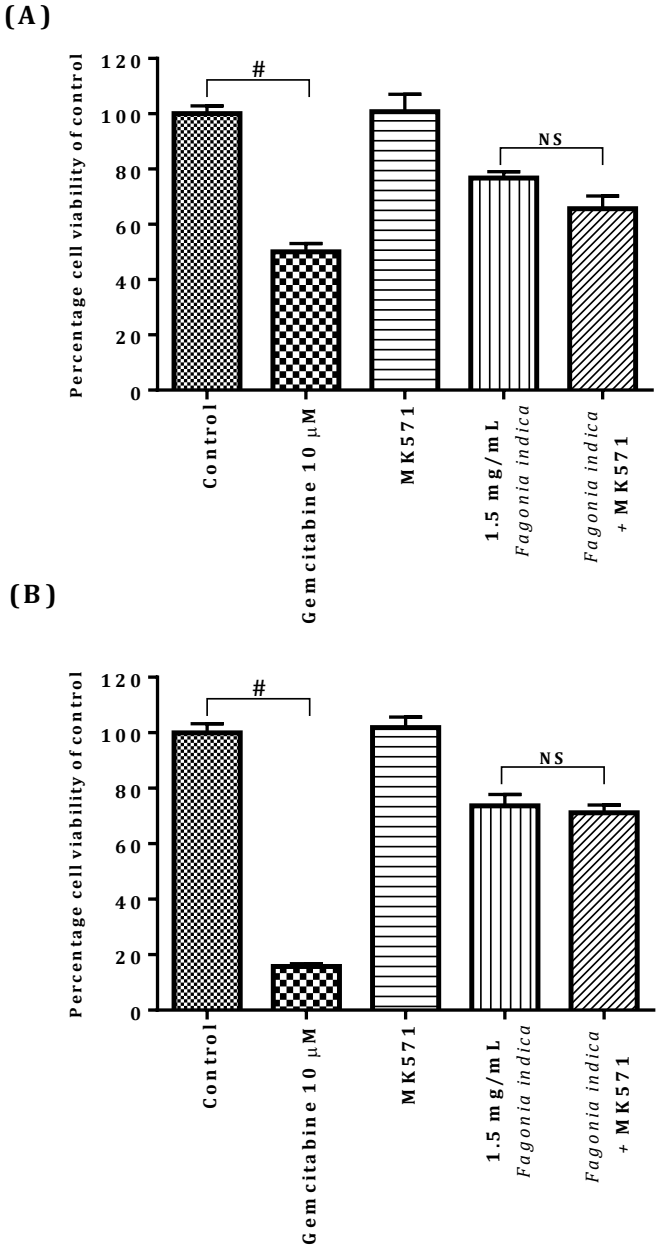

Figure 4.3.29: Effects of ABCC1/4 inhibition on extract induced chemotherapy resistant H630 and RKO cellular cytotoxicity. (A) Gemcitabine resistant H630 cells and (B) tomudex resistant RKO cell lines were treated with 1.5 mg/mL of *Fagonia indica* extract and/or 50 μM of MK571 for 48 hours. 10 μM Gemcitabine was used as a positive control. Cell viability was measured as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted as ** ($p<0.01$) and **** ($p<0.001$), was statistically significant compared to the untreated DMSO vehicle control, analysed using one-way ANOVA with Sidaks's multiple comparison test. All data points are representative of three independent experiments, performed in triplicate and are expressed as mean ± SEM.

Calcein-AM is a hydrophobic compound which diffuses across the cell membrane, where it is subsequently hydrolysed by intracellular esterase's into a hydrophilic, highly fluorescent compound which is retained within the cell. However, the calcein can be transported from the cell via efflux by members of the ABC superfamily of transporters, including ABCB1 and ABCC1. In the present study, Calcein-AM treatment was used to determine the differences in efflux of ABCC transporters between wild-type and chemotherapy resistant H630 and RKO cancer cells. There were no significant changes in intracellular Calcien fluorescence between H630-WT and H630-GM cell lines (Figure 4.3.31A), or between RKO-WT and RKO-TDX cell lines (Figure 4.3.32A) after 30 minutes of Calcein-AM treatment. Secondly, to determine the effect of *Fagonia indica* treatment on efflux of ABCC transporters, wild-type and resistant cell lines were treated with a combination of Calcein-AM and *Fagonia indica* extract for up to 30 minutes. In H630-GM cell lines, Calcein fluorescence was significantly reduced between *Fagonia indica* treated cell lines and the untreated control from 8-30 minutes ($p<0.0001$) of efflux activity (Figure 4.3.31B). A similar effect was noted in the H630-WT cell lines, whereby Calcein fluorescence was significantly reduced between *Fagonia indica* treated cells and the untreated control from 8-30 minutes of efflux activity ($p<0.0001$) (Figure 4.3.31C). In RKO-TDX cell lines, Calcein fluorescence was also significantly reduced between *Fagonia indica* treated cell lines and the untreated control from 5-30 minutes of efflux activity ($p<0.0001$) (Figure 4.3.32B). Similarly, in the RKO-WT cell lines, Calcien fluorescence was significantly reduced between *Fagonia indica* treated cells and the untreated control from 10-30 minutes of efflux activity ($p<0.0001$) (Figure 4.3.32C).

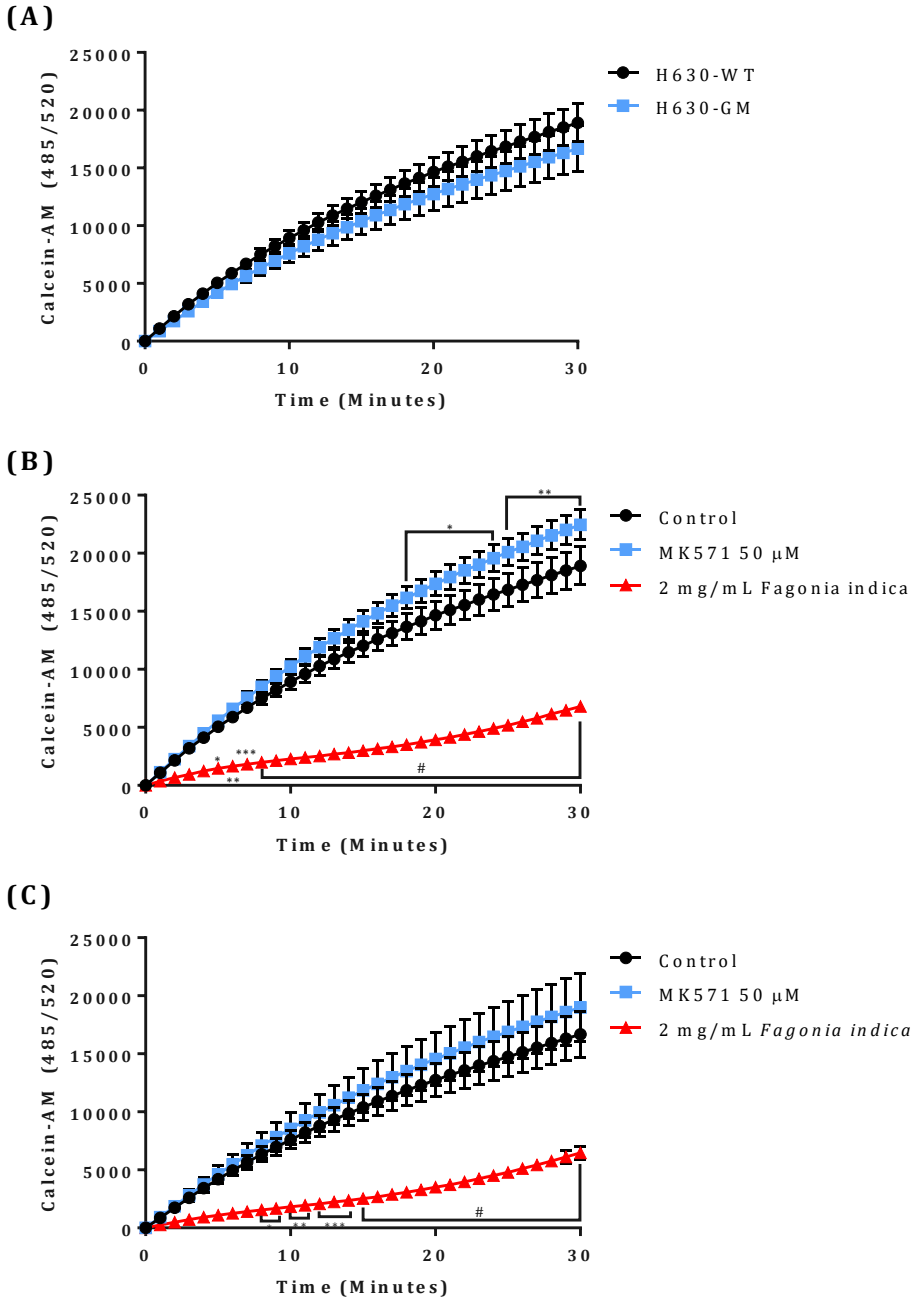

Figure 4.3.30: Fluorometric measurement of ABCC1 transporter protein activity in H630 wt and Gemcitabine resistant colon cancer cell lines. Mean intracellular Calcein fluorescence of (A) wild-type and gemcitabine resistant H630 cells and (B) gemcitabine resistant H630, (C) and wild type H630 cells treated with 2 mg/*mL Fagonia indica*, or 50 µM MK571 inhibitor and 5 mM of Calcein-AM for 30 minutes at 37 ºC. 50 µM MK571 inhibitor was used as a positive control. Data denoted as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and # ($p<0.0001$), was statistically significant compared to the untreated control, analysed using two-way ANOVA with Tukey's multiple comparison test. All data points are representative of three independent experiments, performed in triplicate and are expressed as mean ± SD.

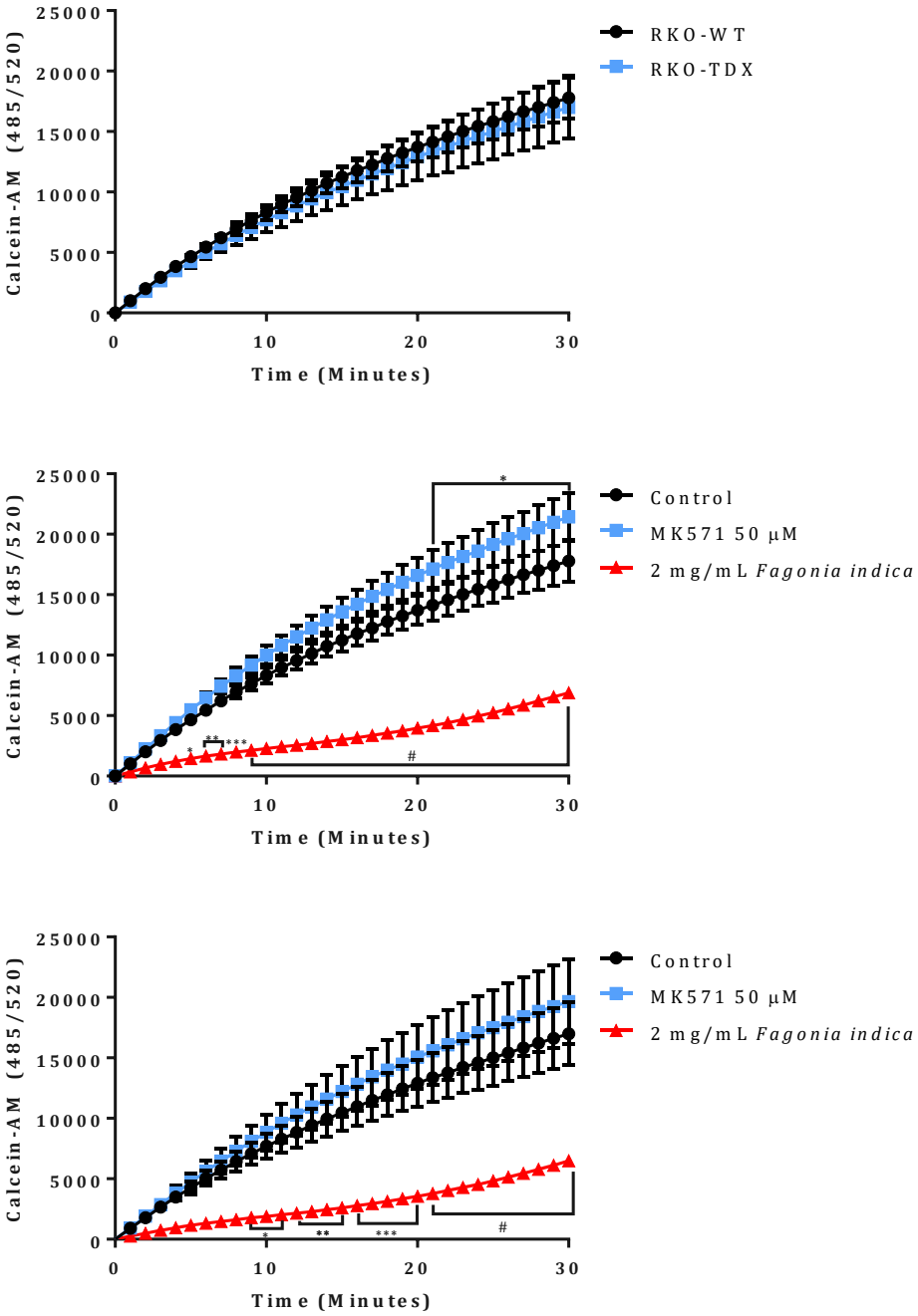

Figure 4.3.31: Fluorometric measurement of ABCC1 transporter protein activity in RKO wt and Tomudex resistant colon cancer cell lines. Mean intracellular Calcein fluorescence of (A) wild-type and tomudex resistant RKO cells and (B) Tomudex resistant RKO, (C) and wild type RKO cells treated with 2 mg/*mL Fagonia indica*, or 50 µM MK571 inhibitor and 5 mM of Calcein-AM for 30 minutes at 37 ºC. 50 µM MK571 inhibitor was used as a positive control. Data denoted as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and # ($p<0.0001$), was statistically significant compared to the untreated control, analysed using two-way ANOVA with Tukey's multiple comparison test. All data points are representative of three independent experiments, performed in triplicate and are expressed as mean ± SD.

4.4 Discussion

Aqueous extracts or 'teas' of herbal medicines have been used for centuries to combat disease and improve health status (Chandrasekara & Shahidi, 2018). For the first time, an aqueous extract of *Fagonia indica* demonstrated a cytotoxic effect against colon cancer cells *in vitro*. It was important to establish the potential of an aqueous extract to reduce cell viability of colon cancer cells, as brewing plant extracts and consuming bioactive compounds as a 'tea' is a popular vehicle of administration (McKay & Blumberg, 2006). Previous studies found that whole n-butanol and ethyl acetate extracts of *Fagonia indica* were able to induce significant reductions in cell viability of colon cancer cell line HCT (Shehab *et al*., 2011). Farheen *et al* (2015), isolated two novel compounds from an aerial extract of *Fagonia indica*; fagonicin and indicacin. Novel compounds; fagonicin and indicacin, demonstrated a 39.3 and 51.4% reduction in colon cancer cell line H-29 after 24 hours. Additionally, a novel steroidal saponin glycoside from an ethanolic extract of *Fagonia indica* was able to induce significant losses in cell viability of Caco-2 colon cancer cells after 24 hours (Waheed *et al*., 2012). Interestingly, there was some disparity between the MTT and neutral red assay with regards to the cytotoxic effect of *Fagonia indica*, with the neutral red assay highlighting much higher degrees of cell death in RKO colon cancer cells. A comparison study of the two cell viability assays, did not highlight any significant differences between the neutral red and MTT assay, when assessing common phenolic compounds from plant materials against human cell lines (Bacanli *et al*., 2017). However, other research has shown the MTT assay to have a higher degree of variability compared to other cell viability assays and was prone to interference from compound inhibitors (Van Tonder, Joubert, & Cromarty, 2015).

Due to rapid escalations in cellular proliferation, cancer cells preferentially shift their metabolism towards glycolysis in a process known as the 'Warburg effect' (Brown, Short, & Williams, 2018). Colon cancer has been considered a neoplasm of the Warburg phenotype, with the rate of ATP generation via glycolysis increasing up to 100 times (Chekulayev *et al*., 2015). In the current study, treatment with *Fagonia indica* extract for 24 hours significantly

reduced intracellular ATP concentration in both H630 and RKO colon cancer cell lines. Other studies have demonstrated the potential of aqueous plant extracts to disrupt metabolic mechanisms. *Scutellaria barbata* extract depleted intracellular ATP, NAD and inhibited glycolysis via mitochondrial dysfunction in colon cancer cell lines (V. Chen *et al*., 2012). Further research is required to determine whether an aqueous extract of *Fagonia indica* is able to inhibit glycolysis directly in colon cancer.

Lam *et al* (2012), found that *Fagonia indica* treatment activated expression of p53 and downstream transcription targets p21 and BAX. Further research has indicated caspase activation as a key element of p53-induced apoptosis. Z-VAD-fluoromethylketone (FMK) is a highly specific caspase inhibitor used to study activation of apoptosis (Van Noorden, 2001). Inhibition of pan-caspases with Z-VAD-FMK significantly reduced the effect of camptothecin treatment in colon cancer, despite activation of p53 and p21 (Han *et al*., 2002). However, in the current study there were no significant differences in the effect of extract treatment with Z-VAD-FMK, this could suggest activation of multiple pathways of apoptosis. Alongside apoptosis, another key feature of a successful therapeutic agent is the ability to inhibit subsequent proliferation (Farhana *et al*., 2020). In the current study, pre-treatment with *Fagonia indica* extract resulted in a subsequent reduction in colony formation in the following 7 days. Pawlak *et al* (2016), found that treatment of colon cancer cell lines with 5-azanculosides exerted long-lasting effects in cell viability; single exposure was sufficient to induce long term sensitisation to other more potent treatments. Further research is required to determine the ability of *Fagonia indica* to cause long term sensitisation to chemotherapeutic compounds such as, 5-FU and oxaliplatin.

The p38-mitogen activated protein kinase (MAPK) signalling pathway is a pivotal feature in stress-induced fate decisions such as cell cycle arrest and apoptosis (Cuenda *et al*., 2007; Thornton & Rincon, 2009). Studies have indicated a pivotal role of MKK3/ p38 MAPK activation and increased cell growth in colon cancer, with increased levels of MKK3 in late stage colon cancer correlating with shorter overall survival (Stramucci *et al*., 2019). In

response to double strand DNA damage, p38 MAPK becomes activated, leading to transcription of cell repair and apoptotic genes, such as p21 (Sui *et al*., 2014). This pathway has been used as a molecular target in the development of other chemotherapeutic agents such as Taxol and small molecule therapies; such as MTBT, which directly induces phosphorylation of p38 (Sui *et al*., 2014; Seidman *et al*., 2001). Despite this, there has been some severe contradictions on the outcome of p38 MAPK activation after chemotherapeutic treatment in colon cancer, with combined roles in apoptosis vs mediating pro-survival signalling and oncogenic growth (Pranteda *et al.*, 2020). Yang *et al* (2011), found that inhibition of p38 MAPK with SB203580 sensitises colon cancer cells to 5-FU via an increase in Bax expression, indicative of a role of p38 in cancer cell survival. In contrasting work, Cruz-Morcillo *et al* (2012) found that inhibition of p38 MAPK correlated with a decrease in 5-FU associated apoptosis, demonstrating a critical role for the signalling pathway in cellular responses to chemotherapy. In the current study, inhibition of p38 with competitive inhibitor SB203580 led to a significant reduction in extract-induced loss of cell viability in H630 colon cancer cell lines. This suggests p38 MAPK activation is critical in propagating apoptosis in *Fagonia indica* treated colon cancer cells. Activation of p38 signalling pathways indirectly inhibits activity of oncogene Akt, targeting Akt signalling pathways has since become of interest as a therapeutic target (Gratton *et al*., 2001). Research has indicated that PI3K/Akt signalling pathways are instrumental in promoting colon cancer stem cell proliferation and survival (S. Chen *et al*., 2017). P13K/Akt signalling is also strongly correlated with 5-FU induced apoptosis in colon cancer (Xu *et al*., 2017). In the current study, activation of Akt with SC79 abrogated the loss in cell viability of *Fagonia indica* in H630 cells. This suggests an important role of p38 MAPK signalling pathways in inducing apoptosis in colon cancer cells, which is reliant on the inhibition of Akt activation.

Vascular endothelial growth factor (VEGF), is a signal protein secreted by cells and associated largely with the formation of new vascularisation. Lin *et al* (2017) described the autocrine production of VEGF by tumour cells, resulting in the increase in tumour proliferation, growth

and metastasis. In a novel finding, we demonstrate a role for VEGF stimulation in *Fagonia indica*-induced cellular cytotoxicity in human colon cancer cell lines. Treatment with *Fagonia indica* led to a significant reduction in mRNA expression of VEGF-A in H630 cancer cell lines after 6 hours. Plant derivates have been utilised in previous studies to inhibit the phosphorylation of VEGF receptors, by inhibiting VEGFR2 directly or reducing transcription of VEGF-A (Kondo *et al.*, 2002; Zhao *et al*., 2017). Inhibition of VEGF-A with selective small molecule inhibitor apatanib, abrogated some of the effects of *Fagonia indica* on cell viability in both RKO and H630 cell lines. Triterpenoids isolated from other species *Celatrus* and *Maytenus spp* have been shown to have prominent angiogenic properties *in vivo* (Mu *et al*., 2012). In the current study, inhibition of VEGF-A led to increased colony formation in H630 cells when compared to *Fagonia indica* treatment alone. Aqueous extracts of other plant extracts; such as *Cinnamomim cassia* have been shown to suppress angiogenesis via the inhibition of VEGFR2 phosphorylation (Kim, Kim, & Kim, 2015). It is possible that *Fagonia indica* extract targets VEGF-A secretion or inhibition of the VEGF receptor site, which could compete with the action of apatanib when used in-tandem. Future research examining the effect of *Fagonia indica* on the direct secretion of VEGF-A into culture medium would be beneficial in determining the scale of this relationship.

VEGF plays a pivotal role in the signalling and up-regulation of target genes such as ICAM-1 via the phosphorylation of PI3K/Akt/NO pathways (Radisavijevic, *et al*., 2000). ICAM-1 has been associated with several tumour types; including colon cancer, due its ability to module inflammation and regulate vascular permeability (Schellerer *et al*., 2019). In the current study, ICAM-1 mRNA expression was determined in extract treated RKO and H630 colon cancer cells. As expected, gene expression of ICAM-1 was reduced to a similar extent as VEGF expression in both cell lines. In RKO cancer cells, this change in gene expression was enhanced at 24 hours, potentially indicating a more prominent role for ICAM-1 in this cell line. VEGF also induces adhesion molecules during inflammatory states, by stimulating expression of ICAM-1 and VCAM-1 in an NFκB-dependent manor (Kim, 2001; Frank &

Listanti, 2008). Patel *et al* (2017) inhibited NF-κB subunits NF-κB2 and RelA and demonstrated a marked decrease in ICAM-1 expression with a corresponding reduction in smooth muscle proliferation and Akt phosphorylation. In the present study, expression of NF-κB subunit p65 was reduced in both RKO and H630 cell lines at both 6 and 24 hours post extract treatment. However, inhibiting NF-κB activation using CAPE did not significantly alter extract-induced cellular cytotoxicity, suggesting that NF-κB signalling is not a key mechanism of *Fagonia indica* but may be related to alternative extract effects.

Despite early prevention and novel treatment options, intrinsic and acquired resistance to chemotherapy remains a significant burden to colon cancer treatment (Van Der Jeught *et al.*, 2018). Approximately half of all metastatic colon cancer patients are now resistant to 5-FU and 5-FU-based chemotherapies (Douillard *et al*., 2000). In the current study, an aqueous extract of *Fagonia indica* was able to cause a significant time and concentration-dependent reduction in cell viability of chemotherapy resistant colon cancer cell lines. There were differences in the extent of extract induced cell death between gemcitabine and 5-FU resistant H630 cells, which could relate to the mechanisms of action of the respective chemotherapeutic agents (Longley, Harkin, & Johnston, 2003; Plunkett *et al*., 1995). Interestingly, treatment with *Fagonia indica* for 24 hours was able to significantly increase sensitivity of resistant H630 and RKO cell lines to chemotherapy. Other research has demonstrated the ability of novel plant extracts to sensitise 5-FU resistant colon cancer cells to the therapeutic activity of the drug (González-Vallinas *et al*., 2013; Shakibaei *et al*., 2014). This demonstrates the potential of *Fagonia indica* as a successful cytotoxic agent against both sensitive and resistant colon cancers. There was also a significance increase in cell death when *Fagonia indica* was used as an adjuvant therapy against wild-type and resistant colon cancer cell line, H630. This is of particular importance, as modern treatment plans utilise a combination chemotherapy approach, with the intention of targeting micro-metastases and reduce recurrence (Morse, 2005).

ABC membrane transporters have been found to be overexpressed in many tumour types displaying multi-drug resistance, where they play key roles in disease development and tumour promotion (Domenichini, Adamska, & Falasca, 2019). ABCG2 and ABCC4 have been previously implicated in the acquisition of colon cancer cells to chemotherapy resistance, as such, they have become a key target of novel pharmacological therapeutics (Hsu *et al*., 2018; J. Hu *et al*., 2017). In the present study, populations of colon cancer cells demonstrating resistance to chemotherapeutic agents tomudex, gemcitabine and 5-FU were isolated and expanded. Gemcitabine and 5-FU resistant H630 colon cancer cells had significantly increased mRNA expression of ABCG2 and ABCC4, compared to the wild-type control. Resistance to 5-FU is well documented in colon cancer and has been correlated with over expression or aberrant expression of ABCG2 and ABCC4 (Chen *et al*., 2017; Qu *et al.*, 2013). Resistant H630 clones were treated with *Fagonia indica* extract, resulting in a significant reduction in both ABCC4 and ABCG2 expression. Previous studies have demonstrated an ability of plant-derived molecules to inhibit ABCG2, sensitising cells to oxaliplatin apoptosis (Chen *et al*., 2018). Further research, correlated treatment with *Evodia rutaecarpa* extract with ABCG2 suppression, via inhibition of NF-κB signalling pathways (Sui *et al*., 2016). In the present study, *Fagonia indica* treatment reduced expression of both ABCG2 and NF-κB, highlighting a potential avenue for combatting resistance in colon cancer cells. Reducing or inhibiting ABCC4 transport has also been shown to be a successful method of drug reversal in colon cancer (Wink, Ashour, & El-Readi, 2012; Zhang *et al*., 2010). In RKO cell lines, only ABCC4 was overexpressed in tomudex resistant clones, with expression of ABCG2 increasing upon treatment with *Fagonia indica*. Treatment with *Fagonia indica* was also able to reduce the expression of ABCG2 in tomudex resistant RKO cell lines, following similar patterns to resistant H630 cell lines.

Plant extracts high in flavonoids have been shown to reverse resistance to common chemotherapeutic agents in colon cancer, via the direct inhibition of ABC transporters 1, 4 and -5 (Wu *et al.*, 2005). ABC transporter efflux assays can be used to measure transporter-

mediated fluorescent substrate activity to evaluate potential inhibitors or competitive substrates (Ansbro *et al.*, 2013). In the present study, transport function of ABCC1 was assessed via the measurement of calcein- extrusion with fluorimetry (Homolya *et al.*, 1996). Calcein fluorescence was significantly lower in both wild-type and chemotherapy resistant colon cancers after treatment with *Fagonia indica* extract, suggesting increased efflux and activity of ABCB1 or ABCC1. Weber *et al* (2004), found that very low concentrations of plant extracted flavonoid quercetin was sufficient to significantly impact ABCB1 activity in several cell lines. Additionally, studies have shown that plant-derived quercetin was able to modulate efflux activity in ABCC1 membrane transporters by markedly increasing ABCC1-mediated ATP hydrolysis, thus increasing the rate of efflux (Chearwae *et al*., 2006). Previous studies have isolated and identified quercetin as a flavonoidal bioactive compound from both ethyl acetate and n-butanol soluble fractions of *Fagonia indica* (Shehab *et al.*, 2011). This suggests that although gene expression of various ABC transporters can be reduced by *Fagonia indica* treatment, the extract has dual action by increasing the rate of efflux immediately upon treatment. Further studies are required to determine the nature of *Fagonia indica* interactions with ABCC1, it is possible that extract compounds directly interfere with calcein-AM in this assay.

Another important consideration regarding the expression and activity of ABC transporters, includes the dependency of intracellular ATP concentration for substrate efflux (Weber *et al*., 2004). In the present study, treatment with *Fagonia indica* extract significantly reduced intracellular ATP concentration in both H630 and RKO colon cancer cell lines. Studies have indicated that reprogrammed glucose metabolism towards increased glycolysis and subsequent ATP production has been directly linked to tumour chemoresistance to 5-FU (Huang *et al*., 2019). Interestingly, other studies have shown that inhibition of glycolysis production of ATP restored the retention of daunorubicin and mitoxantrone in ABCG2 and ABCB1 overexpressing cell lines (Nakano *et al*., 2011). Reduced expression of ABC

transporters in *Fagonia indica* treated colon cancer cell lines could potentially be related to disrupted metabolism of plant treatment.

4.5 Conclusion

This chapter has demonstrated; for the first time, the ability of an aqueous extract of *Fagonia indica* to induce cell death in p53-dependent colon cancer cell lines, H630 and RKO. Cytotoxic mechanisms of *Fagonia indica* were associated with activation of p38/ MAPK signalling pathways, this was demonstrated using inhibitory/activation assays of p38 and Akt. Metabolic dysregulation was also raised as a potential mechanism of action for *Fagonia indica*, with intracellular ATP concentration significantly reduced in both colon cancer cell lines. Future work is required to establish the effect of *Fagonia indica* on the production of ATP and to determine whether the extract inhibits glycolysis in colon cancer.

The effect of *Fagonia indica* on other pathways associated with increased tumorigenesis; including angiogenesis, migration and inflammation, in colon cancer have so far remained elusive. This chapter eluded; for the first time, potential targets of aqueous *Fagonia indica* extract against colon cancer cells. These included a potential target pathway of VEGF, NFκB and ICAM-1. Treatment of colon cancer cell lines with *Fagonia indica* resulted in significantly reduced expression of all three target genes. Activity of VEGFR2 was important for the cytotoxic activity of *Fagonia indica*, this was demonstrated using VEGF inhibitor apatanib. Future work is required to determine the effect of *Fagonia indica* on the release of soluble VEGF-A into culture media.

This chapter also introduced the potential of *Fagonia indica* extract to induce cell death in chemotherapy resistant colon cancers. Aqueous extract of *Fagonia indica* was able to reduce cell viability in all three-chemotherapy resistant colon cancer cell lines and sensitise resistant cell lines to subsequent chemotherapy treatment. One proposed mechanism of action in resistant cell lines was the inhibition or targeting of ABC transporters. The chapter demonstrated the ability of *Fagonia indica* to reduce the expression of both ABCG2 and

ABCC4 membrane transporters. Further work is required to elucidate the mechanisms of action in chemotherapy resistant colon cancers and to determine to what degree the extract is able to reverse acquired resistance mechanisms.

5 Chapter 5: Chemical investigation of a bioactive fraction of Fagonia indica

5.1 5.1 Introduction

The isolation and characterisation of naturally synthesised compounds from medicinal plants has been a topical subject, due to the vast chemical diversity and bioavailability of potential therapeutic compounds (Sasidharan *et al*., 2011). A major advantage of using natural products to source novel compounds is the unmatched diversity, in terms of functional groups and structural complexity (Zhang, *et al*., 2018). As previously described, members of the taxane class of chemotherapy, including paclitaxel, were originally separated and identified from the biomass of Pacific yew tree needles (Y. Wang *et al*., 2011). In regards to paclitaxel, a typical isolation process would involve the production of a 'crude' solvent extract and then subsequent partitioning and quantification using solid-phase extraction and liquid chromatography (LC) (Mattina & MacEachern, 1994). Phytochemicals of particular interest from plant extracts include, flavonoids, triterpenes, tannins, saponins and phenols (Vasil & Constabel, 1989).

Triterpenoids are a phytochemical constituent widely distributed throughout plant and marine kingdoms. Although very diverse in their structures, they are characterised by a common 30 carbon backbone, originating from the intermediate biological precursor, squalene (Ghante & Jamkhande, 2019). These pharmacologically relevant constituents are derived from three terpene units, or six isopentenyl pyrophosphate units (IPP), which are synthesised via the mevalonate pathway (as portrayed in Figure 5.1.1) (Zhao *et al*., 2014). Further characterisation of triterpenoid compounds is difficult, due to the vast diversity of functional group substitutions. Because of which, triterpenoids can fall under the definition of several classes of compound. Broadly speaking, four generalised subdivisions are used to further classify triterpenoids, these include 'true' pentacyclic triterpenoids, steroids, saponins and cardiac glycosides (Stochmal *et al*., 2008).

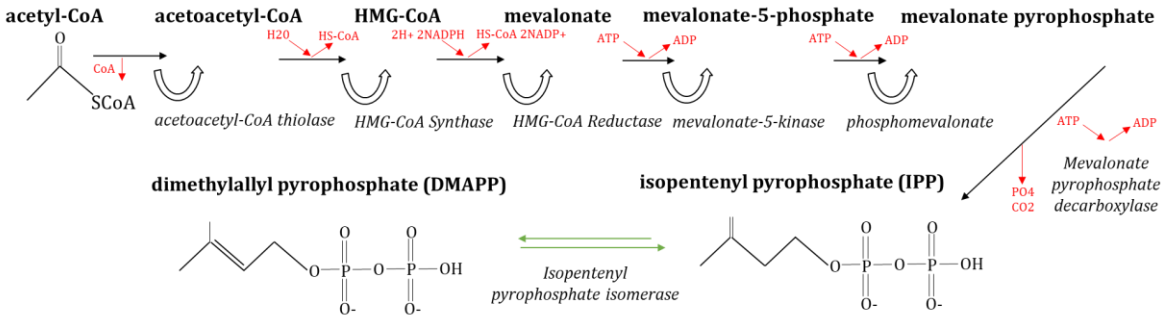

Figure 5.1.1: The mevalonate pathway of isoprenoid precursor biosynthesis. Isopentyl pyrophosphate (IPP) and dimethylallyl (DMAPP) pyrophosphate are precursors of isoprenoids. The mevalonate pathway utilises multiple enzymatic reactions to produce the precursors common to most eukaryotes. These can also be produced in the non-mevalonate pathway (not shown). Information from (Hunter, 2007).

True terpenes or pentacyclic triterpenoids have been the subject of much attention, with several now commercially available as therapeutic agents. In general, these triterpenes can be sub-divided into three of the most common classes: oleanane, ursane and lupane (Alqahtani *et al*., 2013). These compounds are the most widely distributed triterpenes and are typically located in the waxy coatings on leaves and fruits (Harborne, 1984). Derived pentacyclic triterpenoid carboxylic acids, ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) and oleanolic acid (3β-hydroxy-olean-12-en-28-oic acid) are ubiquitous throughout the plant kingdom, with oleanolic acid currently available in China as an over-the-counter medicine for hepatic ailments (Hiramatsu, *et al*., 2015). Both oleanolic and ursolic acid have reported cytotoxic capabilities against both *in vitro* and *in vivo* cancer models (Moldoveanu & Scott, 2016).

The term saponin was coined to classify a subset of triterpenoids which displayed surface-active and detergent properties. The chemical definition describes saponins as naturally occurring secondary metabolites, with surfactant glycosides of triterpenes and steroids. Saponins consist of a non-sugar aglycone attached to one or more sugar units; to exhibit surface activity, at least three of these sugar chains are required (Stochmal *et al*., 2008). The physical properties of saponins are related to their non-polar aglycone and polar sugar groups combined into the overall structure (Moses, *et al*., 2014). The aglycone or sapogenin, contains the triterpene or steroid framework, which remains after the substitution of the glycoside chain with a hydrogen atom (Biswas & Dwivedi, 2019). Saponins of an oleanane sapogenin are the

most abundant in nature, besides which other common sapogenins include those from ursane and dammarane classes (Ikan, 1999). Research on naturally isolated saponins has gained traction in regards to their cytotoxic potential and ability to act as chemo-adjuvants (Koczurkiewicz *et al*., 2019). Saponins are able to inhibit proliferation and induce cell cycle arrest and apoptosis against several cancer cell lines (Man *et al*., 2010).

In attempts to elucidate some of the medicinal properties of *Fagonia indica*, previous studies have attempted to isolate and characterise bioactive compounds from the plant extract. Several triterpenoid compounds have been isolated and identified from *Fagonia indica* extracts (as shown in Table 5.1.1). A select few triterpenoids isolated from *Fagonia indica* have been used in bioassays to determine the cytotoxic potential. Farheen, *et al* (2015), isolated and elucidated a triterpene (3β, 20*S*-dihydroxytaraxastane-28-al/ fagonicin) and triterpenoid glycoside (3-oxo-12-en-23-*O*-β-D-glucopyranosyl-27-hydroxyolean-28-oic acid/ indicacin) from a methanol extract of *Fagonia indica*. Isolated triterpenoids, fagonicin and indicacin inhibited cell growth in HT-29 colon cancer cells at 6.25 μM by 51.4 and 39.4%, respectively. A further study isolated a novel steroidal glycoside from *Fagonia indica* which demonstrated significant growth inhibition against MCF-7 and MDA-MB-231 breast cancer cells and CACO-2 colon cancer cells (Waheed *et al*., 2012).

Triterpenoid	Study
3β-*O*-β-d-glucopyranosyl-20-en-23-hydroxytaraxer-28-oic acid	Farheen *et al* (2015)
3β-hydroxy-23-*O*-β-d-glucopyranosyl-28-carboxy-*O*-β-d-gluco-pyranosyl-taraxer-20-en	Farheen *et al* (2015)
β-amyrin	Farheen *et al* (2015)
Fagonicin	Farheen *et al* (2015)
Indicacin	Farheen *et al* (2015)
Indicasaponin C	Shaker *et al* (2014)
Indicasaponin D	Shaker *et al* (2014)
Indicasaponin A	Shaker *et al* (1999)
Indicasaponin B	Shaker *et al* (1999)
3-*O*-[β-d-glucopyranosyl-(1→3)-α-l-arabinopyranosyl]-ursolic acid-28-*O*-[β-d-glucopyranosyl] ester	Shaker *et al* (1999)
3-*O*-[β-d-glucopyranosyl-(1→3)-α-l-arabinopyranosyl]-oleanolic acid-28-*O*-[β-d-glucopyranosyl] ester	Shaker *et al* (1999)
β-d-glucopyranosyl 3*β*-hydroxy-23-*β*-d-glucopyranosyloxy-lup-20(29)-en-28-oate.	Kanwal *et al* (2017)
β-d-glucopyranosyl 3*β*-*O*-sulfo-23,30-dihydroxy-olean-12-en-28-oate.	Kanwal *et al* (2017)
β-d-glucopyranosyl-(1 → 6)-*β*-d-glucopyranosyl 3*β*-hydroxy-23-*β*-d-glucopyranosyloxy-taraxast-20-en-28-oate.	Kanwal *et al* (2017)
β-d-glucopyranosyl 3*β*-hydroxy-23-(4′-*O*-sulfo-*β*-d-glucopyra-nosyloxy)-taraxast-20-en-28-oate.	Kanwal *et al* (2017)
3*β*-hydroxy-23-(4′-*O*-sulfo-*β*-d-glucopyranosyloxy)-taraxast-20-en-28-oic acid.	Kanwal *et al* (2017)
β-d-glucopyranosyl 3*β*-hydroxy-23-(2′,4′-di-O-sulfo-*β*-d-gluco-pyranosyl oxy)-taraxast-20-en-28-oate.	Kanwal *et al* (2017)
3*β*-hydroxy-23-(2′,4′-di-*O*-sulfo-*β*-d-glucopyranosyloxy)-tarax-ast-20-en-28-oic acid.	Kanwal *et al* (2017)
2-(4-methyl-pent3enoyloxy)-20-(4-methyl-pent-3-enoyloxy)-3b,12b,20b-trihydroxypregnane-3-ylO-b-D-cymapyranosyl-(1 ? 4)-3-methoxy-6-deoxy-bD-glucopyranoside	Waheed *et al* (2012)
Saponin A	Ansari *et al* (1987)
Saponin B	Ansari *et al* (1987)

Table 5.1.1: A consolidation of all known isolated and identified triterpenoid saponins from *Fagonia indica*.

Interestingly, alongside the pentacyclic triterpenoid ursolic acid, several of the isolated saponins from *Fagonia indica* have ursane/ ursolic acid aglycones. Neither ursolic acid triterpenoids or ursane saponins from *Fagonia indica*, have been isolated and tested for their cytotoxic properties. There are multiple studies which suggest ursolic acid or triterpenoids with an ursane aglycone have significant antitumor properties against both breast and colon cancer, including an ability to inhibit proliferation and induce expression of apoptotic-related genes (Mlala *et al*., 2019). Unlike oleanolic acid, ursolic acid is not available as an approved therapeutic medication, despite many studies suggesting significant cytotoxic properties in multiple cancer types. However, it should be noted that phase I trials are currently underway in China to determine the toxicity of ursolic acid (Qian *et al*., 2015; X. H. Wang *et al*., 2013; Zhu *et al*., 2013).

Alongside triterpenoids, further studies have identified high quantities of flavonoids and phenols from extracts of *Fagonia indica* (Shehab *et al*., 2020). Flavonoids are a class of secondary metabolites with a polyphenol structure, found ubiquitously throughout the plant kingdom (Panche *et al*., 2016). In *Fagonia indica*, a high total flavonoid content has been associated with increased hepatotoxicity (Shehab *et al*., 2020). Azam *et al* (2018), found that *Fagonia indica* extracts had an immune regulatory role and were able to alter the expression of pro-inflammatory markers, including IL-1, IL-6, TNF-α and TGF-β. *Fagonia indica* has also been used to enrich Aloe Vera; *Fagonia indica* treatment was able to maintain higher levels of flavonoids and phenols over decay, including ascorbic acid (Khaliq *et al*., 2019). Quercetin is one of the flavonoids that has been characterised from *Fagonia indica* extracts (Shehab *et al*., 2020). Quercetin is a typical flavanol; frequently found within fruits and vegetables, with reported antioxidative, anti-inflammatory and antiproliferative capabilities *in vitro* (Boots *et al*., 2008). Further research has shown that plant-derived quercetin has a prominent role in cancer treatment via the modulation of VEGF expression and VEGF induced translocation of NF-kB, via inhibition of MAPK and Akt (Lee *et al*., 2017).

Shehab *et al* (2020), also noted a high total content of phenols in an extract of *Fagonia indica*. Analysis with thin layer chromatography revealed one of the most abundant phenols in *Fagonia indica* as Gallic acid. Further research used high performance liquid chromatography (HPLC) to screen for high levels of commercially important anticancer secondary metabolites in *Fagonia indica* and revealed the presence of Gallic acid, Myricetin, Caffeic acid, Catechin and Apigenin (Khan *et al*., 2016). Polyphenols are a promising avenue for anticancer therapies; as high phenol diets have been shown to reduce the risk of colon cancer by altering DNA methylation and initiating apoptosis *in vitro*.

As is typical in traditional medicine, *Fagonia indica* extract would be administered aqueously in the form of a tea, as a purgative treatment for common ailments. In rural communities of Pakistan, *Fagonia indica* tea is routinely prescribed for the treatment of breast cancer. It is widely believed that this treatment will improve the condition of the patient. It wasn't until 2012, that a link was established between an aqueous extract of *Fagonia indica* and a cytotoxic effect on breast cancer cells (Lam *et al*., 2012). However, the most dominant confounding issue in the development of a chemotherapeutic agent from a *Fagonia indica* extract, is the isolation and identification of a bioactive compound(s). There has been some effort to isolate and identify the components of *Fagonia indica*, but there has been little progress in linking the cytotoxic mechanisms of these individual compounds.

The most commonly applied approach of isolating compounds from natural products is a bioassay guided fractionation technique, whereby several rounds of extraction and separation processes are used to pinpoint bioactive compounds. Several of the currently isolated compounds from *Fagonia indica*, fall under the category of triterpenoids and triterpenoid saponins. Naturally derived saponins have gained some traction in research, especially in regards to their potential antitumorigenic mechanisms. This chapter aims to utilise solvent extraction to isolate fractions of triterpenoids which are bioactive against breast and colon cancer *in vitro*. The presence of predetermined triterpenoids in the extracted fractions will also be investigated, alongside total triterpenoid content.

Despite ongoing work to identify bioactive compounds from *Fagonia indica*, only few isolated compounds have been tested for their bioactivity against human cancer cell lines. This includes triterpenoids of an ursane/ ursolic acid nature, which have proved a promising treatment avenue for cancer. In this chapter the primary aims were to produce a phytochemical 'rich' fraction of *Fagonia indica* extract and to demonstrate the cytotoxicity of this fraction against breast and colon cancer cell lines. Following this, the chapter aimed to determine the phytochemical properties of *Fagonia indica* extract and determine how these corresponded to the treatment of breast and colon cancer cell lines.

5.2 Methods

5.2.1 *Fagonia indica* solvent extraction

Solvent and aqueous extracts of *Fagonia indica* were prepared as described in Chapter 2.2. Table 5.2.1 displays the polarity of solvents used for *Fagonia indica* extractions in the present study.

Polarity index of solvents	
Solvent	**Polarity**
Water	10.2
Methanol	5.1
Butanol	3.9
Hexane	0.1

Table 5.2.1: Relative polarities of solvents used in the present study. As determined by the Burdick & Jackson polarity index and used to determine solubility of *Fagonia indica* compounds.

5.2.2 MTT assay

The effect of an aqueous, 75% methanolic and butanolic extract of *Fagonia indica* on MDA-MB-231, MCF-7, H630 and RKO cancer cell viability was determined using the MTT assay as described in Chapter 2.5.1. In brief, cells were seeded at $1x10^4$ before being treated with 1 mg/mL of aqueous, 75% methanolic or butanolic *Fagonia indica* extract for up to 48 hours, prior to analysis using MTT.

5.2.3 Phytochemical screening in *Fagonia indica* extracts

5.2.4 Saponins

Phytochemical screening for saponins was conducted in aqueous, 75% methanolic and butanolic *Fagonia indica* extracts with the Froth test, as described in Chapter 2.12.2

5.2.5 Terpenoids

Phytochemical screening for terpenoids was conducted in aqueous, 75% methanolic and butanolic *Fagonia indica* extracts with the Salkowski's test, as described in Chapter 2.12.1

5.2.6 Cardiac glycosides

Phytochemical screening for cardiac glycosides was conducted in aqueous, 75% methanolic and butanolic *Fagonia indica* extracts with the Keller Killiani test, as described in Chapter 2.12.3

5.2.7 Flavonoids

Phytochemical screening for flavonoids was conducted in aqueous, 75% methanolic and butanolic *Fagonia indica* extracts, as described in Chapter 2.12.4

5.2.8 Phenols

Phytochemical screening for phenols was conducted in aqueous, 75% methanolic and butanolic *Fagonia indica* extracts with the Ferric Chloride test, as described in Chapter 2.12.6

5.2.9 Tannins

Phytochemical screening for tannins was conducted in aqueous, 75% methanolic and butanolic *Fagonia indica* extracts with the Ferric Chloride test, as described in Chapter 2.12.5

5.2.10 TLC

Thin layer chromatography was used to indicate the presence of triterpene/ saponin phytochemicals in aqueous and 75% methanolic extracts of *Fagonia indica*, as described in Chapter 2.13.

5.2.11 Total phytochemical content in *Fagonia indica* extracts

5.2.12 Triterpenoids

Total triterpenoid content of aqueous, 75% methanolic and butanolic extracts of *Fagonia indica*, was determined using the Vanillin-sulphuric assay, as described in Chapter 2.14.1.

5.2.13 Flavonoid

Total flavonoid content of aqueous, 75% methanolic and butanolic extracts of *Fagonia indica*, was determined using the Dowd assay, as described in Chapter 2.14.4.

5.2.14 Antioxidants

Total antioxidant capacity of aqueous, 75% methanolic and butanolic extracts of *Fagonia indica*, was determined using the Phosphomolybdate assay, as described in Chapter 2.14.2.

5.2.15 Phenols

Total phenol content of aqueous, 75% methanolic and butanolic extracts of *Fagonia indica*, were determined using the Folin-Ciocalteu assay, as described in Chapter 2.14.3.

5.2.16 DCDFA assay

Intracellular ROS accumulation was determined in breast cancer cell lines; MDA-MB-231 and MCF-7 and colon cancer cell lines; H630 and RKO, after treatment with aqueous and 75% methanolic extracts of *Fagonia indica* using a DCDFA assay, as described in Chapter 2.15.1.

5.2.17 ROS assay/ superoxide assay

ROS/ superoxide accumulation was determined in breast cancer cell lines; MDA-MB-231 and MCF-7 and colon cancer cell lines; H630 and RKO, after treatment with aqueous and 75% methanolic extracts of *Fagonia indica* using a ROS/ superoxide assay, as described in Chapter 2.

5.3 Results

5.3.1 The effect of solvent extraction of *Fagonia indica* on the cytotoxicity against breast and colon cancer

Crude *Fagonia indica* plant was prepared using solvents with varying polarities in order to extract a greater degree of bioactive phytochemicals. Solvent extracts of *Fagonia indica* were tested for their cytotoxic activities against breast cancer cell lines; MDA-MB-231 and MCF-7, and colon cancer cell lines; RKO and H630, using the MTT assay. A 75% methanolic extract of *Fagonia indica* was tested against an aqueous extract to compare cytotoxic effects on breast cancer cell lines; MDA-MB-231 and MCF7 (Figure 5.3.1). After treatment with 1 mg/mL of aqueous *Fagonia indica* extract cell viability of MDA-MB-231 was not significantly changed, compared to an untreated control (Figure 5.3.1A) In contrast, 1 mg/mL of a 75% methanolic *Fagonia indica* extract led to a significant reduction in cell viability by 42.85% ($p>0.05$). There was no significant difference in cell viability between cells treated with an aqueous *Fagonia indica* extract in comparison to those treated with the 75% methanolic extract. In MCF7 cell lines, cell viability was significantly reduced in both aqueous and 75% methanolic *Fagonia indica* treated cells, by 23.76% ($p<0.05$) and 51.87% ($p<0.01$) compared to an untreated control (Figure 5.3.1B). There was also a significant reduced in cell viability of MCF-7 cells treated with a 75% methanolic extract of *Fagonia indica*, by 28.11% ($p<0.05$), compared to the aqueous extract treatment.

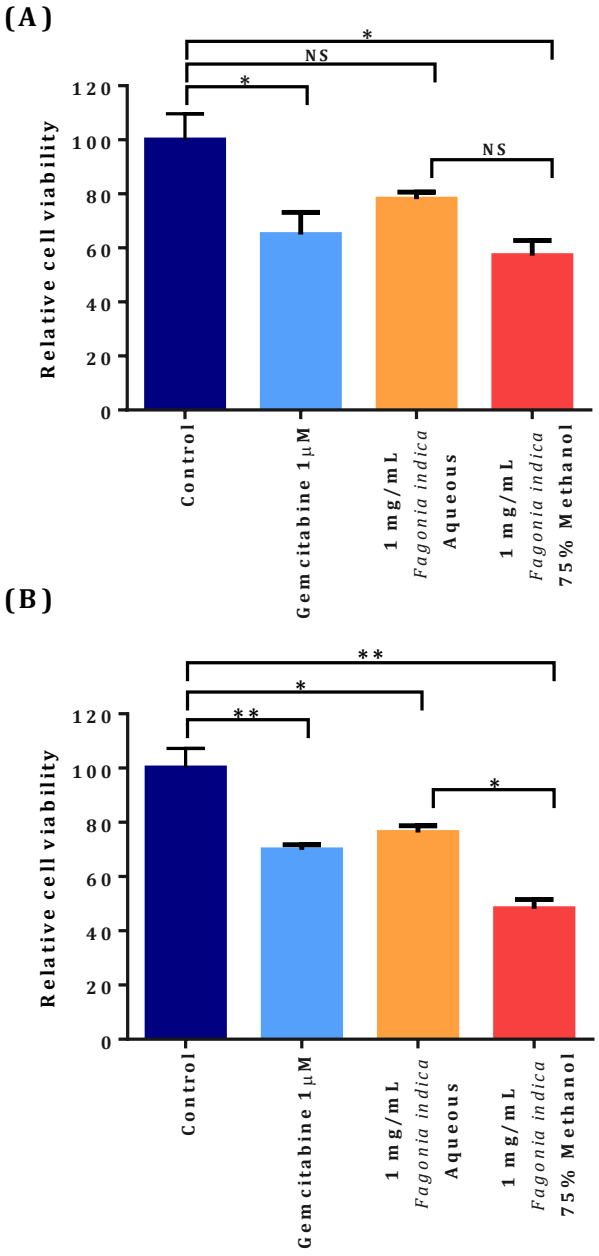

Figure 5.3.1: A methanolic *Fagonia indica* extract treatment reduces *in vitro* cell viability of MDA-MB-231 and MCF7 breast cancer cell lines. (A) MDA-MB-231 and (B) MCF7 cells were treated with 1 mg/mL of aqueous or 75% methanolic *Fagonia indica* extract for 48 hours. 1 µM of gemcitabine was used as a positive control. Cell viability was determined as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted * ($p<0.05$) and ** ($p<0.01$) were significant compared to the untreated control analysed by one-

way ANOVA with Sidak's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

A similar effect was noted in colon cancer cell lines; H630 and RKO, after treatment with an aqueous and 75% methanolic extract of *Fagonia indica* (Figure 5.3.2). After treatment with 1 mg/mL of aqueous *Fagonia indica* extract cell viability of H630 was significantly reduced by 9.12% ($p<0.05$), compared to an untreated control (Figure 5.3.2A). Cell viability was reduced by 33.37% ($p<0.0001$) after treatment with a 75% methanolic extract, compared to an untreated control. There was also a significant increase in extract induced cytotoxicity by 24.25% (0.0001) for cells treated with the 75% methanolic extract of *Fagonia indica*, compared to the aqueous extract. In RKO cell lines, there was no significant changes in cell viability between treatment with an aqueous extract of *Fagonia indica* and the untreated control (Figure 5.3.2B). However, there was a significant reduction in cell viability of RKO cells after treatment with a 75% methanolic extract by 50.69% ($p<0.001$), compared to the untreated control. There was also a significant reduction in cell viability between the 75% methanolic extract and aqueous *Fagonia indica* extract treatment of 32.97 % ($p<0.01$).

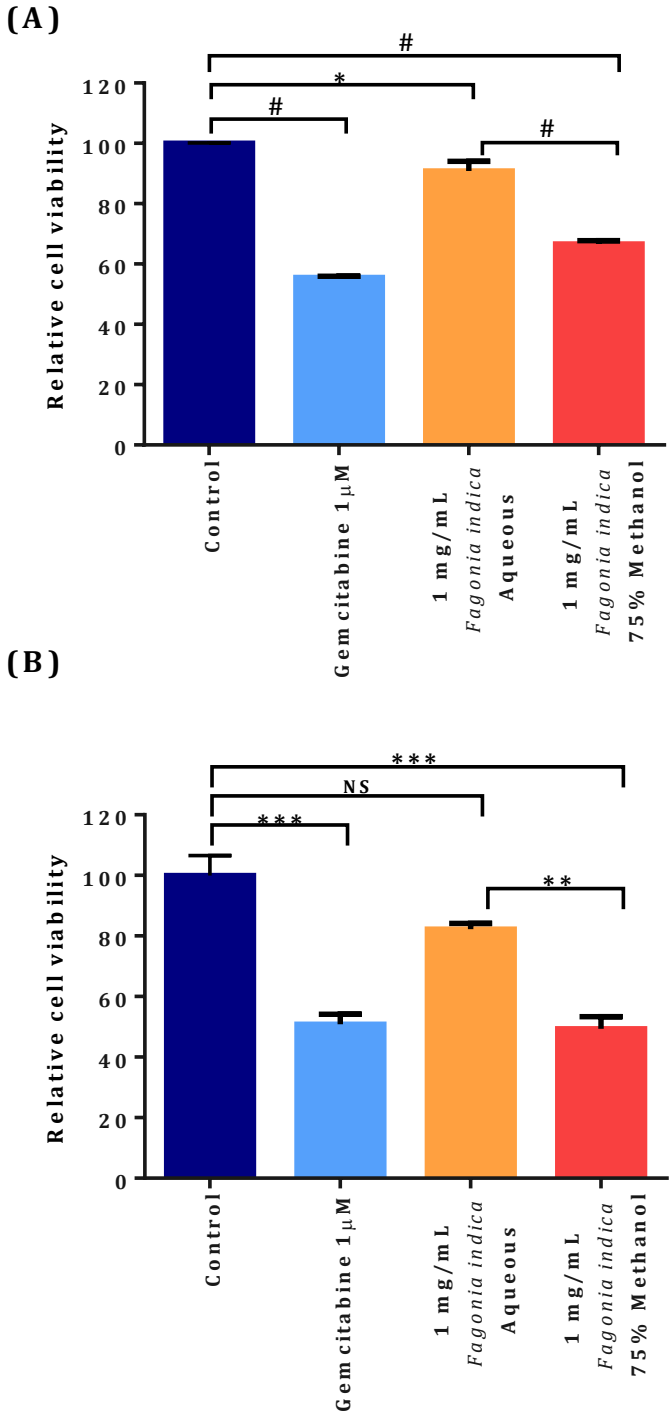

Figure 5.3.2: A methanolic *Fagonia indica* extract treatment reduces *in vitro* cell viability of H630 and RKO colon cancer cell lines. (A) H630 and (B) RKO cells were treated with 1 mg/mL of aqueous or 75% methanolic *Fagonia indica* extract for 48 hours. 1 µM of gemcitabine was used as a positive control. Cell viability was determined as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted * ($p<0.05$), # ($p<0.0001$) were significant compared to the untreated control analysed by one-way ANOVA

with Sidak's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

A butanolic extract of *Fagonia indica* was also tested against an aqueous extract in breast cancer cell lines, MDA-MB-231 and MCF-7 (Figure 5.3.3). In MDA-MB-231 cells there was no significant changes in cell viability and no significant differences between aqueous and butanolic *Fagonia indica* extracts (Figure 5.3.3A). In MCF-7 cells, cell viability was significantly reduced by 24.13% ($p<0.001$) after treatment with 1 mg/mL of aqueous *Fagonia indica*, compared to an untreated control (Figure 5.3.3B). There were no significant changes in cell viability after treatment with the butanolic extract of *Fagonia indica*, compared to an untreated control However, there was a significant increase in cell viability between a butanolic *Fagonia indica* treatment and an aqueous extract of 21.36% ($p<0.01$)

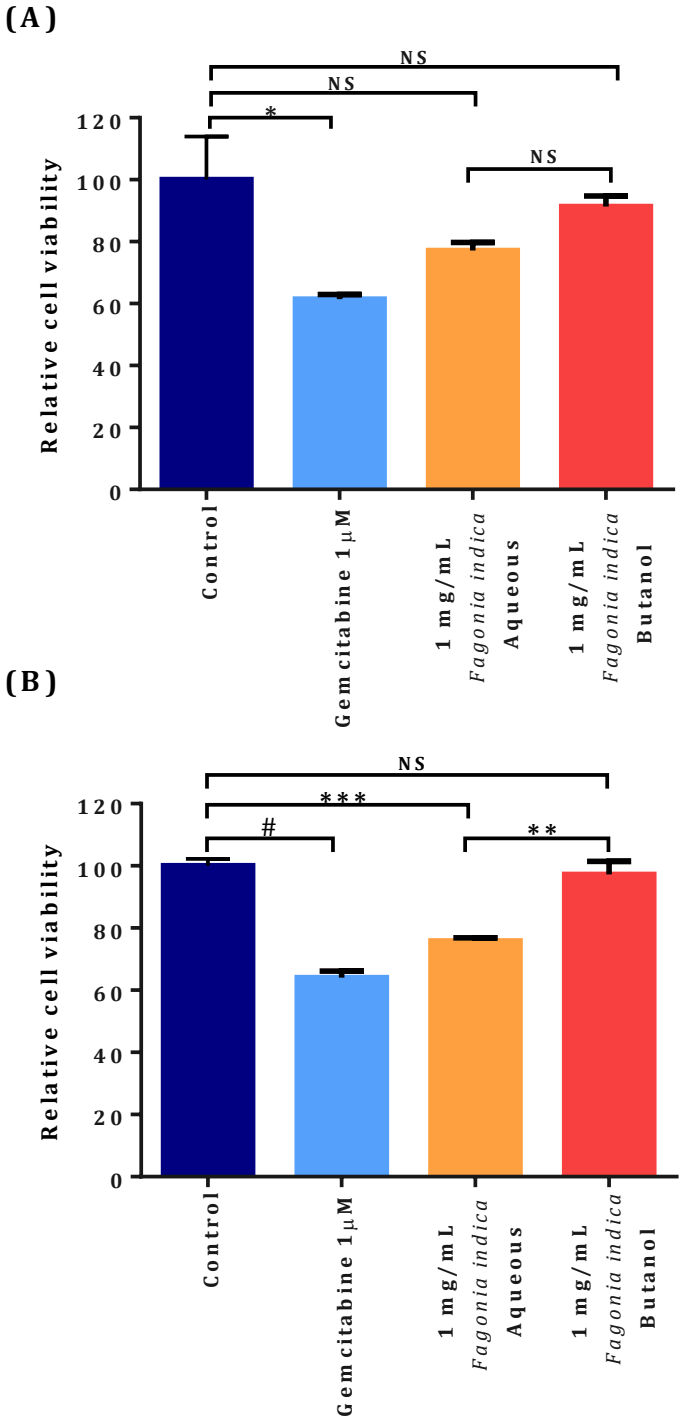

Figure 5.3.3: A butanolic *Fagonia indica* extract treatment reduces *in vitro* cell viability of MDA-MB-231 and MCF7 breast cancer cell lines. (A) MDA-MB-231 and (B) MCF7 cells were treated with 1 mg/mL of aqueous or butanolic *Fagonia indica* extract. 1 µM of gemcitabine was used as a positive control. Cell viability was determined as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and # ($p<0.0001$) were significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test. All data is

representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

A similar effect was noted in colon cancer cell lines, H630 and RKO (Figure 5.3.4). After treatment with 1 mg/mL of aqueous *Fagonia indica* extract, cell viability of H630 cells was reduced by 26.15% ($p>0.001$), against an untreated control (Figure 5.3.4A). However, in H630 cell lines the butanolic extract was also able to significantly reduce cell viability by 35.26% ($p<0.001$) compared to an untreated control. There were no significant differences in cell viability between butanolic and aqueous extract treatments. In RKO cells, there were no significant changes in cell viability after treatment with aqueous or butanolic *Fagonia indica* extracts (Figure 5.3.4B). There was also no significant difference between the two extract treatments.

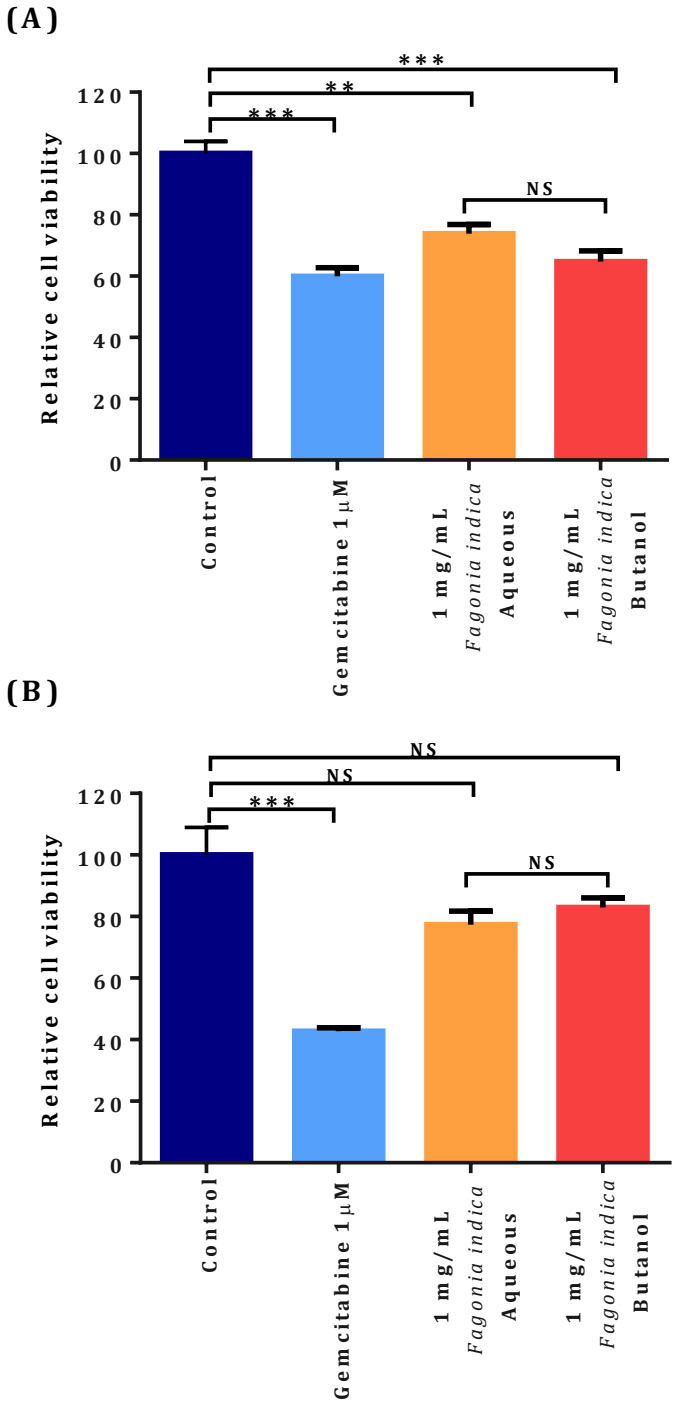

Figure 5.3.4: A butanolic *Fagonia indica* extract treatment reduces *in vitro* cell viability of H630 and RKO colon cancer cell lines. (A) H630 and (B) RKO cells were treated with 1 mg/mL of aqueous or butanolic *Fagonia indica* extract. 1 µM of gemcitabine was used as a positive control. Cell viability was determined as a percentage of an untreated DMSO vehicle control using the MTT assay. Data denoted ** ($p<0.01$) and *** ($p<0.001$) were significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

5.3.2 Phytochemical screening of *Fagonia indica* extracts.

In the present study, aqueous, 75% methanolic and butanolic fractions from a crude *Fagonia indica* extract were screened for the presence of several phytochemicals (Table 5.3.1). All three fractions tested positive for terpenoids and terpenoid derivates; saponins and cardiac glycosides, as measured by a Salkowski, froth and Keller Killiani test, respectively. In phenolic compounds, including, general phenols, flavonoids and tannins, the aqueous and 75% methanolic extracts tested positive, as measured by flavonoid screening and the Ferric Chloride test. There was weak or negligible intensity changes in the butanolic fraction of *Fagonia indica* for tannins and phenols.

Phytochemical screening test			
Phytochemical	**Fraction**		
	Aqueous	75% Methanol	Butanol
Saponin	+	+	+
Terpenoids	+	+	+
Cardiac glycoside	+	+	+
Flavonoid	+	+	+
Tannins	+	+	Weak
Phenols	+	+	Weak

Table 5.3.1 Phytochemical screening of solvent extractions of *Fagonia indica*. Screening tests were conducted determine presence of phytochemicals; saponins, terpenoids, cardiac glycosides, flavonoids, tannins and phenols. Positive samples are indicated by +. All data is representative of three independent experiments (n=3)

Aqueous and 75% methanolic extracts of *Fagonia indica* were analysed for triterpenoid content using TLC (Figure 5.3.5). The purpose of this was to use ursolic acid and triterpene specific staining to determine presence of terpene compounds in *Fagonia indica* extracts. Vanillin staining revealed a level band for both the control; ursolic acid and for the 75% methanolic extract of *Fagonia indica*. There were no visible bands for the aqueous extract of *Fagonia indica*.

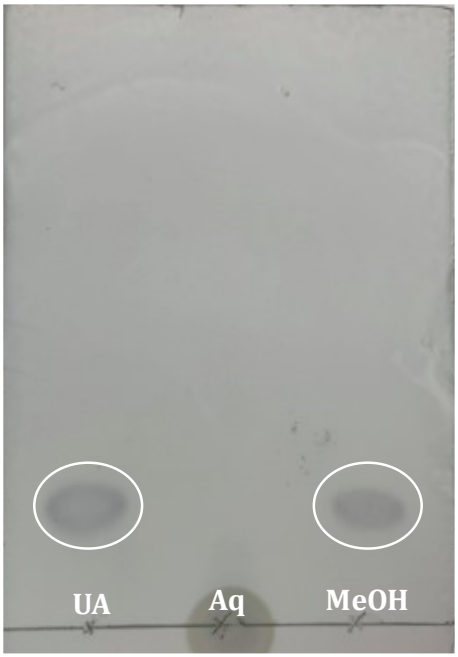

Figure 5.3.5. TLC analysis of *Fagonia indica* solvent extractions. Dried *Fagonia indica* was subjected to solvent extraction in either water or 75% methanol. Extractions were evaporated in a rotary evaporator and dried under vacuum before analysis by TLC (1 mg/mL). TLC was carried out with a mobile phase of an ethyl acetate: methanol (90:10) and visualised with vanillin stain. Ursolic acid (10 µg/mL) was used as a positive control for triterpenoids. Image is representative of two independent experiments.

5.3.3 Determination of total phytochemical contents of *Fagonia indica* extracts

Triterpenoids are polycyclic compounds widely distributed in edible and medicinal plants which exert a multitude of biological and pharmacological activities (Szakiel *et al*., 2012). Total triterpenoid content of aqueous, 75% methanolic and butanolic fractions of *Fagonia indica* was determined using the vanillin-sulphuric assay (Figure 5.3.2). The results were derived from a calibration curve of the standard ($y=0.0016x + 0.1603$, $R_2=0.9219$), ursolic

acid (0-500 µg/mL) and expressed as ursolic acid equivalents (UA) per gram of dry extract. Total triterpenoid content of an aqueous extract of *Fagonia indica* was recorded as 27.45±7 mg UA/ g extract, for the 75% methanolic extract there was a two-fold increase in total triterpenoid content to 56.45±12.31 mg UA/ g dry extract. The butanolic fraction of *Fagonia indica* had the lowest total triterpenoid content at 26.89±1.94 mg UA/ g extract.

Flavonoids are a polyphenol subclass, characterised by the presence of two of more aromatic rings. Flavonoids are found in a range of fruits, vegetables, spices, flowers, tea, etc and are regularly consumed for their anti-inflammatory and anti-cancer effects (García-Lafuente *et al*., 2009). Total flavonoid content of aqueous and 75% methanolic fractions of *Fagonia indica* was determined using the Dowd assay (Figure 5.3.2). The results were derived from a calibration curve ($y=0.0084x +0.0044$, $R_2=0.9823$) of the standard, quercetic acid (0-200 µg/mL) and expressed as quercetin acid equivalents (QC) per gram of dry extract. Total flavonoid content of an aqueous extract of *Fagonia indica* was recorded as 39.77±2.27 mg QC/ g dry extract, for the 75% methanolic extract there was a three-fold increase in total triterpenoid content to 118.89±6.06 mg QC/ g extract.

Plant extracts also typically contain a great degree of redox modulating molecules, which can lead to alterations in the cellular redox state of cells, resulting in a number of downstream consequences. Levels of radical oxygen species can alter the modulation of cell survival responses (Foyer & Noctor, 2013). Total antioxidant capacity of aqueous and 75% methanolic extracts of *Fagonia indica* were assessed using the phosphomolybdate assay (Table 5.3.2) Due to issues with solubility the butanolic extract was not assessed for flavonoidal content. The results were derived from a calibration curve ($y=0.0031x - 0.0798$, $R_2=0.9831$) of the standard, quercetic acid (0-500 µg/mL) and expressed as quercetin acid equivalents (QC) per gram of dry extract. Total antioxidant activity of the aqueous *Fagonia indica* extract was noted as 30.14±3.42 mg QC/ g extract, whilst the 75% methanolic extract had a higher total antioxidant capacity of 43.13±0.56 mg QC/ g extract.

Natural phenolic compounds have been shown to play an important role in health and cancer prevention. Bioactivities of phenolic compounds include antioxidant, anticarcinogenic and anti-inflammatory (Huang *et al*., 2010). With this in mind, the phenolic content of aqueous, 75% methanolic and butanolic extracts of *Fagonia indica* was measured using the Folin-Ciocalteu reagent (Table 5.3.2). The results were derived from a calibration curve ($y=0.002x + 0.0614$, $R_2=0.9829$) of the standard, gallic acid (0-500 μg/mL) and expressed as gallic acid equivalents (GAE) per gram dry extract. The 75% methanolic extract of *Fagonia indica* had the highest total phenol content at 41.95±0.55 mg GAE/ g extract, whereas the butanolic extract had the lowest at 34.29±0.82 mg GAE/ g extract.

Total phytochemical content			
Phytochemical & assay type.	***Fagonia indica* fraction**		
	Aqueous	75% Methanolic	Butanolic
Total triterpenoid content/ mg UA/ g extract	**27.45±*7***	**56.45±*12.31***	**26.89±*1.94***
Total flavonoid content/ mg QC/ g extract	**39.77±*2.27***	**118.89±*6.06***	**-**
Total antioxidant content/ mg QC/ g extract	**30.14±*3.42***	**43.1±*0.56***	**-**
Total phenol content/ mg GAE/ g extract	**37.54±*0.77***	**41.95±*0.55***	**34.29±*0.82***

Table 5.3.2: Total triterpene, flavonoid, phenolic and antioxidant content of solvent extractions of *Fagonia indica*. Data is displayed as mg of standard/ gram of *Fagonia indica* extract. All data are presented as mean ± SD (N=3).

5.3.4 Determining the effect of *Fagonia indica* extracts on intracellular reactive oxygen species.

Plant materials are known to contain a high abundance of redox modulating compounds which cause significant alterations to the redox state of cells and downstream cellular processes. In the current investigation, levels of intracellular ROS were assessed using the DCD-FA assay (Figure 5.3.6). Intracellular ROS was measured in RKO colon cancer cell lines after treatment with 1.5 mg/mL of aqueous and 75% methanolic extracts of *Fagonia indica* for 4 hours (Figure 5.3.6A). There were no significant changes in MDA-MB-231 intracellular ROS after treatment with aqueous and 75% methanolic extracts of *Fagonia indica*, when compared to the untreated control. Similarly, there were also no significant fold-changes in RKO intracellular ROS after treatment with aqueous and 75% methanolic extracts of *Fagonia indica* after 4 hours (Figure 5.3.6B).

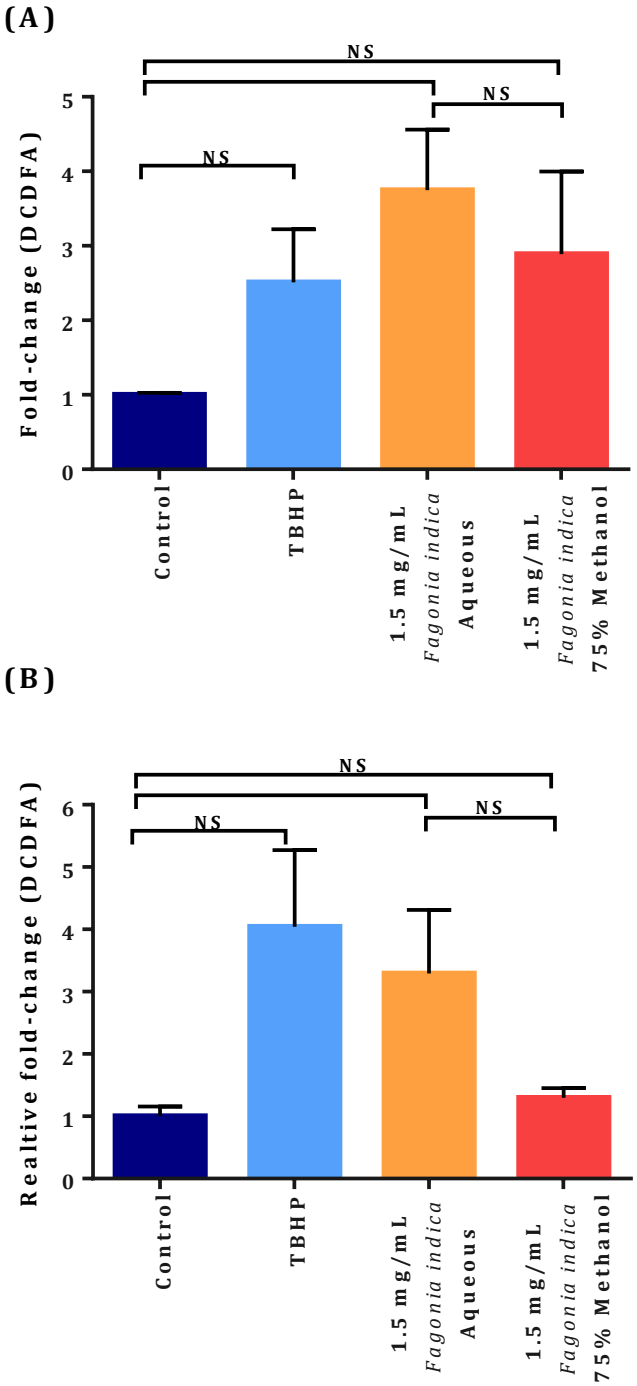

Figure 5.3.6: *Fagonia indica* extract treatment increases intracellular ROS levels in RKO and MDA-MB-231 cancer cells. ROS activity was measured by incubating (A) MDA-MB-231 cells and (B) RKO cells with DCDFDA solution for 45 minutes prior to extract treatment with aqueous or methanolic extracts of *Fagonia indica* for 4 hours. TBHP was used as a positive control. Data denoted NS were not significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

Due to the large variability in replicates in the DCD-FA ASSAY and the lack of significant changes for the positive control, a ROS/Superoxide detection assay kit was used to directly monitor the real-time reactive oxygen species (ROS) in live RKO and MDA-MB-231 cells as they were treated with aqueous and 75% methanolic *Fagonia indica* extracts (Figure 5.3.7). In RKO colon cancer cell lines, intracellular ROS accumulation was significantly increased by an aqueous extract of *Fagonia indica*, by 3.01-fold ($p<0.05$) of an untreated control (Figure 5.3.7A). Treatment with a 75% methanolic extract of *Fagonia indica* was also able to significantly increase intracellular ROS by 4.53-fold ($p<0.001$) of an untreated control. There was no significant difference in fold-change between aqueous and 75% methanolic *Fagonia indica* treatments on ROS accumulation in RKO cells. Superoxide accumulation was also measured in RKO colon cancer cell lines after treatment with *Fagonia indica* extract (Figure 5.3.7B). There were no significant changes in superoxide fold-change in RKO cells treated with an aqueous extract of *Fagonia indica*. However, treatment with a 75% methanolic extract of *Fagonia indica* was able to significantly increase superoxide accumulation by 8.83-fold ($p<0.01$). There were no significant changes in superoxide accumulation between aqueous and 75% methanolic *Fagonia indica* treatments.

In MDA-MB-231 breast cancer cell lines, 1.5 mg/mL aqueous extract of *Fagonia indica* significantly increased intracellular accumulation of ROS by 4.11-fold ($p<0.0001$) of the untreated control (Figure 5.3.7C). Treatment of MDA-MB-231 cells with a 75% methanolic extract *of Fagonia indica* also significantly increased ROS accumulation by 3.27-fold ($p<0.0001$) of the untreated control. Treatment with an aqueous *Fagonia indica* caused a significantly greater fold-increase in ROS than the 75% methanolic extract ($p<0.0001$). Similarly, an aqueous extract of *Fagonia indica* was able to induce a significant increase of 2.84-fold ($p<0.01$) in superoxide in MDA-MB-231 cells, compared to an untreated control (Figure 5.3.7D). The 75% methanolic *Fagonia indica* extract also induced a significant increase of 3.99-fold ($p<0.0001$) of the untreated control. The 75% methanolic extract was

also able to increase superoxide accumulation to a significantly higher degree of the aqueous *Fagonia indica* extract ($p<0.05$).

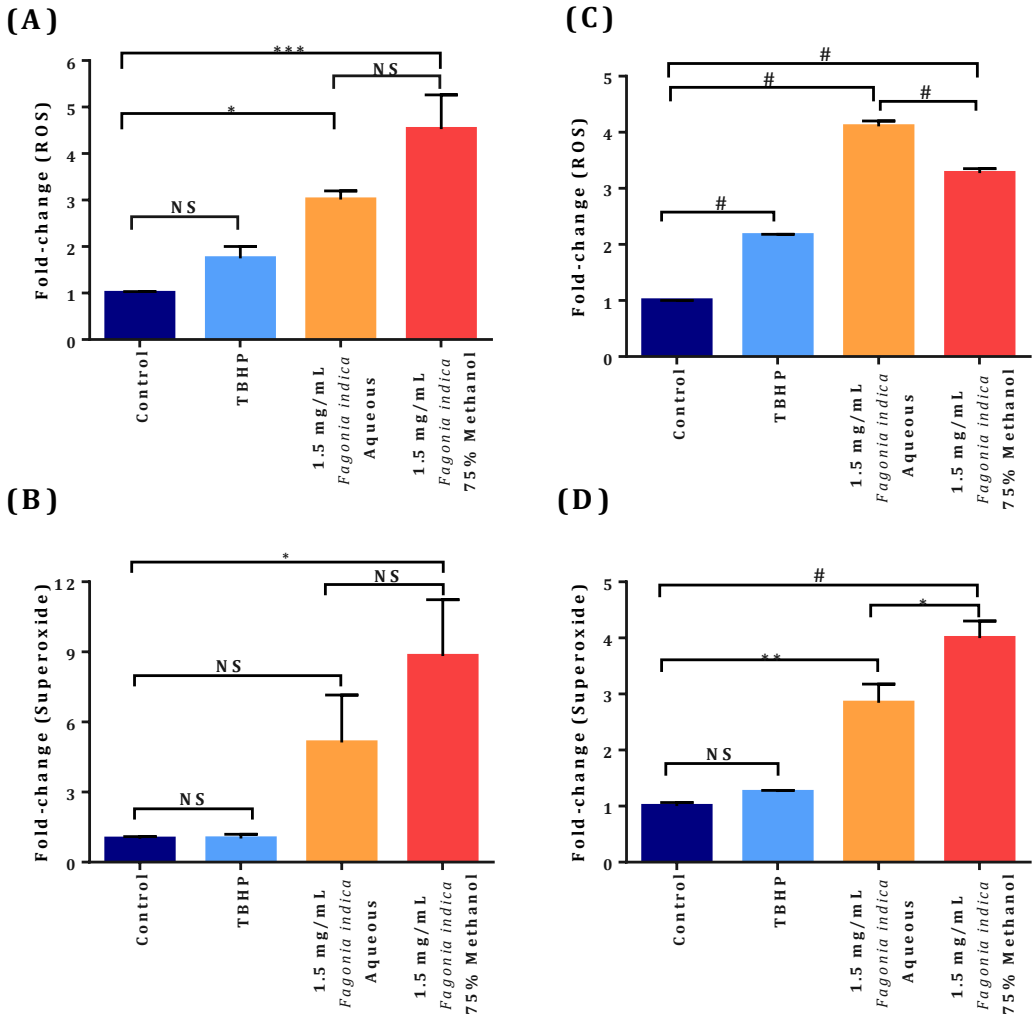

Figure 5.3.7: *Fagonia indica* extract treatment increases intracellular ROS levels in RKO colon cancer cells. (A)ROS activity was measured by incubating RKO cells with DCDFDA solution for 45 minutes prior to extract treatment with aqueous or methanolic extracts of *Fagonia indica* for 4 hours. (B) Total intracellular ROS and (C) superoxide were measured using oxidative stress detection reagent/ superoxide detection reagent for 60 min post treatment with *Fagonia indica* extracts for 4 hours. TBHP was used as a positive control. Data denoted ** ($p<0.01$) and # ($p<0.0001$) were significant compared to the untreated control analysed by one-way ANOVA with Sidak's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

5.4 Discussion

Isolated triterpene glycosides from plant materials have demonstrated *in vitro* and *in vivo* activity with minimal toxicity to healthy cells, highlighting their therapeutic suitability for the treatment of diseases, including cancer (Park *et al*., 2014). *Fagonia* species have gained enormous traction for their diverse phytochemical content, with a particularly high concentration of triterpenoid glycosides and saponins (Kanwal *et al*., 2017). Several triterpenoid saponins have previously been isolated and identified from *Fagonia indica* extracts, *in vitro* assays have demonstrated a degree of cytotoxic activities from these isolated compounds (Table 5.1.1). As such, research has turned to optimising plant growth and regulating factors in a bid to improve the concentration of bioactive compounds in *Fagonia indica* (Tariq Khan *et al*., 2018). Studies have experimented with the interplay between light and other growth factors in attempts to produce a higher quantity of cytotoxic compounds from *Fagonia indica,* including; secondary metabolites such as saponins, phenols, flavonoids and antioxidant compounds (Taimoor Khan, *et al*., 2019; Tariq Khan *et al*., 2018). The current chapter aimed to produce an extract of *Fagonia indica* rich in bioactive compounds, for use against breast and colon cancer cell lines.

Chemical complexity is one of the advantages of screening plant species for bioactive compounds. The presence of biologically relevant compounds including flavonoids, saponins, tannins, glycosides and alkaloids are well established in *Fagonia indica* (Ali & Khan, 2020). *Fagonia indica* has been recognised as a promising avenue for novel therapeutic development from rural medicinal plants in Pakistan; in part, due to high flavonoid and phenolic content (Ahmed *et al*., 2020). This was correlated to increased anti-inflammatory and antioxidant activity. In the current study, phytochemical screening indicated a strong presence of several of these previously identified molecules, including; saponins, cardiac glycosides, terpenoids and flavonoids. As isolated plant triterpenoid saponins have repeatedly demonstrated anti-neoplastic activity, they are a promising avenue for the identification of the bioactive compounds of *Fagonia indica* (Sarikahya *et al*., 2018).

The recovery and isolation of triterpenes from plant tissues has been primarily achieved using solvent partitions of crude extracts followed by column chromatography (Lima *et al*., 2015). Studies have indicated that alcoholic fractions; ethanol and methanol, result in the recovery of a significantly higher amount of triterpene acids (Goulas & Manganaris, 2012). Previously established cytotoxic triterpene acids; ursolic and oleanolic acid, were successfully isolated from *Orthosiphon stamineus* from a methanolic crude extract (Hossain & Ismail, 2013). In the current study a crude extract of *Fagonia indica* was prepared using water, methanol or butanol. A 75% methanolic extract demonstrated significantly higher degrees of cytotoxicity against all four breast and colon cancer cell lines; RKO, H630, MDA-MB-231 and MCF-7. This indicates a higher proportion of bioactive compounds in the methanolic fraction, which could provide some indication of compound structure. This corresponds with previous research highlighting the antimicrobial potency of methanolic extracts of *Fagonia indica* (Rizwan *et a*l., 2020). The present investigation found that a 75% methanolic extract increased total phytochemical content for triterpenes, phenols and flavonoids, compared against an aqueous or butanolic extract, which again could correspond to the increase in cytotoxic activity.

It was hypothesised that the increase in cytotoxicity within the methanolic extract of crude *Fagonia indica* was related to an increase in triterpene/ saponin content. In the current study a methanolic extract of *Fagonia indica* had a higher total triterpenoid content than the aqueous and butanolic extract. This corresponded to a greater level of activity in a methanolic extract of *Fagonia indica*. Gas chromatography/ mass spectroscopy (GC/MS) of methanolic extracts of various medicinal plants have identified promising novel triterpenoids as the bioactive molecules (Hussein *et al*., 2017; Kanthal *et al*., 2014). Previously, Farheen *et al* (2015), isolated and characterised two novel triterpenoid saponins; indicacin and fagonicin, from a methanolic extract of *Fagonia indica*. Both compounds demonstrated significant cytotoxic activity against colon cancer cell line H-29. Despite this, there is large proportion of unknown compounds in the crude extract that are yet to be characterised. Future work is required to further separate and identify novel compounds from *Fagonia indica*. Bio-guided fractionation

is a popular technique, whereby the most active fractions are selected based on their relevant activity over a series of chromatographic separation events (Jallali *et al*., 2020).

In 1984, some of the earliest triterpene acids were isolated and identified from *Fagonia indica* extracts (Ali Ansari & Kenne, 1984), this included the isolation of ursolic acid from an aqueous extract of the ariel plant sections. Ursolic acid has gained traction in recent years due to its ability to reduce cell viability, induce apoptosis and inhibit BCL-2 protein activation (Kassi *et al*., 2007). Previous research has shown prominent cytotoxic activity of ursolic acid in a time and dose dependent manner in both gastric and breast cancer cell lines, associated with the activation of caspase-3 and 8 (Kassi *et al*., 2009; Wang *et al*., 2011). Further research has demonstrated an ability of plant-derived ursolic acid to suppress the activation of NF-κB, this anti-inflammatory effect has been associated with renewed activity of chemotherapeutic agents (Checker *et a*l., 2012; Li *et al*., 2010; Manu & Kuttan, 2008). Huang et al (2011), noted ani-invasive and anti-migratory effects of ursolic acid in human lung cancer cell lines, via the suppression of protein activation of VEGF and ICAM-1 (Huang *et al*., 2011). There is a strong potential that ursolic acid is one of the attributing bioactive compounds of *Fagonia indica*, contributing to the apoptotic, anti-migratory and anti-invasive effects detailed in Chapter 3 and 4. Future work is required to determine the presence and concentration of ursolic acid and other known triterpene acids from the methanolic extract of *Fagonia indica* (Taralkar & Chattopadhyay, 2012). Caligiani *et al* (2013), outlined a simple protocol for screening plant extracts for ursolic acid using GC-MS, which is able to confirm the presence of ursolic acid and its concentration within the extract (Caligiani *et al*., 2013).

Interestingly, quinovic acid was one of the major compounds isolated from the hydrolysed extract of *Fagonia indica* (Kumbi *et al*., 2019). Despite studies identifying quinovic acid in bioactive fractions of plant material and highlighting a potential to induce apoptosis in some cancer types, there is significantly less research into this natural triterpene (Dietrich *et al*., 2014; Kumar *et al*., 2015). A recent study isolated quinovic acid from a *Fagonia indica* extract and demonstrated apoptotic and growth inhibitory activity in breast and lung cancer (Khayam

et al., 2020). Apoptosis was stimulated via the activation of caspases-3 and 8 and via the upregulation of death receptor 5 of the extrinsic pathway of apoptosis. Interestingly, further research has identified a relationship between p38 MAPK signalling activation and promotion of caspases and apoptotic death pathway via death receptor 5. (Yoon *et al*., 2009). Diosgenin, a steroidal saponin present in fenugreek, inhibited the growth of colon cancer cell lines via the activation of p38 MAPK and subsequent initiation of death receptor 5 (Lepage *et al*., 2011). Future work in this area would focus on isolating quinovic acid from a *Fagonia indica* extract and assessing the ability to initiate p38/DR5 apoptotic pathways *in vitro*.

Alongside terpenoids, research has estimated the flavonoid content of *Fagonia indica* to be as high as 3% (Shehab *et al*., 2011). Flavonoids are phenolic substances typically isolated from plant material, which can exhibit a range of biological activity including antiallergenic, antioxidant and anti-inflammatory effects (Pietta, 2000). Studies have identified flavonoidal patterns in *Fagonia indica* based on their systematic significance; these include quercetin and isorhamnetin glycosides (El-hadidi, Al-wakeel, & El-garf, 1988; Shehab *et al*., 2011). A more recent study by Shehab *et al* (2020), found total flavonoid content of *Fagonia indica* to be 31.289 mg per quercetin equivalent, further investigation with TLC revealed the presence of kaempferol and quercetin (Naglaa Gamil Shehab *et al*., 2020). Similarly, in the present study an aqueous extract of *Fagonia indica* was shown to have a total flavonoid content of 39.77 mg per quercetin equivalent, whereas a 75% methanolic extract of *Fagonia indica* had a 3-fold higher total flavonoid content. Further research demonstrated that methanol was the most effective solvent for the extraction of flavonoids, this translated into increased antioxidant and anti-inflammatory activities (Truong *et al*., 2019). Martinez-Perez *et al* (2014) described in detail the potent ability of novel flavonoidal compounds to modulate metabolism, induce anti-proliferative responses, modulate ABC transport proteins and induce apoptosis and cell cycle arrest in breast cancer. Therefore, there is a potential correlation between the increased flavonoid content in the 75% methanolic extract of *Fagonia indica* and the increased apoptotic

activity demonstrated in the present study against both breast and colon cancer, compared against an aqueous extract.

The present study also used total antioxidant capacity; based upon a quercetin equivalent, to estimate the redox potential of phenols and flavonoids in an aqueous and 75% methanolic extract of *Fagonia indica*. Similarly, to the present study, Do *et al* (2014) found that a 75% methanolic extract resulted in an overall higher extraction yield and increased total flavonoid yield compared with 100%, 50% methanolic and aqueous extracts (Do *et al*., 2014). Extracts with high flavonoid contents have been associated with increased cytotoxic activity against cancer cells *in vitro* and subsequent identification of bioactive compounds from plant material (Thomasset *et al*., 2009). As the 75% methanolic extract of *Fagonia indica* had both significantly increased total antioxidant capacity and antineoplastic activity *in vitro*, subsequent analysis of bioactive flavonoids using separation and mass-spectroscopy from this extract could be of pharmacological value (Lei *et al*., 2019).

As many plant isolated flavonoid and polyphenol substances have antioxidant activity, it was important to determine how an 'antioxidant rich' extract would affect production of reactive oxygen species (ROS) in cancer cell lines. Reviews have summarised the dual action of antioxidants in cancer; whereby they are able to decrease the risk of developing cancer via the quenching of free radicals, however this same activity can assist in the progression of cancer cells once malignancy has ensued (Poljsak & Milisav, 2019). In excess, ROS including superoxide and hydroxyl radicals, can cause irreversible cellular and DNA damage, which has corresponded to the initiation of inflammatory states and cancer (Hussain *et al*., 2016; Walton, 2016). However, substances which initiate an excess accumulation of intracellular ROS in malignant cells, have been demonstrated to have therapeutic value via the induction of apoptotic pathways (Ezhilarasan *et al*., 2018; Zaidieh *et al*., 2019). In the current study a DCFDA assay was used to quantitatively asses activity of hydroxyl, peroxyl and other reactive oxygen species within RKO colon and MDA-MB-231 breast cell lines. There were no significant changes in ROS activity between untreated and aqueous/ 75% methanolic *Fagonia*

indica treated cells, due to large variances between replicates. Alternatively, the ROS/superoxide assay was used to directly monitor real time accumulation of ROS and superoxide in live cancer cells. This assay revealed increased intracellular ROS/superoxide in *Fagonia indica* treated colon and breast cancer cell lines, with the most significant increases noted in cells treated with the 75% methanolic extract. Similar research found that a methanolic extract of *Scurrula ferruginea* displayed significantly higher cytotoxic activity against breast cancer cell line MDA-MB-231 than an aqueous extract which corresponded to increased accumulation of intracellular ROS (Marvibaigi *et al*., 2016). In this study it was suggested that increased cytotoxic activity of the methanolic extract was related to higher total flavonoid/ phenol content and antioxidant/ scavenging activity. It is prudent for future work to further isolate bioactive flavonoids from the methanolic extract of *Fagonia indica* for identification and analysis of cytotoxic activity both *in vitro* and *in vivo*.

Despite the promising medicinal potential of naturally derived flavonoids, polyphenols and triterpene substances, barriers such as poor solubility within the gastrointestinal fluid, low absorption and inhibition during metabolism often result in poor bioavailability of these molecules (Furtado *et al*., 2017; Hollman *et al*., 1997). Relative urinary secretion has ranged from 0.35 to 43% for certain flavonoidal intake, highlighting the issue in selecting both bioactive and bioavailable compounds from natural material (Thilakarathna & Vasantha Rupasinghe, 2013). Previous studies have demonstrated bioavailability of widely distributed flavonoid/ triterpene molecules such as quercetin and ursolic acid in *in vivo* studies using dogs and mice. In these studies, concentration of the target molecule in tissue sections increased upon subsequent oral exposure, antioxidant activity of intact molecules also increased alongside exposure (Reinboth *et al*., 2010; Yin *et al*., 2012). The next step of the present study would be to determine the bioavailability of candidate triterpene molecules of *Fagonia indica* in the plasma of human participants voluntarily consuming *Fagonia indica* for supposed health benefits. Ultra-performance liquid chromatography – mass spectroscopy (UPLC-MS) has been used to rapidly quantify ursolic and oleanonic triterpene acids in human plasma

samples and could be used in clinical studies to determine dietary bioavailability of these common natural compounds (Stebounova *et al*., 2018). More recent studies have used LC-MS/MS to successfully evaluate the oral and intravenous bioavailability of novel pentacyclic triterpene; celastrol, from *Tripterygium wilfordii* (Zhan *et al*., 2020).

5.5 Conclusion

This chapter has served as an introduction to deducing the chemical complexity of *Fagonia indica*. The breadth of phytochemicals within the plant extract were detected using chemical screening protocols, which corresponded to previous research into the chemical composition of *Fagonia indica*. A 75% methanolic extract of *Fagonia indica* was produced which displayed significantly increased concentrations of antioxidants, flavonoids and triterpene molecules, corresponding with a higher degree of cytotoxic activity against both breast and colon cancer. This serves as a foundation for future work to further isolate and characterise bioactive molecules of *Fagonia indica.* Future work could further define locations with increased concentrations of biologically relevant phytochemicals by targeting areas of the plant individually; such as the stem, leaves and flowers, or by searching specifically for free or bound compounds (Shi *et al*., 2019). Polarity of extract solvents also provided important indications regarding the structure of compounds within the bioactive extract and provides some basis for subsequent bioguided fractionation of the plant material.

The effect of aqueous and methanolic extracts of *Fagonia indica* on intracellular ROS production were also analysed for the first time. This chapter highlights the ability of a 75% methanolic extract of *Fagonia indica* to induce a significantly higher degree of intracellular ROS and superoxide accumulation in both breast and colon cancer. This highlighted a potential dual action of *Fagonia indica*, in regards to scavenging and ROS inducing activity. The increase of intracellular radicals in breast/ colon cancer cells, post-treatment with the 75% methanolic extract of *Fagonia indica* correlated with increased cytotoxic activity. This has illustrated a potential mechanism of action for *Fagonia indica* induced cytotoxicity in both

breast and colon cancer and future work would aim to explore ROS-induced apoptosis in greater depth.

This chapter was severely limited by restrictions caused by the covid-19 global pandemic and the subsequent isolation and social distancing protocols. The next stages of this chapter were to conduct a human study whereby the bioavailability of candidate molecules from *Fagonia indica* were to be screened from plasma samples using mass-spectroscopy. Ethical approval was also submitted to assess plasma samples of *Fagonia indica* consumers for circulating tumour-associated factors against a negative control group. Despite being unable to conduct this aspect of the study, this chapter has outlined an approach to screen plasma samples for triterpene molecules and has stressed the importance of determining the bioavailability of bioactive molecules in regards to their related activity. Future work would aim to follow up on these areas with a goal to pursue any prospective bioactive molecule from *Fagonia indica* into human trails.

6 Discussion

This investigation; as detailed in the previous chapters, aimed to (1) validate an aqueous extract of *Fagonia indica,* to determine its cytotoxic value against both breast and colon cancer, in a bid to support traditional medicinal information, (2) to detail mechanisms of action of an aqueous extract of *Fagonia indica* and (3) to develop an extract with increased bioactivity and concentrated bioactive compounds, which could be tested for subsequent bioavailability and downstream affects in human studies. The present study demonstrated the ability of an aqueous extract of *Fagonia indica* to induce a time and concentration dependent loss in cell viability of breast cancers (MDA-MB-231 and MCF-7) and for the first time, colon cancers (RKO and H630). Not unlike other medicinal plants, *Fagonia indica* is typically administered orally as a 'tea' in traditional communities (Ahmad *et al*., 2009). There has been a renewed interest in herbal beverages due to their unmatched sources of natural bioactive compounds; including, phenolic acids, flavonoids, alkaloids, saponins and terpenoids (Chandrasekara & Shahidi, 2018). Therefore, it was important to establish the cytotoxicity of an aqueous extract of *Fagonia indica* against cancer cell lines to validate its use as a 'tea' in traditional communities. Lam *et al* (2012), previously described a significant loss in cell viability in MCF-7 and MDA-MB-231 cell lines after treatment with 0.5-2 mg/mL of an aqueous *Fagonia indica* extract over 72 hours. The present study supported this finding and found similar losses in cell viability across colon cancer cell lines RKO and H630. This versatility suggests that bioactive compounds within the extract are targeting key regulated cellular processes, such as replication or the DNA damage response pathway. This is typical of other chemotherapeutic agents derived from natural sources, including paclitaxel which targets mitotic spindle assembly during chromosome segregation (Rajendran & Jain, 2018; Weaver, 2014).

It is important to follow up on the discovery of novel chemotherapeutic agents by understanding and analysing the mechanisms by which they exert antineoplastic affects. Studies have shown that mechanistic evidence is central to all aspects of the drug discovery

and approval process, including assessing efficacy, pharmaceutical quality and determining potential toxicity to healthy cells (Aronson *et al*., 2018). Figure 6.1.1 summarises all of the potential pathways and associated proteins/ molecules implicated in the mechanism-of-action of *Fagonia indica*. This information has been gathered from both the previous literature and the present investigation. These effector molecules have been related back to the hallmarks of cancer, as described by Hanahan & Weinberg (2011). Targeting these pathways therapeutically has been considered a success in regards to controlling the growth of various cancer types and has aided in the application of clinical trials (Al-Bedeary *et al*., 2020). The acquisition of neoplastic cells to resist programmed cell death, via the deregulation of pro and anti-apoptotic molecules, is one of the key hallmarks of cancer (Hersey & Zhang, 2003). Therefore, it is vital that any novel therapeutic agent can demonstrate an ability to overcome these resistance mechanisms.

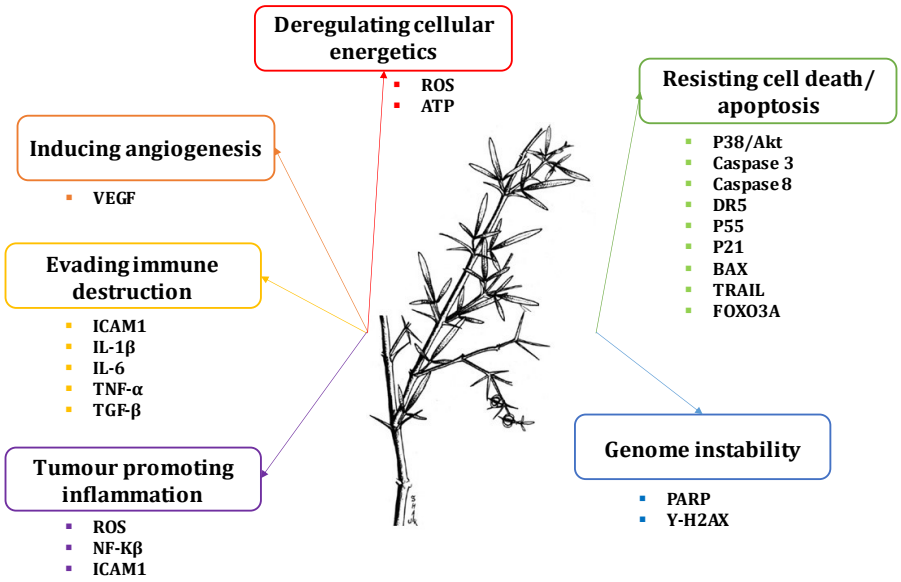

Figure 6.1.1: Effector molecules associated with the *Fagonia indica* 'mechanisms of action'. Antineoplastic molecules associated with *Fagonia indica* treatment. Compiled from the present study and including those described in studies by Lam *et al* (2012) and Azam *et al* (2018), in relation to the hallmarks of cancer as outlined by Hanahan & Weinberg (2011).

In a previous study, Lam *et al* (2012) implicated p53 activation and subsequent activation of downstream signalling molecules; BAX and p21, in *Fagonia indica* induced apoptosis. Activation of p53 via treatment with plant extract, resulted in p53 mediated transcription and

upregulation of target genes associated with growth inhibition of cells with damaged DNA (Gu *et al*., 2012). Further research revealed that quinovic acid isolated from *Fagonia indica* induced apoptosis via the activation of caspases 3/8 and the upregulation of the extrinsic pathway of apoptosis through death receptor 5 (Khayam *et al*., 2020). These pathways of *Fagonia indica* induced apoptosis were expanded upon in the present study, as demonstrated in Figure 6.1.2. In the present study, increased accumulation of intracellular reactive oxygen species (ROS) was associated with treatment with *Fagonia indica* extracts. Reactive oxygen species (ROS) are by-products of normal metabolism; however, accumulation of ROS species can be harmful to cells and DNA integrity. Cancer cells typically have higher ROS levels and are therefore more vulnerable to subsequent ROS insults from exogenous agents (Trachootham *et al*., 2009). Other plant derived compounds have been shown to exert anti-cancer effects via excessive ROS production and subsequent phosphorylation of p38 MAPK (Li *et al*., 2016; Zhang *et al*., 2015). In the present investigation, inhibition of P38 in H630 cells significantly alleviated losses in cellular viability by *Fagonia indica*, indicating a potential mechanism of apoptosis. Furthermore, ROS accumulation in cancer cells has led to inhibition of NF-κB and activation of p53, leading to a reduction in cancer-related inflammation and an increase in cytotoxicity (Yin *et al*., 2015). More recent research has shown how saponins are able to induce cell death through the activation of a ROS/JNK/p53 pathway in established colon cancer cells (Wu *et al*., 2018).

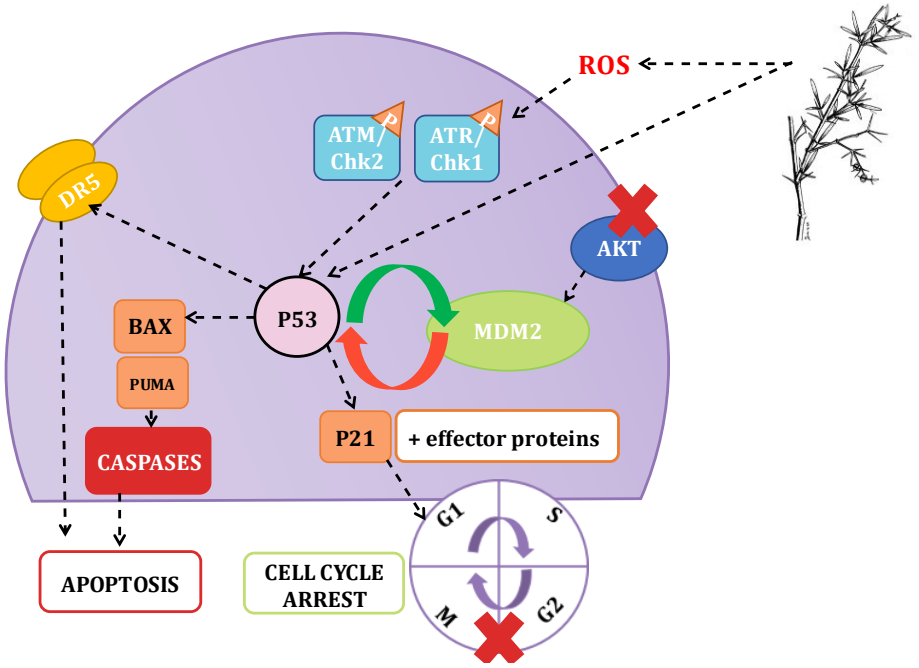

Figure 6.1.2: A potential pathway of *Fagonia indica* induced apoptosis. An outline of a potential pathway for apoptosis in *Fagonia indica* treated cancer cells, based on findings from the present study, Lam *et al* (2012) and Khayam *et al* (2020). *Fagonia indica* extract increased intracellular ROS accumulation which leads to stabilisation of p53 and effector proteins; including cell cycle modulator p21. Activation of p53 and death receptor 5 (DR5), leads to activation of caspase 3 and 8 and subsequent induction of apoptosis.

Alongside increased intracellular ROS, reduced intracellular ATP concentrations were also consistently noted in *Fagonia indica* treated breast and colon cancers. Metabolic dysregulation is a key phenotype in cancer, characterised by increased glycolysis which becomes the predominant pathway of ATP production (Ganapathy-Kanniappan & Geschwind, 2013). Research has identified electron transfer within the mitochondria as a potential target for cancer treatment (Yang *et al*., 2020). Disrupting electron transfer leads to an increase in ROS generation and a decrease in ATP production, leading to a subsequent accumulation of dysfunctional proteins which ultimately impair mitochondrial functioning (Usta *et al*., 2009). Other studies have demonstrated an ability of plant extracts to alter mitochondrial membrane potential in cancer cell lines, resulting in an increase in intracellular ROS and a reduction in ATP (Madi *et al*., 2016). This imbalance led to activation of caspases and cell death in breast cancer cell line MCF-7 (K. Jiang *et al*., 2015). In the present study, increases in intracellular ROS and superoxide levels were consistent with decreases in intracellular ATP concentration

in *Fagonia indica* treated cell lines. These effects were noted in both breast and colon cancer cells, suggesting that ROS and ATP are significant components of *Fagonia indica* mechanisms of action.

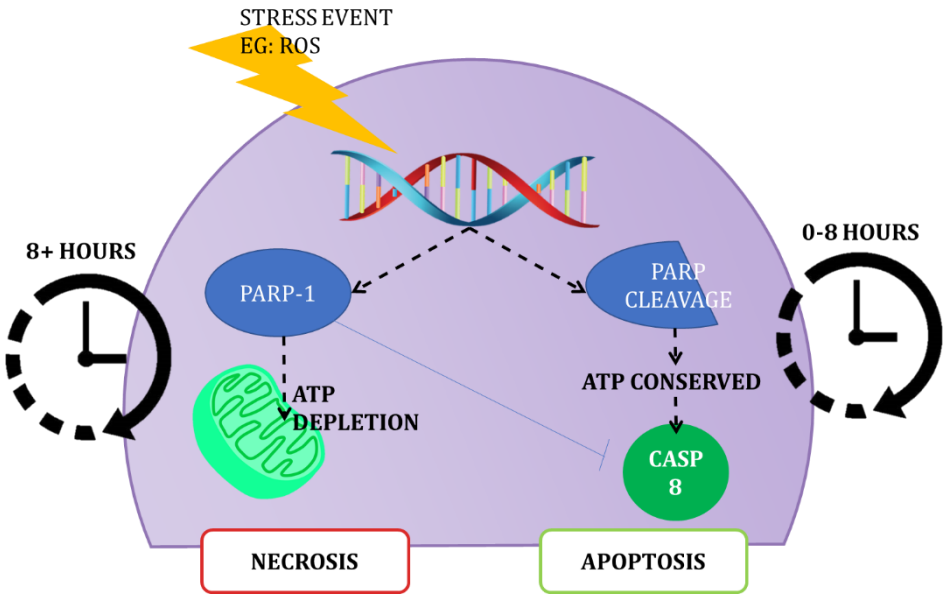

Figure 6.1.3: Proposed pathways of *Fagonia indica* induced apoptosis and necrosis in cancer cell lines. An outline of a potential pathway for apoptosis in *Fagonia indica* treated cancer cells, based on findings from the present study and Lam *et al* (2012). *Fagonia indica* extract increased intracellular ROS accumulation (0-4 hours) leading to irreparable DNA damage and cleavage of PARP1 (4 hours). PARP1 cleavage prevents further depletion of NAD and ATP, required for later stages of apoptosis including activation of caspase 8 (6 hours). After 24 hours PARP-1 remains intact and ATP is depleted during necrosis.

Tumour cells require access to nutrients and oxygen from regulated blood flow in order to survive and replicate (Ferrara, 2005). Angiogenesis, or the formation of new blood vascularisation is fundamental for tumour growth, invasion and metastasis (Niu & Chen, 2010). Among all the proteins involved in this angiogenic switch, VEGF, has been identified in several tumour cells and has demonstrated angiogenic activity *in vitro* and *in vivo* (Shinkaruk *et al*., 2003). In the present study, VEGF expression was reduced in breast and colon cancer cell lines after treatment with *Fagonia indica* extract. Other studies found that plant extracts were able to reduce proliferation of cancer cell lines, in relation to a parallel reduction in VEGF expression or inhibition of VEGF-2 tyrosine kinase receptor (Huang *et al*., 2016; Lamy *et al*., 2014). Further studies have demonstrated a relationship between VEGF

and induction of Akt in cancer cells, Akt was inhibited in a VEGF-dependent manner upon treatment with a Luteolin extract (Bagli *et al*., 2004). Interestingly, research has shown that VEGF activity and angiogenesis is regulated by intracellular ROS (Shinoda *et a*l., 2004; Xia *et al*., 2007). Other studies have shown that angiogenic modulation can be controlled by exogenous antioxidants (Radomska-Lesniewska *et al*., 2017). As phytochemical screening revealed, *Fagonia indica* contained high flavonoid and high total antioxidant capacity. *Fagonia indica* treatment also induced production of reactive oxygen species within cancer cells, whilst also down regulating expression of VEGF. Hassan *et al* (2014), screened plant extracts for those displaying increased biological activity, in regards to antineoplastic and antiangiogenic effects. Increased cytotoxicity and antiangiogenic activity correlated with increased antioxidant capacity and flavonoidal content (Hassan *et al*., 2014).

Expression of transmembrane glycoprotein receptor; ICAM-1, was significantly inhibited in both breast and colon cancer cell lines, after treatment with *Fagonia indica* extract. ICAM-1 is an important molecule for cell-cell and cell-matrix adhesive interactions, which have significant implications for inflammatory, neoplastic and metastatic pathways (Chung *et al*., 2017). A study using an acai phenolic plant extract found that treatment downregulated ICAM-1 as a target of proinflammatory transcription via NF-κB (Dias *et al*., 2014). Ginzburg *et al* (2014), found that NF-κB activity was mediated by *Piper longum* plant extract treatment, alongside cytokines IL-6, IL-8 and ICAM-1. Mediation of this inflammatory pathway prevented development of aggressive growth characteristics associated with cancer progression (Ginzburg *et al*., 2014). In the present study both NF-κB and ICAM-1 expression were down-regulated by extract treatment, highlighting a potential anti-inflammatory pathway for *Fagonia indica*. It is also worth noting that previous studies have demonstrated an ability of *Fagonia indica* to mediate IL-6 and IL-8 in regards to inflammation (Azam *et a*l., 2018). Further research has also correlated natural antioxidant compounds with the inhibition of VEGF-induced activation of NF-κB and subsequent downstream targets, including ICAM-1 (Shukla *et al*., 2019). This pathway is associated with increased angiogenesis and

inflammation and therefore could attribute to the reduction of long-term cell growth and wound healing as shown in the present study.

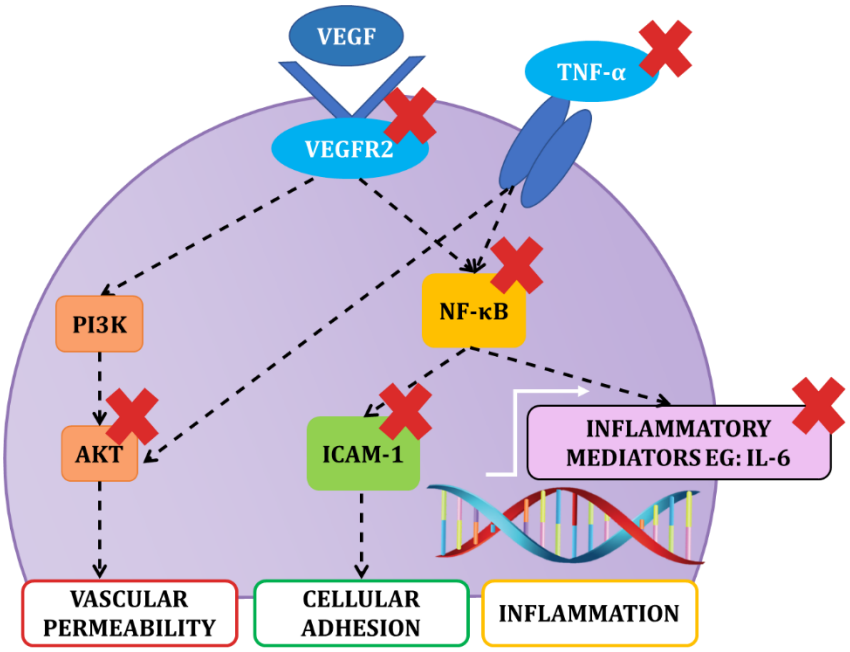

Figure 6.1.4: Potential pathway targets of *Fagonia indica* in cancer related functional mechanisms. An outline of a potential targets for in *Fagonia indica* treated cancer cells, based on findings from the present study and Azam *et al* (2018). *Fagonia indica* extract reduced expression of VEGF and TNF-α. Both VEGF and TNF- α are involved in metastatic pathways including inflammation, cellular adhesion, vascular permeability and angiogenesis. In the current study, VEGF, NF-kB and ICAM-1 gene expression was typically reduced after treatment with Fagonia indica, highlighting a potential pathway for Fagonia indica in inhibiting metastatic pathways in breast and colon cancer. TNF-a, NF-kB and other inflammatory mediators (IL-6) were also reduced in the current study and previous work by Azam *et al* (2018), again highlighting a potential pathway of action for *Fagonia indica*.

The mechanisms of action for *Fagonia indica* can also provide some indications regarding the chemical components of the plant extract. Flavonoids for example, have been shown to affect several targets, including the accumulation of reactive oxygen species, modulation of the cell cycle, inhibition of DNA repair mechanisms, inhibition of metastasis and regulation of inflammatory pathways (Cijo George *et al.*, 2016.). Other aspects include the ability of flavonoids to modulate CYP1 and ABC protein families which have important ramifications for effective drug delivery (Martinez-Perez *et al*., 2014). Several of the flavonoids previously isolated and identified from *Fagonia indica* had a Quercetin aglycone which corresponded to antioxidant activity, typical of compounds of this nature (Shaker *et al*., 2013). Studies have shown that Quercetin is able to strongly induce mitochondrial ROS accumulation leading to

increased p53 protein stability. This occurred specifically in tumours displaying aggressive proliferation (Kim *et al*., 2017). Increased p53 accumulation was also noted in several breast cancer cell lines after treatment with Quercetin. (Tang *et al*., 2020). In the present study, the methanolic extract with increased antioxidant capacity, initiated increased ROS production and apoptosis. Flavonoids; including those with a quercetin backbone, also play an important role in the regulation of inflammatory pathways. Cheng *et al* (2019), identified a potential anti-inflammatory pathway for quercetin. Quercetin inhibited monocyte adherence via the suppression of ICAM-1 via inhibition of NF-κB transcription (Cheng *et al.,* 2019). Additionally, VEGF/Akt signalling has also been implicated as a mechanism in which Quercetin exerts anti-inflammatory and anti-proliferative effects *in vitro* (Sun *et al*., 2018). In the present study, phytochemical screening identified phenolic and flavonoidal compounds in *Fagonia indica* extracts. These could be contributing to some of the mechanisms of action of *Fagonia indica* as described above.

Another important group of phytochemicals present in *Fagonia indica* are triterpenes, including triterpenoids and saponins. Triterpenoids are metabolites of the isopentenyl pathway of steroid metabolism and represent the most abundant group of phytochemicals present in nature (Bishayee *et al*., 2011). Several of the most commonly detected triterpenes; ursolic acid, oleanolic acid, butanolic acid and lupeol, possess potent antitumour and anti-inflammatory properties (Petronellia *et al*., 2009). Phase I trials are currently underway to determine the effects of ursolic acid liposome treatment against subjects with advanced stage tumours (Qian *et al*., 2015). Thus far, ursolic acid has demonstrated limited toxicity, tolerability in accumulated states and improved patient remission rates. In *Fagonia indica*, several triterpenes and triterpenoid saponins have been isolated and characterised, including ursolic acid (Rahman *et al*., 1984). More recently, Farheen *et al* (2015) isolated several established and novel triterpenoids from a methanolic extract of *Fagonia indica*. In the present study a methanolic extract of *Fagonia indica* had an increased concentration of triterpenes than aqueous and butanolic extracts.

Solvent extraction is a key step in the isolation and identification of bioactive compounds from a crude plant extract. Solvent polarity greatly affects the phytochemical/ bioactive compound yields of potent plant extracts (Wakeel *et al*., 2019). Rizhikovs *et al* (2015), found that natural triterpenoids had low solubility in non-polar organic solvents, with extraction becoming less selective with increased polarity. Solvents with the lowest polarity; including hexane (0.009), had the lowest triterpene content (1.5%), whereas methanol (0.762) had the highest extraction of triterpenes (24.3%) (Rizhikovs *et al*., 2015). In the present study, a 75% methanolic extract of *Fagonia indica* displayed increased triterpenoid content, demonstrated by colorimetric screening and TLC. However, as polar solvents also tend to extract higher levels of admixtures; such as phenols and flavonoids, further separation is required to produce a triterpenoid rich fraction. Studies have shown that liquid chromatographic techniques are the most successful for extracting compounds with increased polarity from methanolic extracts (Z. Jiang *et al*., 2016). Bae *et al* (2012), found that methanolic extracts product the greatest triterpene extracts from non-pungent pepper plants; over those with lower polarities, including hexane, ethyl acetate and acetone. A methanolic extract also contained higher levels of flavonoids, antioxidants and compounds with DPPH activity (Bae *et al*., 2012). Interestingly, a similar effect was noted in the present investigation, with the methanolic extract displaying significantly higher flavonoids and total antioxidant capacity. As the methanolic extract displayed increased cytotoxicity against breast and colon cancer, it is prudent to further establish the phytochemical make-up of the extract in order to isolate bioactive compounds. It is likely that *Fagonia indica* has several bioactive compounds, made up of various secondary metabolites working synergistically (Caesar & Cech, 2019).

Multidrug resistance is one of the most confounding issues facing cancer pharmacology. A common mechanism in MDR is the overexpression of ATP-binding cassette (ABC) efflux transporters, caused by continued exposure to anticancer therapies (Hee Choi & Yu, 2014). In the present investigation, ABCC4 was consistently overexpressed across all resistant breast and colon cancer cells. ABCC4 has been reported to transport a range of substrates including

antivirals, antibiotics, anti-inflammatories and antitumour drugs (Tsukamoto *et al*., 2019). In regards to natural products, plant metabolites; such as flavonoids, can pass through efflux pumps competing with other drugs or even inhibiting their transport (Aszalos, 2008; Lu *et al*., 2021). *Fagonia indica* extract significantly reduced the expression of ABCC4 in both breast and colon cancer and it was hypothesised that extract treatment could reverse multidrug resistance. Interestingly, Zhao *et a*l (2014) found that suppression of ABCC4 in lung cancer inhibited cell growth and increased the percentage of cells in the G1 phase of the cell cycle (Zhao *et al*., 2014). Inhibition of ABCC4 expression could be a potential mechanism of action for *Fagonia indica* induced apoptosis in chemotherapy resistant cell lines.

Reprogramming of cellular metabolism is another potential cause of decreased ABC transporter expression in cancer (Ma & Zong, 2020). The 'Warburg' effect describes the prioritisation of glycolysis as the main source of ATP production in malignant cells. Therefore, the inhibition of glycolysis provides a method to overcome resistance in ABC overexpressing cancer cells (Nakano *et al*., 2011). Akins *et al* (2018), inhibited key enzymes of glycolysis, resulting in reduced proliferation and expression of ABC transporters and drug trafficking in tumour cells (Akins *et al*., 2018). Other studies noted similar effects when glycolysis was switched to oxidative phosphorylation. Expression of several ABC transporters; including ABCC1, ABCC5 and ABCG2, were downregulated in p53 wild-type cells (Belkahla *et al*., 2018). In the present investigation, intracellular ATP concentration was significantly reduced in all four cancer cell lines after treatment with *Fagonia indica*. Reducing available ATP for active transport via ABC membrane transporters, could result in inhibited expression of over-expressed transporter proteins.

6.1 Future work

This thesis made some significant progress in the characterisation of a *Fagonia indica* extract in the treatment of breast and colon cancer. Building on previous studies, other key effector molecules; including caspase-8, p38 and Akt, were proposed as components of the apoptotic

pathway of *Fagonia indica*. However, these effector molecules were not consistently affected across all four cell lines. Therefore, key questions regarding the mechanisms of action of *Fagonia indica* remain unanswered and therefore warrant further investigation. For example, flow cytometry could help differentiate between the type of cell death initiated post-treatment, including apoptosis, necrosis and autophagy Determining the type of cell death is important when postulating the mechanisms of action. Autophagy is typically induced prior to apoptosis upon stress stimuli, whereby apoptosis rather than necrosis is induced when autophagy is impaired (Chen *et al*., 2018). Also, it is worth considering that two or even three types of cell death can be induced simultaneously or successively depending on the exposure to a particular stimulus, which can be detected using flow cytometry.

A promising avenue for *Fagonia indica* treatment, is the potential ability to regulate metabolic reprogramming in cancer cells. It was postulated that metabolic dysfunction was responsible for the loss in cell viability from *Fagonia indica* treatment. Intracellular ATP concentrations were significantly reduced in all four cell lines after treatment with an aqueous extract. It is known that prolonged metabolic stress can trigger autophagy in cancer cells, in relation to stabilisation of p53. As part of the 'Warburg' effect malignant cells become reliant on glycolysis for ATP generation, as such glycolytic activity is essential for survival and growth (Pelicano *et al*., 2006). In the present investigation, the mechanism in which *Fagonia indica* reduces ATP concentration is unclear. Glycolytic metabolism assay kits are available to measure the activity of several key enzymes involved in glycolysis, this could provide some indication of whether *Fagonia indica* extract inhibits the process of ATP formation in cancer. As reactive oxygen species accumulation was also associated with *Fagonia indica* treatment, it would be worthwhile to undertake an assay determining the effect of extract treatment on mitochondrial membrane potential (Marchi *et al*., 2012). Understanding the role of *Fagonia indica* in reprogramming metabolic dysregulation in cancer is important in establishing its potential use as an antineoplastic treatment.

A role of *Fagonia indica* in regulating inflammatory pathways was also reported. Gene expression analysis revealed a down-regulation of key effector molecules; VEGF, NF-κB and ICAM-1, after treatment with *Fagonia indica* extract. Future work would aim to determine how this reduction in mRNA expression translated to protein expression, via western blotting analysis. As a growth factor; VEGF is secreted from the cell and can therefore be measured from culture medium. An enzyme-linked immunosorbent assay (ELISA) would be useful to investigate the concentration of VEGF into the secreted cell supernatant (Jensen *et al*., 2006). Determining the effect of *Fagonia indica* on expression and secretion of VEGF on tumour associated fibroblasts, would be useful in determining how these effects translate into the tumour microenvironment. Studies have shown that latent angiogenic activity can be activated via the secretion of VEGF from stromal fibroblasts surrounding the tumour, in a process known as the 'angiogenic' switch (Ito *et al*., 2007).

The original aims of this study included an element of *in vivo* testing of *Fagonia indica*, in terms of a human study. Aston University ethical approval was obtained to recruit a cohort of British Pakistani women who frequently consumed *Fagonia indica* tea via a member of the supervisory team, Prof Carmichael. Plasma samples were to be then screened for circulating tumour risk factors (eg: biomarkers such as interleukin-6, leptin and vascular endothelial growth factor) associated with breast and colon cancer, using enzyme-linked immunosorbent assays (ELISA). However, due to the restrictions on social interactions that were sanctioned in light of the Covid-19 pandemic, any in person investigation had to be cancelled. Future studies would aim to pursue this line of investigation, in a bid to determine the effectiveness of *Fagonia indica* in a non-clinical environment (Ried *et al*., 2017).

A key question surrounding this project was 'what are the bioactive compounds of *Fagonia indica*?'. Although this work went some way to producing a fraction of *Fagonia indica* with increased cytotoxic activity, there is still a significant amount of work required to isolate and identify bioactive compounds. Solvent extraction was utilised to separate compounds from the crude extract of *Fagonia indica* based on their polarity and solubility. Phytochemical

screening revealed increased concentration of triterpenes, flavonoids and antioxidant compounds in a methanolic extract. Important follow-on work is required to further isolate individual compounds from the methanolic extract and identify their structure and function. Typically, gas chromatography- mass spectrometry is used as an extraction and quantification protocol (Medina-Meza *et al*., 2016). After selection of candidate antineoplastic compounds, it is important to determine the bioavailability of the natural compound. In order to exert cytotoxic effects, the compound needs to be able to withstand consumption, be bio-accessible within the gastrointestinal tract, undergo metabolism and then reach the tumour target (Rein *et al*., 2013).

6.2 Limitations

As mentioned in previously, there were some limiting factors which impacted on the progress of particular aspects of this project. The announcement of a national lockdown in response to the Covid-19 pandemic, resulted in the closure and subsequent introduction of social distancing methods within the laboratory. These restrictions severely impacted on planned experiments in Chapter 5. Ethical approval was sought; prior to the lockdown, for a human based study whereby plasma samples of women regularly drinking the tea were to be taken and assessed for biomarkers of disease. However, due to the face-to-face nature of this study and the safety of participants under the current guidelines, the study was postponed as a future project falling outside the time scale of the current project. Similarly, access to specialist equipment and training from technical staff for chemical evaluation of the *Fagonia indica* extract was also severely limited, due to social distancing measures and access requirements in individual laboratories. With these factors taken into consideration, it was decided that the focus of Chapter 5 would be limited to providing a more generalised introduction of the phytochemical content of *Fagonia indica*, which could instead provide a basis for future identification of novel compounds.

6.3 Conclusion

This thesis describes the use of an aqueous extract of *Fagonia indica* against phenotypically distinct breast and colon cancer cell lines. *Fagonia indica* is an herbaceous plant used frequently in rural communities in Pakistan, as a treatment for a variety of diseases including cancer. An aqueous extract of *Fagonia indica* was prepared and tested for its cytotoxic potential, in a bid to replicate and validate traditional administration of the extract by medicinal healers. This investigation confirms that an aqueous extract is cytotoxic against breast cancer cell lines; MCF-7 and MDA-MB-231, and for the first time, colon cancer cell lines; RKO and H630. Mechanisms of action for *Fagonia indica* were expanded upon from previous studies which identified p53, FOXO3a and downstream transcription targets p21 and BAX, as important features of *Fagonia indica* induced apoptosis. Several molecular targets were proposed as effectors of *Fagonia indica* induced apoptosis; including caspase-8, p38 and Akt. However, these weren't consistently affected across all four cell lines, demonstrating some disparity between cancer types with varying molecular phenotypes. For the first time, accumulation of reactive oxygen species (ROS) was associated with treatment of *Fagonia indica* and subsequent cell death in both breast and colon cancer cell lines. This also corresponded to reduced intracellular ATP, highlighting a role for *Fagonia indica* in overcoming dysregulated metabolism *in vitro*. The role of *Fagonia indica* to disrupt other key pathways in tumorigenesis; angiogenesis and inflammation, was also investigated in breast and colon cancer. Treatment with an aqueous extract significantly reduced long-term proliferation of breast and colon cancer cell lines and inhibited wound healing potential i*n vitro*. For the first time, VEGF, NF-κB and ICAM-1 downregulation was associated with treatment with *Fagonia indica*, highlighting a role of extract treatment in the reduction of tumour-associated inflammation and angiogenesis.

This thesis also explored; for the first time, the effects of *Fagonia indica* treatment on chemotherapy resistant breast and colon cancer cell lines. An aqueous extract reduced cell viability in gemcitabine, paclitaxel and tomudex resistant breast and colon cancers, in a time

and dose dependent manner. The ability of *Fagonia indica* treatment to overcome resistance mechanisms in chemotherapy resistant cell lines was investigated. Gene expression analysis revealed an overexpression of ABCG2 and ABCC4 in resistant cell lines, which was largely alleviated upon treatment with *Fagonia indica*. Despite this, investigation revealed that it was unlikely that *Fagonia indica* acted as a direct inhibitor of ABC transporters, in fact treatment could initially increase efflux activity. As *Fagonia indica* reduces intracellular accumulation of ATP in both breast and colon cancer, it was proposed that reduced ATP availability could contribute to reduced expression of ABC transporters *in vitro*.

Finally, phytochemical screening revealed a diverse chemical backdrop in a crude *Fagonia indica* extract. Previous research had proposed high flavonoidal and triterpenoid content as a key component in the bioactivity of *Fagonia indica*. The present investigation produced a methanolic extract of *Fagonia indica* which provided a significantly higher concentration of triterpene and flavonoidal compounds and molecules capable of antioxidant activity. The methanolic extract also demonstrated a higher level of cytotoxic activity in breast and colon cancer, when compared to aqueous and butanolic extracts. This provides a good basis for subsequent isolation and identification of bioactive compounds.

7 Chapter 7: Appendix

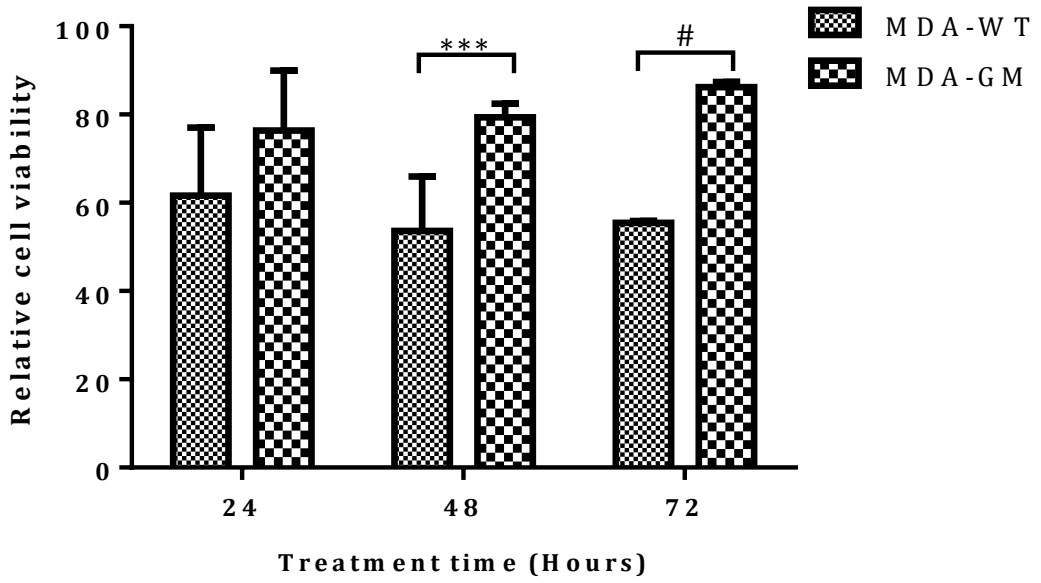

Figure 7.1 Treatment of wild-type and chemotherapy resistant MDA-MB-231 cells with gemcitabine. Wild-type MDA-MB-231 (MDA-WT) and gemcitabine resistant MDA-MB-231 (MDA-GM) cells were treated with 1 μM gemcitabine for 24-72 hours. Cell viability was determined as a percentage of an untreated DMSO vehicle control using MTT assay. Data denoted *** ($p<0.001$) and # ($p<0.0001$) were significant compared to the untreated control analysed by two-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SD.

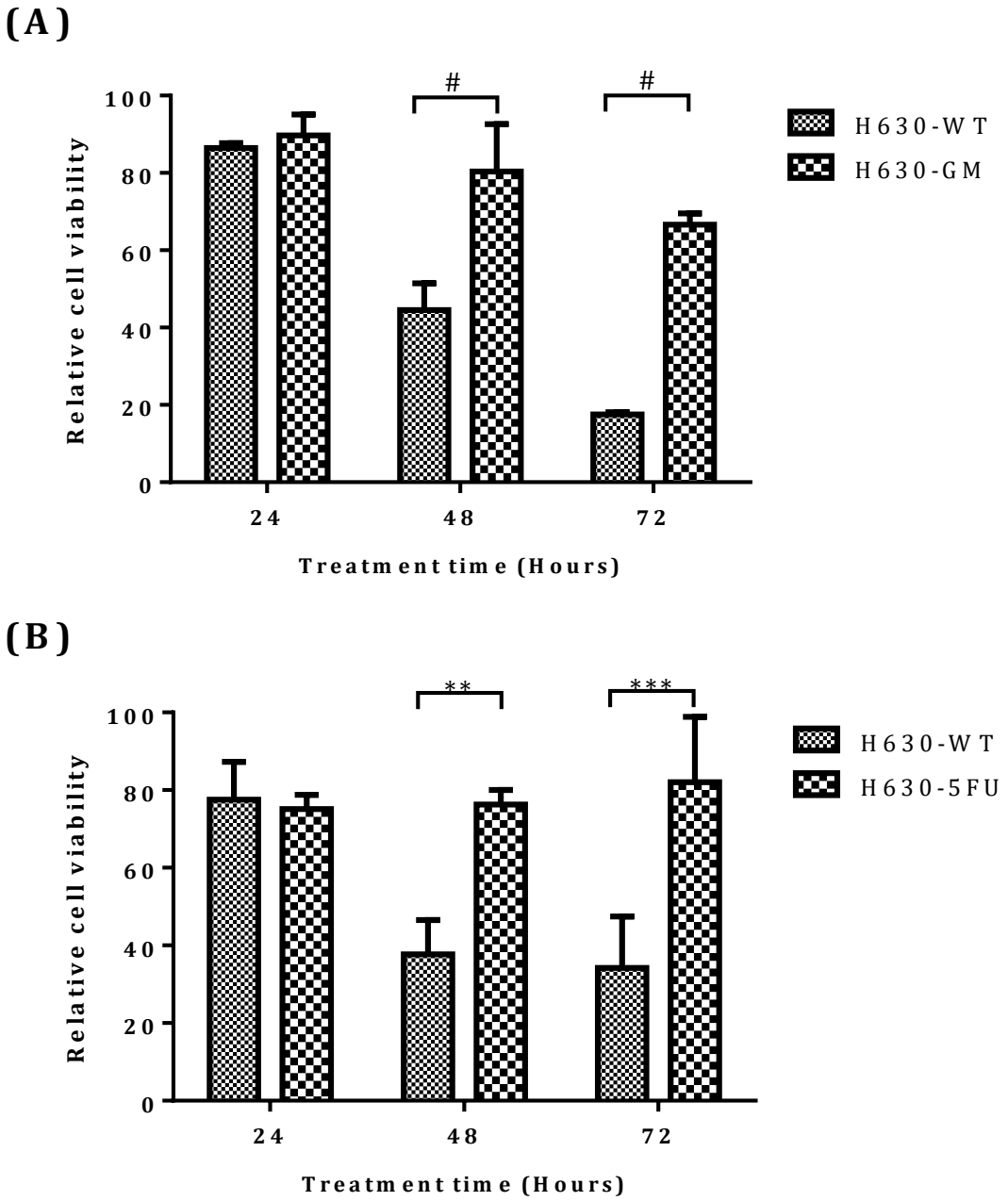

Figure 7.2: Treatment of wild-type and chemotherapy resistant H630 cells with gemcitabine and 5-fluorouracil. (A) Wild-type H630 (H630-WT) and gemcitabine resistant H630 (H630-GM) cells were treated with 1 μM gemcitabine and (B) 5-fluorouracil resistant H630 (H630-5FU) cells were treated with 10 μM 5-fluorouracil for 24-72 hours. Cell viability was determined as a percentage of an untreated DMSO vehicle control using MTT assay. Data denoted ** ($p<0.01$), *** ($p<0.001$) and # ($p<0.0001$) were significant compared to the untreated control analysed by two-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SD.

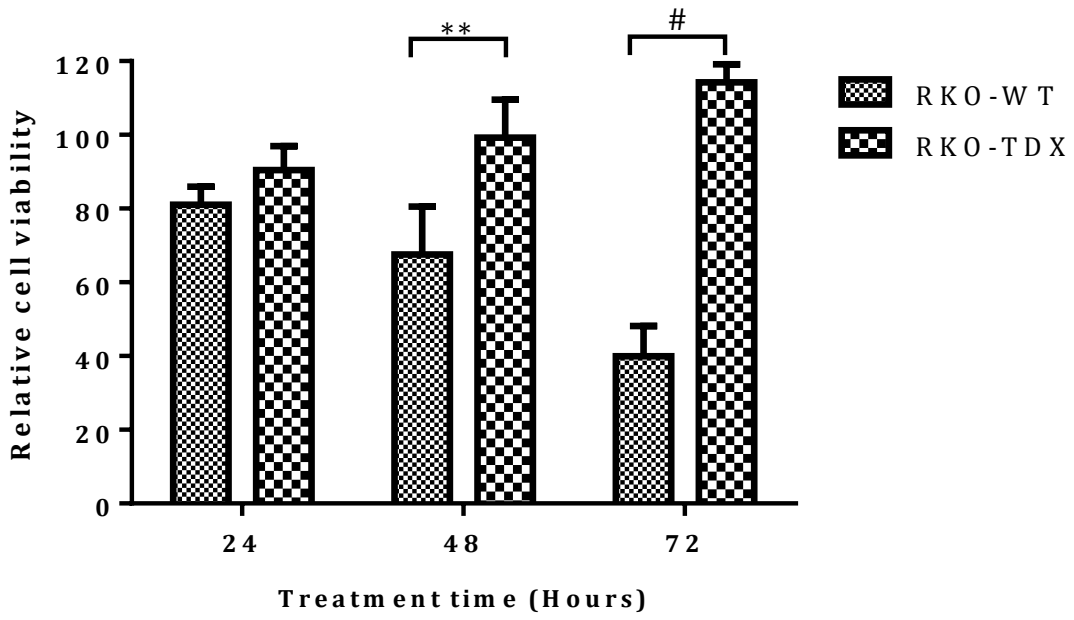

Figure 7.3: Treatment of wild-type and chemotherapy resistant RKO cells with tomudex. Wild-type RKO (RKO-WT) and tomudex resistant RKO (RKO-TDX) cells were treated with 2 μM tomudex for 24-72 hours. Cell viability was determined as a percentage of an untreated DMSO vehicle control using MTT assay. Data denoted ** ($p<0.01$), *** ($p<0.001$) and # ($p<0.0001$) were significant compared to the untreated control analysed by two-way ANOVA with Dunnett's multiple comparison test. All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SD.

GENE OF INTEREST	CELL-LINE TYPE	REPEAT NO	AVERAGE CT VALUE	ΔCT	ΔΔCT
VEGF	H630 WT	1	22.35667	5.009337	-0.49666
		2	23.40333	6.056003	0.550003
		3	22.8	5.45267	-0.05333
	H630 6 HR	1	25.13333	8.536393	3.003723
		2	23.695	7.09806	1.56539
		3	23.75333	7.156393	1.623723
	H630 24 HR	1	23.255	5.84556	0.31289
		2	22.58	5.17056	-0.36211
		3	24.25333	6.843893	1.311223
	RKO WT	1	24.98667	7.731387	0.242778
		2	25.065	7.80972	0.321111
		3	24.18	6.92472	-0.56389
	RKO 6 HR	1	25.23	8.2511	0.76249
		2	25.8	8.8211	1.33249
		3	25.38667	8.407767	0.919157
	RKO 24 HR	1	24.8	7.8211	0.33249
		2	25.69667	8.717767	1.229157
		3	24.46333	7.484433	-0.00418
	MCF7 WT	1	23.24333	6.271633	0.925555
		2	22.21667	5.244967	-0.10111
		3	21.76333	4.791633	-0.55444
	MCF7 6 HR	1	24.32	6.9789	1.54282
		2	25.78	8.4389	3.00282
		3	25.295	7.9539	2.51782
	MCF7 24 HR	1	22.055	4.7066	-0.72948
		2	23.125	5.7766	0.34052
		3	25.72	8.3716	2.93552
	MDA-MB-231 WT	1	23.325	5.80972	0.217778
		2	23.05	5.53472	-0.05722
		3	22.94667	5.431387	-0.16056
	MDA-MB-231 6 HR	1	23.535	6.98583	1.273888
		2	23.83333	7.284163	1.572221
		3	24.69333	8.144163	2.432221
	MDA-MB-231 24HR	1	25.47333	8.406633	2.694691
		2	24.69	7.6233	1.911358
		3	25.305	8.2383	2.526358
ICAM-1	H630 WT	1	22.21	4.8627	-0.69333

		2	22.8	5.4527	-0.10333
		3	23.7	6.3527	0.79667
	H630 6 HR	1	25.18	8.58306	3.02703
		2	24.27667	7.679727	2.123697
		3	24.59	7.99306	2.43703
	H630 24 HR	1	22.57667	5.167227	-0.3888
		2	22.74	5.33056	-0.22547
		3	23.62333	6.213893	0.657863
	RKO WT	1	22.08	4.82472	-0.03889
		2	21.99333	4.738053	-0.12556
		3	22.28333	5.028053	0.164444
	RKO 6 HR	1	22.83667	5.860837	0.997228
		2	24.24667	7.270837	2.407228
		3	22.61667	5.640837	0.777228
	RKO 24 HR	1	23.32	6.55042	1.686811
		2	25.10333	8.333753	3.470144
		3	24.23333	7.463753	2.600144
	MCF7 WT	1	23.24333	6.271633	0.835555
		2	22.21667	5.244967	-0.19111
		3	21.76333	4.791633	-0.64444
	MCF7 6 HR	1	25.875	8.5339	3.09782
		2	27.00667	9.665567	4.229487
		3	27.68667	10.34557	4.909487
	MCF7 24 HR	1	24.16667	6.818267	1.382187
		2	24.64	7.2916	1.85552
		3	26.59	9.2416	3.80552
	MDA-MB-231 WT	1	20.29667	2.771487	-0.18333
		2	19.33	1.80482	-1.15
		3	20.66333	3.138153	0.183333
	MDA-MB-231 6 HR	1	22.98	6.43083	3.47601
		2	22.84	6.29083	3.33601
		3	23.61	7.06083	4.10601
	MDA-MB-231 24HR	1	25.07667	8.009967	5.055147
		2	25.43	8.3633	5.40848
		3	24.51333	7.446633	4.491813
NFKB	H630 WT	1	25.555	8.2077	-0.23222
		2	26.23	8.8827	0.442778
		3	25.57667	8.229367	-0.21056
	H630 6 HR	1	28.21	11.61306	3.173138
		2	30.465	13.86806	5.428138

		3	29.13667	12.53973	4.099804
	H630 24 HR	1	28.73333	11.32389	2.995637
		2	27.54	10.13056	1.802304
		3	26.625	9.21556	0.887304
	RKO WT	1	26.41667	9.161387	-0.48833
		2	26.88333	9.628053	-0.02167
		3	27.415	10.15972	0.51
	RKO 6 HR	1	28.66	11.6811	2.03138
		2	29.67667	12.69777	3.048047
		3	29.81	12.8311	3.18138
	RKO 24 HR	1	27.82	11.05042	1.4007
		2	27.595	10.82542	1.1757
		3	27.18	10.41042	0.7607
	MCF7 WT	1	29.13	12.1583	0.1889
		2	29.44333	12.47163	0.502233
		3	28.25	11.2783	-0.6911
	MCF7 6 HR	1			
		2	29.09333	11.75223	-0.21717
		3	29.355	12.0139	0.0445
	MCF7 24 HR	1	28.42333	11.07493	1.858923
		2	28.80333	11.45493	1.428466
		3	28.03	10.6816	2.441555
	MDA-MB-231 WT	1	24.52667	7.001487	-0.09055
		2	24.18	6.65482	-0.43722
		3	25.145	7.61982	0.52778
	MDA-MB-231 6 HR	1	26.33333	9.784163	2.692123
		2	25.91333	9.364163	2.272123
		3	27.3	10.75083	3.65879
	MDA-MB-231 24HR	1	28.52333	11.45663	4.364593
		2	28.01	10.9433	3.85126
		3	28.54	11.4733	4.38126
AUF-1	MCF7 WT	1	21.11	3.59472	0.587778
		2	20.35667	2.841387	-0.16556
		3	20.1	2.58472	-0.42222
	MCF7 6 HR	1	23.20333	6.654163	3.647221
		2	24.215	7.66583	4.658888
		3	24.78	8.23083	5.223888
ABCG2	H630 GM	1	24.065	5.6003	-0.35722

		2	24.26667	5.801967	-0.15555
		3	24.935	6.4703	0.51278
	H630 GM 6 HR	1	26.11	8.2011	2.24358
		2	25.97333	8.064433	2.106913
		3	24.84	6.9311	0.97358
	H630 GM 24 HR	1	25.92667	6.276667	0.319147
		2	26.84667	7.196667	1.239147
		3	27.43	7.78	1.82248
	H630 5FU	1	27.32667	8.420833	0.805556
		2	26.42	7.514167	-0.10111
		3	25.81667	6.910833	-0.70444
	H630 5FU 6 HR	1	24.55	8.3794	2.18969
		2	24.14	7.9694	1.77969
		3	24.65333	8.482733	2.293023
	H630 5FU 24 HR	1	24.03333	7.895033	1.705323
		2	24.01333	7.875033	1.685323
		3	24.09667	7.958367	1.768657
	MDA-MB-231 GM 6 HOUR	1	24.085	4.65111	-1.265
		2	25.48	6.04611	0.13
		3	26.485	7.05111	1.135
	MDA-MB-231 GM 6 HOUR	1	27.9	9.2314	0.83919
		2	26.53	7.8614	2.16906
		3	27.48667	8.818067	1.1176
	MDA-MB-231 GM 24 HR	1	31.045	12.602	3.62353
		2	27.9	9.457	0.47853
		3	29.905	11.462	2.48353
	RKO TDX	1	26.79	9.69	0.874444
		2	25.51667	8.416667	-0.39889
		3	25.44	8.34	-0.47556
	RKO TDX 6HR	1	24.32333	6.852663	-1.96294
		2	25.90333	8.432663	-0.38294
		3	25.83333	8.362663	-0.45294
	RKO TDX 24HR	1	24.17	6.874	-1.9416
		2	25.19333	7.897333	-0.91827
		3	24.03667	6.740667	-2.07493
ABCC4	H630 GM	1	25.67	7.2053	-0.73222
		2	27.52667	9.061967	1.124445
		3	26.01	7.5453	-0.39222
	H630 GM 6 HR	1	28.7	10.7911	2.85358
		2	28.78	10.8711	2.93358
		3	29.53333	11.62443	3.686913
	H630 GM 24 HR	1	32.04	12.39	4.45248
		2	32.29	12.64	4.70248
		3	31.53333	11.88333	3.945813
	H630 5FU	1	25.59	6.6827	-0.40056

		2	26.17667	7.269367	0.186107
		3	26.205	7.2977	0.21444
	H630 5FU 6 HR	1	27.4	11.2617	4.17844
		2	28.17	12.0317	4.94844
		3	24.33333	8.195033	1.111773
	H630 5FU 24 HR	1	26.55667	10.38607	3.302807
		2	25.845	9.6744	2.59114
		3	26.28667	10.11607	3.032807
	MDA-MB-231 GM 6 HOUR	1	26.94	8.3208	-0.66667
		2	28.08	9.4608	0.473333
		3	27.8	9.1808	0.193333
	MDA-MB-231 GM 6 HOUR	1	29.97	11.3014	2.32293
		2	29.22	10.5514	1.57293
		3	30.88667	12.21807	3.239597
	MDA-MB-231 GM 24 HR	1	29.855	11.412	2.43353
		2	31.185	12.742	3.76353
		3	31.2	12.757	3.77853
	RKO TDX	1	24.91	7.81	0.023333
		2	24.575	7.475	-0.31167
		3	25.175	8.075	0.288333
	RKO TDX 6HR	1	25.295	7.82433	0.037663
		2	27.03667	9.565997	1.77933
		3	27.31333	9.842663	2.055996
	RKO TDX 24HR	1	25.925	8.629	0.842333
		2	26.82333	9.527333	1.740666
		3	26.69667	9.400667	1.614

Table 7.1: Raw Ct Values and ΔCt, ΔΔCt calculations for RT-PCR.

8 Chapter 8: References

Adnan, M., Ullah, I., Tariq, A., Murad, W., Azizullah, A., Khan, A. L., & Ali, N. (2014). Ethnomedicine use in the war affected region of northwest Pakistan. *Journal of Ethnobiology and Ethnomedicine*, *10*(1), 1-16.

Aguilera, O., & Serna-Blasco, R. (2018). Targeting KRAS mutant CMS3 subtype by metabolic inhibitors. *In Advances in Experimental Medicine and Biology, 1110*, 23–34.

Ahmad, M., Qureshi, R., Arshad, M., Ajab Khan, M., & Zafar, M. (2009). Traditional herbal remedies used for the treatment of diabetes from district Attock (Pakistan). *International Pak. J. Bot, 41*(6), 2777-2782.

Ahmed, A., Hameed, A., & Saeed, S. (2020). Biochemical profile and bioactive potential of thirteen wild folk medicinal plants from Balochistan, Pakistan. *PLoS ONE*, *15*(8), e0231612.

Ahmed, N., Mahmood, A., Tahir, S. S., Bano, A., Naseem Malik, R., Hassan, S., & Ashraf, A. (2014). Ethnomedicinal knowledge and relative importance of indigenous medicinal plants of Cholistan desert, Punjab Province, Pakistan. *Journal of Ethnopharmacology*, *155*(2), 1263–1275.

Aiello, P., Sharghi, M., Mansourkhani, S. M., Ardekan, A. P., Jouybari, L., Daraei, N., … Kooti, W. (2019). Medicinal plants in the prevention and treatment of colon cancer. *Oxidative Medicine and Cellular Longevity*, *2019*, 1-52.

Akins, N. S., Nielson, T. C., & Le, H. V. (2018). Inhibition of Glycolysis and Glutaminolysis: An Emerging Drug Discovery Approach to Combat Cancer. *Current Topics in Medicinal Chemistry*, *18*(6), 494–504.

AlAhmari, M. M., Al-Khalaf, H. H., Al-Mohanna, F. H., Ghebeh, H., & Aboussekhra, A. (2020). AUF1 promotes stemness in human mammary epithelial cells through stabilization of the EMT transcription factors TWIST1 and SNAIL1. *Oncogenesis*, *9*(8), 70.

Al-Bedeary, S., Getta, H., & Al-Sharafi, D. (2020). The hallmarks of cancer and their therapeutic targeting in current use and clinical trials. *Iraqi Journal of Hematology*, *9*(1), 1.

Al-Eitan, L. N., Rababa'h, D. M., Alghamdi, M. A., & Khasawneh, R. H. (2019). Role of Four ABC Transporter Genes in Pharmacogenetic Susceptibility to Breast Cancer in Jordanian Patients. *Journal of Oncology*, *2019,* 6425708.

Albuquerque, C., & Pebre Pereira, L. (2018). Wnt signalling-targeted therapy in the CMS2 tumour subtype: A new paradigm in CRC treatment? In *Advances in Experimental Medicine and Biology, 1110*, 75–100.

Ali Ansari, A., Kenne, L., Rahman, A. (1984). Hederagenin, Ursolic Acid and Pinatol from *Fagonia indica*. , *Journal of Natural Products*, 47(1), 186-187.

Ali, K., & Khan, H. (2020). *Fagonia indica*; A Review on Chemical Constituents, Traditional Uses and Pharmacological Activities. *Current Pharmaceutical Design*, *27()*.

Ali, M. A., Abul Farah, M., Al-Hemaid, F. M., & Abou-Tarboush, F. M. (2014). In vitro cytotoxicity screening of wild plant extracts from Saudi Arabia on human breast adenocarcinoma cells. *Genetics and Molecular Research*. *13*(2), 3891-90.

Ali, S. S., Kasoju, N., Luthra, A., Singh, A., Sharanabasava, H., Sahu, A., & Bora, U. (2008). Indian medicinal herbs as sources of antioxidants. *Food Research International*, *41*, 1–15.

Alqahtani, A., Hamid, K., Kam, A., Wong, K. H., Abdelhak, Z., Razmovski-Naumovski, V., … Li, G. Q. (2013). The Pentacyclic Triterpenoids in Herbal Medicines and Their Pharmacological Activities in Diabetes and Diabetic Complications. *Current Medicinal Chemistry*, *20*(7), 908–931.

Amico, D., Barbui, A. M., Erba, E., Rambaldi, A., Introna, M., & Golay, J. (2003). Differential response of human acute myeloid leukemia cells to gemtuzumab ozogamicin in vitro: Role of Chk1 and Chk2 phosphorylation and caspase 3. *Blood*, *101*(11), 4589–4597.

Anil, P., Nikhil, B., Manoj, G., & Prakash, N. (2012). Phytochemicals and biological activities of Fagonia indica. *International Research Journal of Pharmacy*, *3*(5), 56–59.

Ansbro, M. R., Shukla, S., Ambudkar, S. V., Yuspa, S. H., & Li, L. (2013). Screening Compounds with a Novel High-Throughput ABCB1-Mediated Efflux Assay Identifies Drugs with Known Therapeutic Targets at Risk for Multidrug Resistance Interference. *PLoS ONE*, *8*(4), e60334.

Appella, E., & Anderson, C. W. (2001). Post-translational modifications and activation of p53 by genotoxic stresses. *European Journal of Biochemistry*, *268*(10), 2764–2772.

Arnold, M., Sierra, M. S., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2017). Global patterns and trends in colorectal cancer incidence and mortality. *Gut*, *66*(4), 683–691.

Aronson, J. K., La Caze, A., Kelly, M. P., Parkkinen, V. P., & Williamson, J. (2018). The use of mechanistic evidence in drug approval. *Journal of Evaluation in Clinical Practice*, *24*(5), 1166–1176.

Aryal, S., Baniya, M. K., Danekhu, K., Kunwar, P., Gurung, R., & Koirala, N. (2019). Total Phenolic content, Flavonoid content and antioxidant potential of wild vegetables from western Nepal. *Plants*, *8*(4), 96.

Aszalos, A. (2008). Role of ATP-Binding Cassette (ABC) Transporters in Interactions Between Natural Products and Drugs. *Current Drug Metabolism*, *9*(10), 1010–1018.

Azam, F., Sheikh, N., Ali, G., & Tayyeb, A. (2018). *Fagonia indica* Repairs Hepatic Damage through Expression Regulation of Toll-Like Receptors in a Liver Injury Model. *Journal of Immunology Research*, *2018* (), 7967135

Bae, H., Jayaprakasha, G. K., Crosby, K., Jifon, J. L., & Patil, B. S. (2012). Influence of Extraction Solvents on Antioxidant Activity and the Content of Bioactive Compounds in Non-pungent Peppers. *Plant Foods for Human Nutrition, 67*(2), 120-8

Bacanli, M., Anlar, H. G., Başaran, A. A., & Başaran, N. (2017). Fenoliklerin sitotoksisite profillerinin değerlendirilmesi: Farklı hücrelerde farklı zaman aralıklarında nötral kırmızı ve MTT Yöntemlerinin karşılaştırılması. *Turkish Journal of Pharmaceutical Sciences*, *14*(2), 95–107.

Bagban, I. M., Roy, S. P., Chaudhary, A., Das, S. K., Gohil, K. J., & Bhandari, K. K. (2012). Hepatoprotective activity of the methanolic extract of *Fagonia indica* Burm in carbon tetra chloride induced hepatotoxicity in albino rats. *Asian Pacific Journal of Tropical Biomedicine*, *2*(3), S1457-60

Bagli, E., Stefaniotou, M., Morbidelli, L., Ziche, M., Psillas, K., Murphy, C., & Fotsis, T. (2004). Luteolin inhibits vascular endothelial growth factor-induced angiogenesis;

inhibition of endothelial cell survival and proliferation by targeting phosphatidylinositol 3′-kinase activity. *Cancer Research*, *64*(21), 7936–7946.

Bajerski, F., Stock, J., Hanf, B., Darienko, T., Heine-Dobbernack, E., Lorenz, M., … Overmann, J. (2018). ATP Content and Cell Viability as Indicators for Cryostress Across the Diversity of Life. *Frontiers in Physiology*, *9*(), 921.

Bartek, J., & Lukas, J. (2003). Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell*, *3*(5), 421–429.

Beier, B. A. (2001). Two new unifoliolate species of Fagonia (Zygophyllaceae) from the Horn of Africa region, and the resurrection of F. subinermis from Iran. *Nordic Journal of Botany*, *21*(5), 449–455.

Belahsen, R. (2014). Nutrition transition and food sustainability. *Proceedings of the Nutrition Society*, *73*(3), 385–388.

Belkahla, S., Khan, A. U. H., Gitenay, D., Alexia, C., Gondeau, C., Vo, D. N., … Gonzalez, M. V. (2018). Changes in metabolism affect expression of ABC transporters through ERK5 and depending on p53 status. *Oncotarget*, *9*(1), 1114–1129.

Berger, E., & Happel, L. (1974). Different Mechanisms of Energy Coupling for the Shock-sensitive and Shock-resistant Amino Acid Permeases of Escherichia coli. *Journal of Biological Chemistry*, *249*(24*)*, 7747–7755.

Bernardo, A., Palumbo, R., Pedersini, R., Rota Caremoli, E., Gambaro, A. R., Ferzi, A., … Cazzaniga, M. E. (2017). Nab-Paclitaxel in Advanced HER2-negative Breast Cancer Patients: Efficacy and Safety Beyond Clinical Trials. *Clinical Breast Cancer*, *17*(6), 433–440.

Berry, D., Cirrincione, C., Henderson, C., Citron, M., Budman, D., Goldstein, L., Martino, S., Perez, E., Muss, H., Norton, L., Hudis, C. and Winer, E. (2006). Estrogen-Receptor Status and Outcomes of Modern Chemotherapy for Patients With Node-Positive Breast Cancer—Correction. *JAMA*, 295(20), 2356.

Bhattacharya, R., Fan, F., Wang, R., Ye, X., Xia, L., Boulbes, D., & Ellis, L. M. (2017). Intracrine VEGF signalling mediates colorectal cancer cell migration and invasion. *British Journal of Cancer*, *117*(6), 848–855.

Bishayee, A., Ahmed, S., Brankov, N., & Perloff, M. (2011). Triterpenoids as potential agents for the chemoprevention and therapy of breast cancer. *Frontiers in Bioscience*, *16*(1). 980–996.

Biswas, D. K., Shi, Q., Baily, S., Strickland, I., Ghosh, S., Pardee, A. B., & Iglehart, J. D. (2004). NF-κB activation in human breast cancer specimens and its role in cell proliferation and apoptosis. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(27), 10137–10142.

Biswas, T., & Dwivedi, U. N. (2019). Plant triterpenoid saponins: biosynthesis, in vitro production, and pharmacological relevance. *Protoplasma*, *256*, 1463–1486.

Boots, A. W., Haenen, G. R. M. M., & Bast, A. (2008). Health effects of quercetin: From antioxidant to nutraceutical. *European Journal of Pharmacology*, *585*(2-3). 325–337.

Boveri, T. (1902). Uber mehrpolige Mitosen als Mittle zur Analyse des Zellkerns. *Verhandl Phys-Med Ges (Wulzburg) NF*, *35*, 67–90.

Braga, F., Ayres-Saraiva, D., Gattass, C. R., & Capella, M. A. M. (2007). Oleanolic acid inhibits the activity of the multidrug resistance protein ABCC1 (MRP1) but not of the ABCB1 (P-glycoprotein): Possible use in cancer chemotherapy. *Cancer Letters*,

248(1), 147–152.

Brambilla, E., Gazzeri, S., Lantuejoul, S., Luc Coil, J., Moro, D., Negoescu, A., … Chu, C. J. F. (1998). Advances in Brief p53 Mutant Immunophenotype and Deregulation of p53 Transcription Pathway (Bc12, Bax, and Waft) in Precursor Bronchial Lesions of Lung Cancer. *Clinical Cancer Research*, *4*(7)1609-18.

Bray, F., McCarron, P., & Parkin, D. M. (2004). The changing global patterns of female breast cancer incidence and mortality. *Breast Cancer Research*, *6*(6), 229–239.

Bretthauer, M., Kaminski, M. F., Løberg, M., Zauber, A. G., Regula, J., Kuipers, E. J., … Author, C. (2016). Population-Based Colonoscopy Screening for Colorectal Cancer A Randomized Clinical Trial. *JAMA Intern Med*, *176*(7), 894–902.

Brown, R. E., Short, S. P., & Williams, C. S. (2018). Colorectal Cancer and Metabolism. *Current Colorectal Cancer Reports*, 14(6), 226–241.

Caesar, L. K., & Cech, N. B. (2019). Synergy and antagonism in natural product extracts: When 1 + 1 does not equal 2. *Natural Product Reports*, *36*(6), 869–888.

Calcagno, A. M., Fostel, J. M., To, K. K. W., Salcido, C. D., Martin, S. E., Chewning, K. J., … Ambudkar, S. V. (2008). Single-step doxorubicin-selected cancer cells overexpress the ABCG2 drug transporter through epigenetic changes. *British Journal of Cancer*, *98*(9), 1515–1524.

Cameron, D., Piccart-Gebhart, M. J., Gelber, R. D., Procter, M., Goldhirsch, A., de Azambuja, E., … Jackisch, C. (2017). 11 years' follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive early breast cancer: final analysis of the HERceptin Adjuvant (HERA) trial. *The Lancet*, *389*(10075), 1195–1205.

Caligiani, A., Malavasi, G., Palla, G., Marseglia, A., Tognolini, M., & Bruni, R. (2013). A simple GC-MS method for the screening of betulinic, corosolic, maslinic, oleanolic and ursolic acid contents in commercial botanicals used as food supplement ingredients. *Food Chemistry*, *136*(2), 735–741.

Chandrasekara, A., & Shahidi, F. (2018). Herbal beverages: Bioactive compounds and their role in disease risk reduction - A review. *Journal of Traditional and Complementary Medicine*, *8*(4), 451–458.

Chavez, K. J., Garimella, S. V., & Lipkowitz, S. (2010). Triple negative breast cancer cell lines: One tool in the search for better treatment of triple negative breast cancer. *Breast Disease*, *32*(1–2), 35–48.

Chearwae, W., Wu, C. P., Chu, H. Y., Lee, T. R., Ambudkar, S. V., & Limtrakul, P. (2006). Curcuminoids purified from turmeric powder modulate the function of human multidrug resistance protein 1 (ABCC1). *Cancer Chemotherapy and Pharmacology*, *57*(3), 376–388.

Checker, R., Sandur, S. K., Sharma, D., Patwardhan, R. S., Jayakumar, S., Kohli, V., … Sainis, K. B. (2012). Potent Anti-Inflammatory Activity of Ursolic Acid, a Triterpenoid Antioxidant, Is Mediated through Suppression of NF-κB, AP-1 and NF-AT. *PLoS ONE*, *7*(2), e31318.

Chekulayev, V., Mado, K., Shevchuk, I., Koit, A., Kaldma, A., Klepinin, A., … Kaambre, T. (2015). Metabolic remodeling in human colorectal cancer and surrounding tissues: Alterations in regulation of mitochondrial respiration and metabolic fluxes. *Biochemistry and Biophysics Reports*, *4*, 111–125.

Chen, M.-C., Hsu, H.-H., Chu, Y.-Y., Cheng, S.-F., Shen, C.-Y., Lin, Y.-J., … Huang, C.-Y. (2018). Lupeol alters ER stress-signaling pathway by downregulating ABCG2

expression to induce Oxaliplatin-resistant LoVo colorectal cancer cell apoptosis. *Environmental Toxicology*, *33*(5), 587–593.

Chen, Q., Kang, J., & Fu, C. (2018). The independence of and associations among apoptosis, autophagy, and necrosis. *Signal Transduction and Targeted Therapy*, *3*(18), 1–11.

Chen, Q., Meng, F., Wang, L., Mao, Y., Zhou, H., Hua, D., … Wang, W. (2017). A polymorphism in ABCC4 is related to efficacy of 5-FU/capecitabine-based chemotherapy in colorectal cancer patients. *Scientific Reports*, *7*(1), 1–7.

Chen, S., Fisher, R. C., Signs, S., Molina, L. A., Shenoy, A. K., Lopez, M. C., … Huang, E. H. (2017). Inhibition of PI3K/Akt/mTOR signaling in PI3KR2-overexpressing colon cancer stem cells reduces tumor growth due to apoptosis. *Oncotarget*, *8*(31), 50476–50488.

Chen, W., Liu, L., Luo, Y., Odaka, Y., Awate, S., Zhou, H., … Huang, S. (2012). Cryptotanshinone activates p38/JNK and inhibits Erk1/2 leading to caspase-independent cell death in tumor cells. *Cancer Prevention Research*, *5*(5), 778–787.

Chen, W. T. L., Yang, T. S., Chen, H. C., Chen, H. H., Chiang, H. C., Lin, T. C., … Kuo, M. L. (2014). Effectiveness of a novel herbal agent MB-6 as a potential adjunct to 5-fluoracil-based chemotherapy in colorectal cancer. *Nutrition Research*, *34*(7), 585–594.

Cheng, D., & Liang, B. (2015). Intercellular adhesion molecule-1 (ICAM-1) polymorphisms and cancer risk: A meta- analysis. *Iranian Journal of Public Health*, *44*(5), 615–624.

Cheng, S. C., Wu, Y. H., Huang, W. C., Pang, J. H. S., Huang, T. H., & Cheng, C. Y. (2019). Anti-inflammatory property of quercetin through downregulation of ICAM-1 and MMP-9 in TNF-α-activated retinal pigment epithelial cells. *Cytokine*, *116*, 48–60.

Choi, M., Kipps, T., & Kurzrock, R. (2016). ATM mutations in cancer: Therapeutic implications. *Molecular Cancer Therapeutics*, *15*(8), 1781–1791.

Chung, T. W., Choi, H., Lee, J. M., Ha, S. H., Kwak, C. H., Abekura, F., … Kim, C. H. (2017). Oldenlandia diffusa suppresses metastatic potential through inhibiting matrix metalloproteinase-9 and intercellular adhesion molecule-1 expression via p38 and ERK1/2 MAPK pathways and induces apoptosis in human breast cancer MCF-7 cells. *Journal of Ethnopharmacology*, *195*, 309–317.

Chuu, C. P., Lin, H. P., Ciaccio, M. F., Kokontis, J. M., Hause, R. J., Hiipakka, R. A., … Jones, R. B. (2012). Caffeic acid phenethyl ester suppresses the proliferation of human prostate cancer cells through inhibition of p70S6K and Akt signaling networks. *Cancer Prevention Research*, *5*(5), 788–797.

Cijo George, V., Vijayakumaran Vijesh, V., Inoka Madumani Amararathna, D., Ayshwarya Lakshmi, C., Anbarasu, K., Ragupathi Naveen Kumar, D., … P. Vasantha Rupasinghe, H. (2016). Mechanism of Action of Flavonoids in Prevention of Inflammation-Associated Skin Cancer. *Current Medicinal Chemistry*, *23*(32), 3697-3716

Crowley, L. C., Christensen, M. E., & Waterhouse, N. J. (2016). Measuring survival of adherent cells with the Colony-forming assay. *Cold Spring Harbor Protocols*, *2016*(8), 721–724.

CRUK. (2020). *Breast cancer statistics | Cancer Research UK*. Retrieved March 30, 2020, from https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/breast-cancer#heading-Zero

CRUK. (2019). *Cancer in the UK 2019*. Retrieved from https://www.cancerresearchuk.org/sites/default/files/state_of_the_nation_april_2019.pdf

Cserni, G., Chmielik, E., Cserni, B., & Tot, T. (2018). The new TNM-based staging of breast cancer. *Virchows Archive*, *472*, 697–703.

Cuenda, A., & Rousseau, S. (2007). p38 MAP-Kinases pathway regulation, function and role in human diseases. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1773*(8), 1358–1375.

Cui, J., Shen, Y., & Li, R. (2013). Estrogen synthesis and signaling pathways during aging: From periphery to brain. *Trends in Molecular Medicine*, *19*(3), 197–209.

Dafni, U., Tsourti, Z., & Alatsathianos, I. (2019). Breast Cancer Statistics in the European Union: Incidence and Survival across European Countries. *Breast Care*, *14*(6), 344–353.

Dagogo-Jack, I., & Shaw, A. T. (2018). Tumour heterogeneity and resistance to cancer therapies. *Nature Reviews Clinical Oncology*, *15*, 81–94.

Daniyal, M., Akram, M., Zainab, R., Munir, R., Sharif, A., Shah, S., … Wang, W. (2019). Prevalence and current therapy in chronic liver disorders. *Inflammopharmacology*, *27*(2), 213–231.

Davies, C., Pan, H., Godwin, J., Gray, R., Arriagada, R., Raina, V., Abraham, M., Alencar, V., Badran, A., Bonfill, X., Bradbury, J., Clarke, M., Collins, R., Davis, S., Delmestri, A., Forbes, J., Haddad, P., Hou, M., Inbar, M., Khaled, H., Kielanowska, J., Kwan, W., Mathew, B., Mittra, I., Müller, B., Nicolucci, A., Peralta, O., Pernas, F., Petruzelka, L., Pienkowski, T., Radhika, R., Rajan, B., Rubach, M., Tort, S., Urrútia, G., Valentini, M., Wang, Y. and Peto, R. (2013). Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. *The Lancet*, 381(9869), pp.805-816.

De La Cruz-Morcillo, M. A., Valero, M. L. L., Callejas-Valera, J. L., Arias-González, L., Melgar-Rojas, P., Galán-Moya, E. M., … Sánchez-Prieto, R. (2012). P38MAPK is a major determinant of the balance between apoptosis and autophagy triggered by 5-fluorouracil: Implication in resistance. *Oncogene*, *31*(9), 1073–1085.

Dermauw, W., & Van Leeuwen, T. (2014). The ABC gene family in arthropods: Comparative genomics and role in insecticide transport and resistance. *Insect Biochemistry and Molecular Biology*, *45*, 89–110.

Deschesnes, R. G., Huot, J., Valerie, K., & Landry, J. (2001). Involvement of p38 in apoptosis-associated membrane blebbing and nuclear condensation. *Molecular Biology of the Cell*, *12*(6), 1569–1582.

Dias, M. M. D. S., Noratto, G., Martino, H. S. D., Arbizu, S., Peluzio, M. D. C. G., Talcott, S., … Mertens-Talcott, S. U. (2014). Pro-apoptotic activities of polyphenolics from açai (Euterpe oleracea Martius) in human SW-480 colon cancer cells. *Nutrition and Cancer*, *66*(8), 1394–1405.

Dietrich, F., Kaiser, S., Rockenbach, L., Figueiró, F., Bergamin, L. S., Cunha, F. M. da, … Battastini, A. M. O. (2014). Quinovic acid glycosides purified fraction from Uncaria tomentosa induces cell death by apoptosis in the T24 human bladder cancer cell line. *Food and Chemical Toxicology*, *67*, 222–229.

Do, Q. D., Angkawijaya, A. E., Tran-Nguyen, P. L., Huynh, L. H., Soetaredjo, F. E., Ismadji, S., & Ju, Y. H. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of Limnophila aromatica. *Journal of Food and Drug Analysis*, *22*(3), 296–302.

Dodd, R. H., Kobayashi, L. C., & von Wagner, C. (2019). Perceived life expectancy and

colorectal cancer screening intentions and behaviour: A population-based UK study. *Preventive Medicine Reports*, *16*, 101002

Domenichini, A., Adamska, A., & Falasca, M. (2019). ABC transporters as cancer drivers: Potential functions in cancer development. *Biochimica et Biophysica Acta - General Subjects*, *1863*(1), 52-60

Douillard, J. Y., Cunningham, D., Roth, A. D., Navarro, M., James, R. D., Karasek, P., … Rougier, P. (2000). Irinotecan combined with fluorouracil compared with fluorouracil alone. as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. *Lancet*, *355*(9209), 1041–1047.

Drenberg, C., Hu, S., Li, L., Buelow, D., Orwick, S., Gibson, A., … Baker, S. (2016). ABCC4 Is a Determinant of Cytarabine-Induced Cytotoxicity and Myelosuppression. *Clinical and Translational Science*, *9*(1), 51–59.

Drew, D. A., Chin, S. M., Gilpin, K. K., Parziale, M., Pond, E., Schuck, M. M., … Chan, A. T. (2017). ASPirin Intervention for the REDuction of colorectal cancer risk (ASPIRED): A study protocol for a randomized controlled trial. *Trials*, *18*(1), 50.

Dupont, W. D., & Page, D. L. (1985). Risk Factors for Breast Cancer in Women with Proliferative Breast Disease. *New England Journal of Medicine*, *312*(3), 146–151.

Dymicka-Piekarska, V., Guzinska-Ustymowicz, K., Kuklinski, A., & Kemona, H. (2012). Prognostic significance of adhesion molecules (sICAM-1, sVCAM-1) and VEGF in colorectal cancer patients. *Thrombosis Research*, *129*(4), e47-50.

Eccles, S. A., Aboagye, E. O., Ali, S., Anderson, A. S., Armes, J., Berditchevski, F., … Thompson, A. M. (2013). Critical research gaps and translational priorities for the successful prevention and treatment of breast cancer. *Breast Cancer Research*, *15*(5), R92.

El-hadidi, M. N., Al-wakeel, S. A. M., & El-garf, I. A. (1988). Systematic significance of the flavonoid constituents in the Fagonia indica-complex. *Biochemical Systematics and Ecology*, *16*(3), 293–297.

El kinany, K., Deoula, M., Hatime, Z., Bennani, B., & El Rhazi, K. (2018). Dairy products and colorectal cancer in middle eastern and north African countries: A systematic review. *BMC Cancer*, *18*(1), 233.

Engel, N., Ali, I., Adamus, A., Frank, M., Dad, A., Ali, S., … Ahmad, V. U. (2016). Antitumor evaluation of two selected Pakistani plant extracts on human bone and breast cancer cell lines. *BMC Complementary and Alternative Medicine*, *16*(1), 244.

Ezhilarasan, D., Apoorva, V. S., & Ashok Vardhan, N. (2018). *Syzygium cumini* extract induced reactive oxygen species-mediated apoptosis in human oral squamous carcinoma cells. *Journal of Oral Pathology & Medicine*, *48*(2), 12806.

Fallah, A., Sadeghinia, A., Kahroba, H., Samadi, A., Heidari, H. R., Bradaran, B., … Molavi, O. (2019). Therapeutic targeting of angiogenesis molecular pathways in angiogenesis-dependent diseases. *Biomedicine and Pharmacotherapy*, *110*, 775–785.

Farhana, L., Sarkar, S., Nangia-Makker, P., Yu, Y., Khosla, P., Levi, E., … Majumdar, A. P. N. (2020). Natural agents inhibit colon cancer cell proliferation and alter microbial diversity in mice. *PLOS ONE*, *15*(3), e0229823.

Farheen, R., Siddiqui, B. S., Mahmood, I., Simjee, S. U., & Majeed, S. (2015). Triterpenoids and triterpenoid saponins from the aerial parts of Fagonia indica Burm. *Phytochemistry Letters*, *13*, 256–261.

Ferlay, J., Colombet, M., Soerjomataram, I., Dyba, T., Randi, G., Bettio, M., … Bray, F. (2018). Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. *European Journal of Cancer*, *103*, 356–387.

Ferrara, N. (2005). VEGF as a Therapeutic Target in Cancer. *Oncology*, *69*(3), 11–16.

Fessler, E., Drost, J., Hooff, S. R., Linnekamp, J. F., Wang, X., Jansen, M., … Medema, J. P. (2016). TGFβ signaling directs serrated adenomas to the mesenchymal colorectal cancer subtype. *EMBO Molecular Medicine*, *8*(7), 745–760.

Fessler, E., & Medema, J. P. (2016). Colorectal Cancer Subtypes: Developmental Origin and Microenvironmental Regulation. *Trends in Cancer*, *2*(9), 505–518.

Folkman, J., & Hanahan, D. (1991). Switch to the angiogenic phenotype during tumorigenesis. *Princess Takamatsu Symposia*, *22*, 339–347.

Fotakis, G., & Timbrell, J. A. (2006). In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters*, *160*(2), 171–177.

Foyer, C. H., & Noctor, G. (2013). Redox signaling in plants. *Antioxidants and Redox Signaling*, *18*(16), 2087–2090.

Frank, P. G., & Lisanti, M. P. (2008). ICAM-1: Role in inflammation and in the regulation of vascular permeability. *American Journal of Physiology - Heart and Circulatory Physiology*, *295*(3), 926-27.

Fujisawa, S., Romin, Y., Barlas, A., Petrovic, L. M., Turkekul, M., Fan, N., … Sofocleous, C. T. (2014). Evaluation of YO-PRO-1 as an early marker of apoptosis following radiofrequency ablation of colon cancer liver metastases. *Cytotechnology*, *66*(2), 259–273.

Furtado, N. A. J. C., Pirson, L., Edelberg, H., Miranda, L. M., Loira-Pastoriza, C., Preat, V., … André, C. M. (2017). Pentacyclic triterpene bioavailability: An overview of in vitro and in vivo studies. *Molecules*, *22*(3), 400.

Ganapathy-Kanniappan, S., & Geschwind, J. F. H. (2013). Tumor glycolysis as a target for cancer therapy: Progress and prospects. *Molecular Cancer*, *12(*152), 1–11.

García-Lafuente, A., Guillamón, E., Villares, A., Rostagno, M. A., & Martínez, J. A. (2009). Flavonoids as anti-inflammatory agents: Implications in cancer and cardiovascular disease. *Inflammation Research*, *58*(9), 537–552.

Garrido-Castro, A. C., Lin, N. U., & Polyak, K. (2019). Insights into molecular classifications of triple-negative breast cancer: Improving patient selection for treatment. *Cancer Discovery*, *9*(2), 176–198.

Ginzburg, S., Golovine, K. V., Makhov, P. B., Uzzo, R. G., Kutikov, A., & Kolenko, V. M. (2014). Piperlongumine inhibits NF-κB activity and attenuates aggressive growth characteristics of prostate cancer cells. *Prostate*, *74*(2), 177–186.

Gatalica, Z., Vranic, S., Xiu, J., Swensen, J., & Reddy, S. (2016). High microsatellite instability (MSI-H) colorectal carcinoma: a brief review of predictive biomarkers in the era of personalized medicine. *Familial Cancer*, *15*(3), 405–412.

George, A. T., Aggarwal, S., Dharmavaram, S., Menon, A., Dube, M., Vogler, M., … Field, A. (2018). Regional variations in UK colorectal cancer screening and mortality. *The Lancet*, *392*(10144), 277–278.

Ghante, M. H., & Jamkhande, P. G. (2019). Role of pentacyclic triterpenoids in

chemoprevention and anticancer treatment: An overview on targets and underling mechanisms. *Journal of Pharmacopuncture*, *22*(2), 55–67.

Gierach, G. L., Burke, A., & Anderson, W. F. (2010). Epidemiology of triple negative breast cancers. *Breast Disease*, *32*(1–2), 5–24.

Ginzburg, S., Golovine, K. V., Makhov, P. B., Uzzo, R. G., Kutikov, A., & Kolenko, V. M. (2014). Piperlongumine inhibits NF-κB activity and attenuates aggressive growth characteristics of prostate cancer cells. *Prostate*, *74*(2), 177–186.

González-Vallinas, M., Molina, S., Vicente, G., De La Cueva, A., Vargas, T., Santoyo, S., … Ramírez De Molina, A. (2013). Antitumor effect of 5-fluorouracil is enhanced by rosemary extract in both drug sensitive and resistant colon cancer cells. *Pharmacological Research*, *72*, 61–68.

Goulas, V., & Manganaris, G. A. (2012). Towards an Efficient Protocol for the Determination of Triterpenic Acids in Olive Fruit: A Comparative Study of Drying and Extraction Methods. *Phytochemical Analysis*, *23*(5), 444–449.

Gradilone, A., Pulcinelli, F., Lotti, L., Trifiro, E., Martino, S., Gandini, O., … Gazzaniga, P. (2008). Celecoxib Upregulates Multidrug Resistance Proteins in Colon Cancer: Lack of Synergy with Standard Chemotherapy. *Current Cancer Drug Targets*, *8*(5), 414–420.

Graham, T. A., & Sottoriva, A. (2017). Measuring cancer evolution from the genome. *Journal of Pathology*, *241*(2), 183-191.

Gratton, J. P., Morales-Ruiz, M., Kureishi, Y., Fulton, D., Walsh, K., & Sessa, W. C. (2001). Akt Down-regulation of p38 Signaling Provides a Novel Mechanism of Vascular Endothelial Growth Factor-mediated Cytoprotection in Endothelial Cells. *Journal of Biological Chemistry*, *276*(32), 30359–30365.

Gu, G., Barone, I., Gelsomino, L., Giordano, C., Bonofiglio, D., Statti, G., … Andò, S. (2012). Oldenlandia diffusa extracts exert antiproliferative and apoptotic effects on human breast cancer cells through ERα/Sp1-mediated p53 activation. *Journal of Cellular Physiology*, *227*(10), 3363–3372.

Guestini, F., McNamara, K. M., & Sasano, H. (2017). The use of chemosensitizers to enhance the response to conventional therapy in triple-negative breast cancer patients. *Breast Cancer Management*, *6*(4), 127–131.

Gulshan, A. B., Dasti, A. A., Hussain, S., Atta, M. I., & Amin, M. (2012). Indigenous uses of medicinal plants in rural areas of Dera Ghazi Khan, Punjab, Pakistan. *ARPN Journal of Agricultural and Biological Science*, *7*(9), 750-762.

Gustavsson, B., Carlsson, G., MacHover, D., Petrelli, N., Roth, A., Schmoll, H. J., … Gibson, F. (2015). A review of the evolution of systemic chemotherapy in the management of colorectal cancer. *Clinical Colorectal Cancer*, *14*(1), 1–10.

Haidari, R. El, Abbas, L. A., Nerich, V., & Anota, A. (2020). Factors associated with health-related quality of life in women with breast cancer in the middle east: A systematic review. *Cancers*, *12*(3), 696.

Hajdu, S. I. (2011). A note from history: Landmarks in history of cancer, part 1. *Cancer*, *117*(5), 1097–1102.

Han, X., Chen, H., Zhou, J., Steed, H., Postovit, L. M., & Fu, Y. (2018). Pharmacological inhibition of p38 MAPK by SB203580 increases resistance to carboplatin in A2780cp cells and promotes growth in primary ovarian cancer cells. *International Journal of Molecular Sciences*, *19*(8), 2184.

Han, Z., Wei, W., Dunaway, S., Darnowski, J. W., Calabresi, P., Sedivy, J., … Wyche, J. H. (2002). Role of p21 in apoptosis and senescence of human colon cancer cells treated with camptothecin. *Journal of Biological Chemistry*, *277*(19), 17154–17160.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, *100*(1), 57–70.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*, *144*(5), 646–674.

Hansen, S. N., Ehlers, N. S., Zhu, S., Thomsen, M. B. H., Nielsen, R. L., Liu, D., … Gupta, R. (2016). The stepwise evolution of the exome during acquisition of docetaxel resistance in breast cancer cells. *BMC Genomics*, *17*(1), 442.

Hansford, S., & Huntsman, D. G. (2014). Boveri at 100: Theodor Boveri and genetic predisposition to cancer. *Journal of Pathology*, *234*(2), 142–145.

Harbeck, N. (2013). *HER2-positive breast cancer: Adjuvant and neoadjuvant therapy. In Handbook of HER2-Targeted Agents in Breast Cancer*. Springer Healthcare, Tarporley, 25–42.

Harborne, J. (B). (1984). *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis - J. B. Harborne - Google Books* (2nd ed.). New York: Chapman and Hall.

Harris, S. L., & Levine, A. J. (2005). The p53 pathway: Positive and negative feedback loops. *Oncogene*, *24*, 2899–2908.

Hassan, L. E. A., Khadeer Ahamed, M. B., Abdul Majid, A. S., Baharetha, H. M., Muslim, N. S., Nassar, Z. D., & Abdul Majid, A. M. S. (2014). Correlation of antiangiogenic, antioxidant and cytotoxic activities of some Sudanese medicinal plants with phenolic and flavonoid contents. *BMC Complementary and Alternative Medicine*, *14*(1), 406.

Haupt, Y., Maya, R., Kazaz, A., & Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. *Nature*, *387*(6630), 296–299

He, X.-K., Wu, W., Ding, Y.-E., Li, Y., Sun, L.-M., & Si, J. (2018). Different Anatomical Subsites of Colon Cancer and Mortality: A Population-Based Study. *Gastroenterology Research and Practice*, *2018*, 7153685.

Hee Choi, Y., & Yu, A.-M. (2014). ABC Transporters in Multidrug Resistance and Pharmacokinetics, and Strategies for Drug Development. *Current Pharmaceutical Design*, *20*(5), 793–807.

Hendrayani, S. F., Al-Khalaf, H. H., & Aboussekhra, A. (2014). The cytokine il-6 reactivates breast stromal fibroblasts through transcription factor STAT3-dependent up-regulation of the RNA-binding protein AUF1. *Journal of Biological Chemistry*, *289*(45), 30962–30976.

Hernández, J. F., Urueña, C. P., Sandoval, T. A., Cifuentes, M. C., Formentini, L., Cuezva, J. M., & Fiorentino, S. (2017). A cytotoxic Petiveria alliacea dry extract induces ATP depletion and decreases β-F1-ATPase expression in breast cancer cells and promotes survival in tumor-bearing mice. *Revista Brasileira de Farmacognosia*, *27*(3), 306–314.

Hersey, P., & Zhang, X. D. (2003). Overcoming resistance of cancer cells to apoptosis. *Journal of Cellular Physiology*, *196*(1), 9–18.

Higgins, C. F., Hiles, I. D., Salmond, G. P. C., Gill, D. R., Downie, J. A., Evans, I. J., … Hermodson, M. A. (1986). A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. *Nature*, *323*(6087), 448–450.

Hiramatsu, R., Fukuhara, S., Mitsuda, S., Yokomichi, T., & Kataoka, T. (2015). Betulinic

acid and oleanolic acid, natural pentacyclic triterpenoids, interfere with N-linked glycan modifications to intercellular adhesion molecule-1, but not its intracellular transport to the cell surface. *European Journal of Pharmacology*, *767*, 126–134.

Hollenstein, K., Dawson, R. J., & Locher, K. P. (2007). Structure and mechanism of ABC transporter proteins. *Current Opinion in Structural Biology*, *17*(4), 412–418.

Hollman, P. C. H., Tijburg, L. B. M., & Yang, C. S. (1997). Bioavailability of flavonoids from tea. *Critical Reviews in Food Science and Nutrition*, *37*(8), 719–738.

Homolya, L., Holló, Z., Müller, M., Mechetner, E. B., & Sarkadi, B. (1996). A new method for quantitative assessment of P-glycoprotein-related multidrug resistance in tumour cells. *British Journal of Cancer*, *73*(7), 849–855.

Hong, Y. K., McMasters, K. M., Egger, M. E., & Ajkay, N. (2018). Ductal carcinoma in situ current trends, controversies, and review of literature. *American Journal of Surgery*, *216*(5), 998–1003.

Hossain, M. A., & Ismail, Z. (2013). Isolation and characterization of triterpenes from the leaves of Orthosiphon stamineus. *Arabian Journal of Chemistry*, *6*(3), 295–298.

Housman, G., Byler, S., Heerboth, S., Lapinska, K., Longacre, M., Snyder, N., & Sarkar, S. (2014). Drug resistance in cancer: An overview. *Cancers*, *6*(3), 1769–1792.

Hsu, H. H., Chen, M. C., Baskaran, R., Lin, Y. M., Day, C. H., Lin, Y. J., … Huang, C. Y. (2018). Oxaliplatin resistance in colorectal cancer cells is mediated via activation of ABCG2 to alleviate ER stress induced apoptosis. *Journal of Cellular Physiology*, *233*(7), 5458–5467.

Hu, J., Li, J., Yue, X., Wang, J. cang, Wang, J. feng, Liu, J. zhong, & Kong, D. lu. (2017). Targeting BCRP/ABCG2 by RNA interference enhances the chemotherapy sensitivity of human colon cancer side population cells. *Journal of Huazhong University of Science and Technology - Medical Science*, *37*(2), 231–236.

Hu, T., Li, Z., Gao, C. Y., & Cho, C. H. (2016). Mechanisms of drug resistance in colon cancer and its therapeutic strategies. *World Journal of Gastroenterology*, *22*(30), 6876–6889.

Hu, Q., Wang, G., Peng, J., Qian, G., Jiang, W., Xie, C., … Wang, X. (2017). Knockdown of SIRT1 Suppresses Bladder Cancer Cell Proliferation and Migration and Induces Cell Cycle Arrest and Antioxidant Response through FOXO3a-Mediated Pathways. *BioMed Research International*, *2017,* 3781904

Huang, C. Y., Lin, C. Y., Tsai, C. W., & Yin, M. C. (2011). Inhibition of cell proliferation, invasion and migration by ursolic acid in human lung cancer cell lines. *Toxicology in Vitro*, *25*(7), 1274–1280.

Huang, C.-Y., Huang, C.-Y., Pai, Y.-C., Lin, B.-R., Lee, T.-C., Liang, P.-H., & Yu, L. C.-H. (2019). Glucose Metabolites Exert Opposing Roles in Tumor Chemoresistance. *Frontiers in Oncology*, *9*, 1282.

Huang, W. C., & Hung, M. C. (2009). Induction of Akt activity by chemotherapy confers acquired resistance. *Journal of the Formosan Medical Association*, *108*(3), 180–194.

Huang, W., Liang, Y., Wang, J., Li, G., Wang, G., Li, Y., & Chung, H. Y. (2016). Anti-angiogenic activity and mechanism of kaurane diterpenoids from Wedelia chinensis. *Phytomedicine*, *23*(3), 283–292.

Huang, W. Y., Cai, Y. Z., & Zhang, Y. (2010). Natural phenolic compounds from medicinal herbs and dietary plants: Potential use for cancer prevention. *Nutrition and Cancer*,

62(1), 1–20.

Hunter, W. N. (2007). The non-mevalonate pathway of isoprenoid precursor biosynthesis. *Journal of Biological Chemistry, 282*(30), 21573–21577.

Hussain, A., Zia, M., & Mirza, B. (2007). Cytotoxic and Antitumor Potential of *Fagonia cretica* L. *Turkish Journal of Biology*, *31*, 19–24.

Hussain, T., Tan, B., Yin, Y., Blachier, F., Tossou, M. C. B., & Rahu, N. (2016). Oxidative Stress and Inflammation: What Polyphenols Can Do for Us? *Oxidative Medicine and Cellular Longevity*, *2016*, 7432797.

Hussein, H. J., Hameed, I. H., & Hadi, M. Y. (2017). Using Gas Chromatography-Mass Spectrometry (GC-MS) Technique for Analysis of Bioactive Compounds of Methanolic Leaves extract of Lepidium sativum drug evaluation. *Research Journal of Pharmacy and Technology*, *10*(11), 3981-89.

Iacobuzio-Donahue, C. A. (2009). Epigenetic Changes in Cancer. *Annual Review of Pathology: Mechanisms of Disease*, *4*(1), 229–249.

Ikan, R. (1999). *Naturally occurring glycosides*. John Wiley.

Ito, T.-K., Ishii, G., Chiba, H., & Ochiai, A. (2007). The VEGF angiogenic switch of fibroblasts is regulated by MMP-7 from cancer cells. *Oncogene*, *26*(5), 7194–7203.

Iqbal, J., Bano, K., Saeed, A., Akram, M., & Aziz, Z. (2010). Survival of women with locally advanced breast cancer at a teaching hospital in Lahore. *Journal of the Pakistan Medical Association*, *60*(9), 721–725.

Itatani, Y., Kawada, K., & Sakai, Y. (2019). Transforming growth factor-β signaling pathway in colorectal cancer and its tumor microenvironment. *International Journal of Molecular Sciences*, *20*(23), 5822.

Jallali, I., waffo Téguo, P., Smaoui, A., Mérillon, J. M., Abdelly, C., & Ksouri, R. (2020). Bio-guided fractionation and characterization of powerful antioxidant compounds from the halophyte Inula crithmoïdes. *Arabian Journal of Chemistry*, *13*(1), 2680–2688.

Jensen, R. L., Ragel, B. T., Whang, K., & Gillespie, D. (2006). Inhibition of hypoxia inducible factor-1a (HIF-1a) decreases vascular endothelial growth factor (VEGF) secretion and tumor growth in malignant gliomas. *Journal of neuro-oncology, 78*(3), 233-47.

Jia, D., Lu, M., Jung, K. H., Park, J. H., Yu, L., Onuchic, J. N., … Levine, H. (2019). Elucidating cancer metabolic plasticity by coupling gene regulation with metabolic pathways. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(9), 3909–3918.

Jiang, K., Wang, W., Jin, X., Wang, Z., Ji, Z., & Meng, G. (2015). Silibinin, a natural flavonoid, induces autophagy via ROS-dependent mitochondrial dysfunction and loss of ATP involving BNIP3 in human MCF7 breast cancer cells. *Oncology Reports*, *33*(6), 2711–2718.

Jiang, Z., Kempinski, C., & Chappell, J. (2016). Extraction and Analysis of Terpenes/Terpenoids. *Current Protocols in Plant Biology*, *1*(2), 345–358.

Joerger, A. C., & Fersht, A. R. (2007). Structure-function-rescue: The diverse nature of common p53 cancer mutants. *Oncogene*, *26*, 2226–2242.

Johnson, K. E., & Wilgus, T. A. (2014). Vascular Endothelial Growth Factor and Angiogenesis in the Regulation of Cutaneous Wound Repair. *Advances in Wound*

Care, *3*(10), 647–661.

Johnson, M. C. (2010*). Anatomy and physiology of the breast*. Management of Breast Diseases, Springer, Cham, 1–36.

Jones, P. M., & George, A. M. (2002). Mechanism of ABC transporters: A molecular dynamics simulation of a well characterized nucleotide-binding subunit. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(20), 12639–12644.

Jonker, L. (2014). TGF- β & BMP Receptors Endoglin and ALK1: Overview of their Functional Role and Status as Antiangiogenic Targets. *Microcirculation*, *21*(2), 93–103.

Jordan, V., Obiorah, I., Fan, P., Kim, H., Ariazi, E., Cunliffe, H. and Brauch, H. (2011). The St. Gallen Prize Lecture 2011: Evolution of long-term adjuvant anti-hormone therapy: consequences and opportunities. *The Breast*, 20, S1-S11.

Kadhim, B., Rasool, A., Shehab, N. G., Khan, S. A., Bayoumi, F. A., Bazigha Kadhim Abdul, R., … Fatehia Aly, B. (2014). A new natural gel of Fagonia indica Burm f. extract for the treatment of burn on rats. *Pakistan Journal of Pharmaceutical sciences, 27*(1), 73-81.

Kanthal, L. K., Dey, A., Satyavathi, K., & Bhojaraju, P. (2014). GC-MS analysis of bio-active compounds in methanolic extract of Lactuca runcinata DC. *Pharmacognosy Research*, *6*(1), 58–61.

Kanwal, N., Adhikari, A., Hameed, A., Hafizur, R. M., & Musharraf, S. G. (2017). Isolation and characterization of non-sulfated and sulfated triterpenoid saponins from *Fagonia indica*. *Phytochemistry*, *143*, 151–159.

Karar, J., & Maity, A. (2011). PI3K/AKT/mTOR Pathway in Angiogenesis. *Frontiers in Molecular Neuroscience*, *4*, 51.

Kartal-Yandim, M., Adan-Gokbulut, A., & Baran, Y. (2016). Molecular mechanisms of drug resistance and its reversal in cancer. *Critical Reviews in Biotechnology*, *36*(4), 716–726.

Kassi, E., Papoutsi, Z., Pratsinis, H., Aligiannis, N., Manoussakis, M., & Moutsatsou, P. (2007). Ursolic acid, a naturally occurring triterpenoid, demonstrates anticancer activity on human prostate cancer cells. *Journal of Cancer Research and Clinical Oncology*, *133*(7), 493–500.

Kassi, E., Sourlingas, T. G., Spiliotaki, M., Papoutsi, Z., Pratsinis, H., Aligiannis, N., & Moutsatsou, P. (2009). Ursolic acid triggers apoptosis and Bcl-2 downregulation in MCF-7 breast cancer cells. *Cancer Investigation*, *27*(7), 723–733.

Katiyar, S. K., Roy, A. M., & Baliga, M. S. (2005). Silymarin induces apoptosis primarily through a p53-dependent pathway involving Bcl-2/Bax, cytochrome c release, and caspase activation. *Molecular Cancer Therapeutics*, *4*(2), 207–216.

Khalik, S. M. A., Miyase, T., El-Ashaal, H. A., & Melek, F. R. (2000). Triterpenoid saponins from Fagonia cretica. *Phytochemistry*, *54*(8), 853–859.

Khaliq, G., Ramzan, M., & Baloch, A. H. (2019). Effect of Aloe vera gel coating enriched with Fagonia indica plant extract on physicochemical and antioxidant activity of sapodilla fruit during postharvest storage. *Food Chemistry*, *286*, 346–353.

Khan, Taimoor, Ullah, M. A., Garros, L., Hano, C., & Abbasi, B. H. (2019). Synergistic effects of melatonin and distinct spectral lights for enhanced production of anti-

cancerous compounds in callus cultures of Fagonia indica. *Journal of Photochemistry and Photobiology B: Biology*, *190*, 163–171.

Khan, Tariq, Abbasi, B. H., & Khan, M. A. (2018). The interplay between light, plant growth regulators and elicitors on growth and secondary metabolism in cell cultures of *Fagonia indica*. *Journal of Photochemistry and Photobiology B: Biology*, *185*, 153–160.

Khan, Tariq, Abbasi, B. H., Khan, M. A., & Shinwari, Z. K. (2016). Differential Effects of Thidiazuron on Production of Anticancer Phenolic Compounds in Callus Cultures of *Fagonia indica*. *Applied Biochemistry and Biotechnology*, *179*(1), 46–58.

Khan, Tariq, Ali, M., Khan, A., Nisar, P., Jan, S. A., Afridi, S., & Shinwari, Z. K. (2020). Anticancer plants: A review of the active phytochemicals, applications in animal models, and regulatory aspects. *Biomolecules*, *10*(1), 47.

Khatoon, M., Islam, E., Islam, R., Rahman, A. A., Alam, A. H. M. K., Khondkar, P., … Parvin, S. (2013). Estimation of total phenol and in vitro antioxidant activity of Albizia procera leaves. *BMC Research Notes*, *6*(1), 121.

Khayam, A. U., Patel, H., Faiola, N. A., Figueroa Milla, A. E., Dilshad, E., Mirza, B., … Sheikh, M. S. (2020). Quinovic acid purified from medicinal plant Fagonia indica mediates anticancer effects via death receptor 5. *Molecular and Cellular Biochemistry*, *474*(1–2), 159–169.

Kim, E. C., Kim, H. J., & Kim, T. J. (2015). Water extract of Cinnamomum cassia suppresses angiogenesis through inhibition of VEGF receptor 2 phosphorylation. *Bioscience, Biotechnology and Biochemistry*, *79*(4), 617–624.

Kim, I., Moon, S. O., Kim, S. H., Kim, H. J., Koh, Y. S., & Koh, G. Y. (2001). Vascular Endothelial Growth Factor Expression of Intercellular Adhesion Molecule 1 (ICAM-1), Vascular Cell Adhesion Molecule 1 (VCAM-1), and E-selectin through Nuclear Factor-κB Activation in Endothelial Cells. *Journal of Biological Chemistry*, *276*(10), 7614–7620.

Kim, K. L., & Suh, W. (2017). Apatinib, an inhibitor of vascular endothelial growth factor receptor 2, suppresses pathologic ocular neovascularization in mice. *Investigative Ophthalmology and Visual Science*, *58*(9), 3592–3599.

Kim, S. Y., Jeong, H. C., Hong, S. K., Lee, M. O., Cho, S. J., & Cha, H. J. (2017). Quercetin induced ROS production triggers mitochondrial cell death of human embryonic stem cells. *Oncotarget*, *8*(39), 64964–64973.

Kleinsmith, L. J. (2006). *Principles of cancer biology*. Pearson Benjamin Cummings.

Koblinski, J. E., Kaplan-Singer, B. R., VanOsdol, S. J., Wu, M., Engbring, J. A., Wang, S., … Kleinman, H. K. (2005). Endogenous osteonectin/SPARC/BM-40 expression inhibits MDA-MB-231 breast cancer cell metastasis. *Cancer Research*, *65*(16), 7370–7377.

Kochel, T. J., Reader, J. C., Ma, X., Kundu, N., & Fulton, A. M. (2017). Multiple drug resistance-associated protein (MRP4) exports prostaglandin E2 (PGE2) and contributes to metastasis in basal/ triple negative breast cancer. *Oncotarget*, *8*(4), 6540–6554.

Koczurkiewicz, P., Klaś, K., Grabowska, K., Piska, K., Rogowska, K., Wójcik-Pszczoła, K., … Pękala, E. (2019). Saponins as chemosensitizing substances that improve effectiveness and selectivity of anticancer drug—Minireview of in vitro studies. *Phytotherapy Research*, *33*(9), 2141–2151.

Kondo, T., Ohta, T., Igura, K., Hara, Y., & Kaji, K. (2002). Tea catechins inhibit

angiogenesis in vitro, measured by human endothelial cell growth, migration and tube formation, through inhibition of VEGF receptor binding. *Cancer Letters*, *180*(2), 139–144.

Kummar, S., Sitki Copur, M., Rose, M., Wadler, S., Stephenson, J., O'Rourke, M., … Chu, E. (2011). A phase I study of the chinese herbal medicine PHY906 as a modulator of irinotecan-based chemotherapy in patients with advanced colorectal cancer. *Clinical Colorectal Cancer*, *10*(2), 85–96.

Kumar, D., Tejaswi, C., Rasamalla, S., Mallick, S., & Pal, B. C. (2015). Bio-assay Guided Isolation of Anti-cancer Compounds from *Anthocephalus Cadamba* Bark. *Natural Product Communications*, *10*(8), 1934578X1501000.

Kumbi, Y., Miranda, C., Wu, W., & Stevens, J. (2019). *Phytochemical characterization of Fagonia indica and its effects on MCF-7 breast cancer cell line*. Oregon State University, Corvallis.

Kuo, M. T. (2007). *Roles of multidrug resistance genes in breast cancer chemoresistance. Advances in Experimental Medicine and Biology,* Springer, New York, 608.

Lakhtakia, R. (2014). A brief history of breast cancer: Part I: Surgical domination reinvented. *Sultan Qaboos University Medical Journal*, *14*(2) 166-69

Lam, M., Carmichael, A. R., & Griffiths, H. R. (2012). An Aqueous Extract of Fagonia cretica Induces DNA Damage, Cell Cycle Arrest and Apoptosis in Breast Cancer Cells via FOXO3a and p53 Expression. *PLoS ONE*, *7*(6), e40152.

Lamy, S., Ouanouki, A., Béliveau, R., & Desrosiers, R. R. (2014). Olive oil compounds inhibit vascular endothelial growth factor receptor-2 phosphorylation. *Experimental Cell Research*, *322*(1), 89–98.

Lauby-Secretan, B., Vilahur, N., Bianchini, F., Guha, N., & Straif, K. (2018). The IARC Perspective on Colorectal Cancer Screening. *New England Journal of Medicine*, *378*(18), 1734–1740.

Lee, E. Y. H. P., & Muller, W. J. (2010). Oncogenes and tumor suppressor genes. *Cold Spring Harbor Perspectives in Biology, 2(*10), a003236

Lee, M., Yun, S., Lee, H., & Yang, J. (2017). Quercetin mitigates inflammatory responses induced by vascular endothelial growth factor in mouse retinal photoreceptor cells through suppression of nuclear factor Kappa B. *International Journal of Molecular Sciences*, *18*(11), 2497.

Lei, Z., Sumner, B. W., Bhatia, A., Sarma, S. J., & Sumner, L. W. (2019). UHPLC-MS Analyses of Plant Flavonoids. *Current Protocols in Plant Biology*, *4*(1), e20085.

Lepage, C., Léger, D. Y., Bertrand, J., Martin, F., Beneytout, J. L., & Liagre, B. (2011). Diosgenin induces death receptor-5 through activation of p38 pathway and promotes TRAIL-induced apoptosis in colon cancer cells. *Cancer Letters*, *301*(2), 193–202.

Lerebours, F., Vacher, S., Andrieu, C., Espie, M., Marty, M., Lidereau, R., & Bieche, I. (2008). NF-kappa B genes have a major role in Inflammatory Breast Cancer. *BMC Cancer*, *8*(1), 41.

Levy-Lahad, E., & Friedman, E. (2007). Cancer risks among BRCA1 and BRCA2 mutation carriers. *British Journal of Cancer*, *96*(1), 11–15.

Lewin, M. H., Bailey, N., Bandaletova, T., Bowman, R., Cross, A. J., Pollock, J., … Bingham, S. A. (2006). Red meat enhances the colonic formation of the DNA adduct O 6-carboxymethyl guanine: Implications for colorectal cancer risk. *Cancer Research*,

66(3), 1859–1865.

Li, J., Liang, H., Liu, J., & Wang, Z. (2018). Poly (amidoamine) (PAMAM) dendrimer mediated delivery of drug and pDNA/siRNA for cancer therapy. *International Journal of Pharmaceutics*, *546*(1-2), 215–225.

Li, Q., Liang, Y., Tian, Y., & Hu, G. (2016). Arctigenin induces apoptosis in colon cancer cells through ROS / p 38 MAPK pathway. *Jouranl of B.U.O.N*, *21*(1), 87-94.

Li, Y., Revalde, J. L., Reid, G., & Paxton, J. W. (2010). Interactions of dietary phytochemicals with ABC transporters: Possible implications for drug disposition and multidrug resistance in cancer. *Drug Metabolism Reviews*, *42*(4), 590–611.

Li, Ying, Wang, M., Zhi, P., You, J., & Gao, J. (2018). Metformin synergistically suppress tumor growth with doxorubicin and reverse drug resistance by inhibiting the expression and function of P-glycoprotein in MCF7/ADR cells and xenograft models. *Oncotarget*, *9*(2), 2158–2174.

Liang, Z., Brooks, J., Willard, M., Liang, K., Yoon, Y., Kang, S., & Shim, H. (2007). CXCR4/CXCL12 axis promotes VEGF-mediated tumor angiogenesis through Akt signaling pathway. *Biochemical and Biophysical Research Communications*, *359*(3), 716–722.

Lima, A. M. B., Siani, A. C., Nakamura, M. J., & D'Avila, L. A. (2015). Selective and cost effective protocol to separate bioactive triterpene acids from plant matrices using alkalinized ethanol: Application to leaves of Myrtaceae species. *Pharmacognosy Magazine*, *11*(43), 470–476.

Limtrakul, P. (2007). Curcumin as chemosensitizer. *Advances in Experimental Medicine and Biology*, *595*, 269-300.

Lin, A. B., McNeely, S. C., & Beckmann, R. P. (2017). Achieving precision death with cell-cycle inhibitors that target DNA replication and repair. *Clinical Cancer Research*, *23*(13), 3232–3240.

Lin, C. Y., Ström, A., Vega, V. B., Kong, S. L., Yeo, A. L., Thomsen, J. S., … Liu, E. T. (2004). Discovery of estrogen receptor alpha target genes and response elements in breast tumor cells. *Genome Biology*, *5*(9), R66.

Lin, Y., Zhai, E., Liao, B., Xu, L., Zhang, X., Peng, S., … Chen, M. (2017). Autocrine VEGF signaling promotes cell proliferation through a PLC-dependent pathway and modulates Apatinib treatment efficacy in gastric cancer. *Oncotarget*, *8*(7), 11990–12002.

Liu, D. P., Song, H., & Xu, Y. (2010). A common gain of function of p53 cancer mutants in inducing genetic instability. *Oncogene*, *29*(7), 949–956.

Liu, J., Uematsu, H., Tsuchida, N., & Ikeda, M. A. (2011). Essential role of caspase-8 in p53/p73-dependent apoptosis induced by etoposide in head and neck carcinoma cells. *Molecular Cancer*, *10*(1), 95.

Liu, T., Zhang, L., Joo, D., & Sun, S. C. (2017). NF-κB signaling in inflammation. *Signal Transduction and Targeted Therapy*, *2*, 17023.

Liu, Y., Peng, H., & Zhang, J. T. (2005). Expression profiling of ABC transporters in a drug-resistant breast cancer cell line using AmpArray. *Molecular Pharmacology*, *68*(2), 430–438.

Longley, D. B., Harkin, D. P., & Johnston, P. G. (2003). 5-Fluorouracil: Mechanisms of action and clinical strategies. *Nature Reviews Cancer*, *3*(5), 330–338.

López-Gómez, L., Díaz-Ruano, S., Girón, R., López-Pérez, A. E., Vera, G., Herradón Pliego, E., … Abalo, R. (2018). Preclinical evaluation of the effects on the gastrointestinal tract of the antineoplastic drug vincristine repeatedly administered to rats. *Neurogastroenterology & Motility*, *30*(11), e13399.

Low, F. G., Shabir, K., Brown, J. E., Bill, R. M., & Rothnie, A. J. (2020). Roles of abcc1 and abcc4 in proliferation and migration of breast cancer cell lines. *International Journal of Molecular Sciences*, *21*(20), 1–21.

Lu, Q., Chen, W., Ji, Y., Liu, Y., & Xue, X. (2021). Ursolic Acid Enhances Cytotoxicity of Doxorubicin-Resistant Triple-Negative Breast Cancer Cells via *ZEB1-AS1* / *miR-186-5p* / *ABCC1* Axis. *Cancer Biotherapy and Radiopharmaceuticals*, *2020*, 4147.

Lugli, A., Zlobec, I., Minoo, P., Baker, K., Tornillo, L., Terracciano, L., & Jass, J. R. (2007). Prognostic significance of the wnt signalling pathway molecules APC, ?-catenin and E-cadherin in colorectal cancer?a tissue microarray-based analysis. *Histopathology*, *50*(4), 453–464.

Ma, L., & Zong, X. (2020). Metabolic Symbiosis in Chemoresistance: Refocusing the Role of Aerobic Glycolysis. *Frontiers in Oncology*, *10*(5), 5.

Maddams, J., Utley, M., & Møller, H. (2012). Projections of cancer prevalence in the United Kingdom, 2010-2040. *British Journal of Cancer*, *107*(7), 1195–1202.

Madi, N., Dany, M., Abdoun, S., & Usta, J. (2016). Moringa oleifera's Nutritious Aqueous Leaf Extract Has Anticancerous Effects by Compromising Mitochondrial Viability in an ROS-Dependent Manner. *Journal of the American College of Nutrition*, *35*(7), 604–613.

Mahato, S. B., Sarkar, S. K., & Poddar, G. (1988). Triterpenoid saponins. *Phytochemistry*, *27*(10), 3037–3067.

Mahdy, A., Ahmed, N. G., & Shehab, N. G. (2015). Hypoglycemic activity of Fagonia indica and Aloe vera in Alloxan-induced hyperglycemia in mice. *EC Pharmaceutical Science*, *4,* 239-244.

Maida, M., Macaluso, F. S., Ianiro, G., Mangiola, F., Sinagra, E., Hold, G., … Scarpulla, G. (2017). Screening of colorectal cancer: present and future. *Expert Review of Anticancer Therapy*, *17*(12), 1131-1146.

Malik, I. A., Khan, N. A., & Khan, W. (1999). Use of Unconventional Methods of Therapy by Cancer Patients in Pakistan. *European Journal of Epidemiology*, 16, 155–160.

Man, S., Gao, W., Zhang, Y., Huang, L., & Liu, C. (2010). Chemical study and medical application of saponins as anti-cancer agents. *Fitoterapia*, *81*(7), 703–714.

Mansoori, B., Mohammadi, A., Davudian, S., Shirjang, S., & Baradaran, B. (2017). The different mechanisms of cancer drug resistance: A brief review. *Advanced Pharmaceutical Bulletin*, *7*(3), 339–348.

Manu, K. A., & Kuttan, G. (2008). Ursolic acid induces apoptosis by activating p53 and caspase-3 gene expressions and suppressing NF-κB mediated activation of bcl-2 in B16F-10 melanoma cells. *International Immunopharmacology*, *8*(7), 974–981.

Marchi, S., Giorgi, C., Suski, J. M., Agnoletto, C., Bononi, A., Bonora, M., … Pinton, P. (2012). Mitochondria-Ros Crosstalk in the Control of Cell Death and Aging. *Journal of Signal Transduction*, *329635,* 1–17.

Martinet, W., Schrijvers, D. M., Herman, A. G., & De Meyer, G. R. Y. (2006). z-VAD-fmk-induced non-apoptotic cell death of macrophages: Possibilities and limitations for

atherosclerotic plaque stabilization. *Autophagy*, *2*(4), 312–314.

Martinez-Perez, C., Ward, C., Cook, G., Mullen, P., McPhail, D., Harrison, D. J., & Langdon, S. P. (2014). Novel flavonoids as anti-cancer agents: Mechanisms of action and promise for their potential application in breast cancer. *Biochemical Society Transactions*, *42*(4), 1017–1023.

Martino, E., Casamassima, G., Castiglione, S., Cellupica, E., Pantalone, S., Papagni, F., … Collina, S. (2018). Vinca alkaloids and analogues as anti-cancer agents: Looking back, peering ahead. *Bioorganic and Medicinal Chemistry Letters*, *28*(17), 2816–2826.

Marvibaigi, M., Amini, N., Supriyanto, E., Majid, F. A. A., Jaganathan, S. K., Jamil, S., … Nasiri, R. (2016). Antioxidant activity and ROS-dependent apoptotic effect of scurrula ferruginea (jack) danser methanol extract in human breast cancer cell MDA-MB-231. *PLoS ONE*, *11*(7), 158942.

Mattina, M. J. I., & MacEachern, G. J. (1994). Extraction, purification by solid-phase extraction and high-performance liquid chromatographic analysis of taxanes from ornamental Taxus needles. *Journal of Chromatography A*, *679*(2), 269–275.

McCleary, N. J., Niedzwiecki, D., Hollis, D., Saltz, L. B., Schaefer, P., Whittom, R., … Meyerhardt, J. A. (2010). Impact of smoking on patients with stage iii colon cancer: Results from cancer and leukemia group B 89803. *Cancer*, *116*(4), 957–966.

McDevitt, M. A., Glidewell-Kenney, C., Jimenez, M. A., Ahearn, P. C., Weiss, J., Jameson, J. L., & Levine, J. E. (2008). New insights into the classical and non-classical actions of estrogen: Evidence from estrogen receptor knock-out and knock-in mice. *Molecular and Cellular Endocrinology*, *290*(1-2), 24–30.

McKay, D. L., & Blumberg, J. B. (2006). A Review of the bioactivity and potential health benefits of chamomile tea (Matricaria recutita L.). *Phytotherapy Research*, *20*(7), 519–530.

Medina-Meza, I. G., Aluwi, N. A., Saunders, S. R., & Ganjyal, G. M. (2016). GC-MS Profiling of Triterpenoid Saponins from 28 Quinoa Varieties (Chenopodium quinoa Willd.) Grown in Washington State. *Journal of Agricultural and Food Chemistry*, *64*(45), 8583–8591.

Menchón, S. A. (2015). The Effect of Intrinsic and Acquired Resistances on Chemotherapy Effectiveness. *Acta Biotheoretica*, *63*(2), 113–127.

Meng, F., Zou, L., Zhang, T., Jiang, L., Ding, Y., Yu, P., & Peng, J. (2018). Using LC-MS/MS- based targeted proteomics to monitor the pattern of ABC transporters expression in the development of drug resistance. *Cancer Management and Research*, *10*, 2859–2870.

Mishra, R., & Kaur, G. (2013). Aqueous Ethanolic Extract of Tinospora cordifolia as a Potential Candidate for Differentiation Based Therapy of Glioblastomas. *PLoS ONE*, *8*(10), e78764.

Mlala, S., Oyedeji, A. O., Gondwe, M., & Oyedeji, O. O. (2019). Ursolic Acid and Its Derivatives as Bioactive Agents. *Molecules (Basel, Switzerland)*, *24*(15), 2751.

Moldoveanu, S. C., & Scott, W. A. (2016). Analysis of four pentacyclic triterpenoid acids in several bioactive botanicals with gas and liquid chromatography and mass spectrometry detection. *Journal of Separation Science*, *39*(2), 324–332.

Moll, U. M., & Petrenko, O. (2003). The MDM2-p53 Interaction. *Molecular Cancer Research*, *1*(14), 1001–1008.

Mooi, J. K., Wirapati, P., Asher, R., Lee, C. K., Savas, P., Price, T. J., … Tebbutt, N. C. (2018). The prognostic impact of consensus molecular subtypes (CMS) and its predictive effects for bevacizumab benefit in metastatic colorectal cancer: molecular analysis of the AGITG MAX clinical trial. *Annals of Oncology : Official Journal of the European Society for Medical Oncology*, *29*(11), 2240–2246.

Moradi, B., Abbaszadeh, S., Shahsavari, S., Alizadeh, M., & Beyranvand, F. (2018). The most useful medicinal herbs to treat diabetes. *Biomedical Research and Therapy*, *5*(8), 2538–2551.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1–2), 55–63.

Moses, T., Papadopoulou, K. K., & Osbourn, A. (2014). Metabolic and functional diversity of saponins, biosynthetic intermediates and semi-synthetic derivatives. *Critical Reviews in Biochemistry and Molecular Biology*, *49*(6), 439–462.

Morse, M. A. (2005). Adjuvant Therapy of Colon Cancer: Current Status and Future Developments. *Clinics in Colon and Rectal Surgery*, *18*(3), 224.

Mousavi, S. H., Tavakkol-Afshari, J., Brook, A., & Jafari-Anarkooli, I. (2009). Role of caspases and Bax protein in saffron-induced apoptosis in MCF-7 cells. *Food and Chemical Toxicology*, *47*(8), 1909–1913.

Müller, M. F., Ibrahim, A. E. K., & Arends, M. J. (2016). Molecular pathological classification of colorectal cancer. *Virchows Archiv*, *469*(2), 125–134.

Nakano, A., Tsuji, D., Miki, H., Cui, Q., Sayed, S. M. El, Ikegame, A., … Abe, M. (2011). Glycolysis Inhibition Inactivates ABC Transporters to Restore Drug Sensitivity in Malignant Cells. *PLoS ONE*, *6*(11), e27222.

Nishida, N., Yano, H., Nishida, T., Kamura, T., & Kojiro, M. (2006). Angiogenesis in cancer. *Vascular Health and Risk Management*, *2*(3), 213–219.

Niu, G., & Chen, X. (2010.). Vascular Endothelial Growth Factor as an Anti-Angiogenic Target for Cancer Therapy. *Current Drug Targets, 11*(8), 1000-17.

Olivier, M., Langerød, A., Carrieri, P., Bergh, J., Klaar, S., Eyfjord, J., … Børresen-Dale, A. L. (2006). The clinical value of somatic TP53 gene mutations in 1,794 patients with breast cancer. *Clinical Cancer Research*, *12*(4), 1157–1167.

Pabla, N., Huang, S., Mi, Q.-S., Daniel, R., & Dong, Z. (2007). ATR-Chk2 Signaling in p53 Activation and DNA Damage Response during Cisplatin-induced Apoptosis. *The Journal of Biological Chemistry, 283*(10), 6752-83.

Pahnke, J., Fröhlich, C., Krohn, M., Schumacher, T., & Paarmann, K. (2013). Impaired mitochondrial energy production and ABC transporter function-A crucial interconnection in dementing proteopathies of the brain. *Mechanisms of Ageing and Development*, *134*(10), 506–515.

Panche, A. N., Diwan, A. D., & Chandra, S. R. (2016). Flavonoids: An overview. *Journal of Nutritional Science*, *5*, 1–15.

Park, J. I., Bae, H. R., Kim, C. G., Stonik, V. A., & Kwak, J. Y. (2014). Relationships between chemical structures and functions of triterpene glycosides isolated from sea cucumbers. *Frontiers in Chemistry*, *2*, 77.

Parsekar, K., Howard Wilsher, S., Sweeting, A., Patel, A., & Fordham, R. (2021). Societal costs of chemotherapy in the UK: an incidence-based cost-of-illness model for early

breast cancer. *BMJ Open*, *11*(1), e039412.

Patel, H., Zaghloul, N., Lin, K., Liu, S. F., Miller, E. J., & Ahmed, M. (2017). Hypoxia-induced activation of specific members of the NF-kB family and its relevance to pulmonary vascular remodeling. *International Journal of Biochemistry and Cell Biology*, *92*, 141–147.

Pawlak, A., Ziolo, E., Fiedorowicz, A., Fidyt, K., Strzadala, L., & Kalas, W. (2016). Long-lasting reduction in clonogenic potential of colorectal cancer cells by sequential treatments with 5-azanucleosides and topoisomerase inhibitors. *BMC Cancer*, *16*(1), 893.

Pelicano, H., Martin, D. S., Xu, R. H., & Huang, P. (2006). Glycolysis inhibition for anticancer treatment. *Oncogene*, *25*, 4633–4646.

Perez, E. A., Romond, E. H., Suman, V. J., Jeong, J., Sledge, G., Geyer, C. E., . . . Wolmark, M. (2014). Trastuzumab Plus Adjuvant Chemotherapy for Human Epidermal Growth Factor Receptor 2–Positive Breast Cancer: Planned Joint Analysis of Overall Survival From NSABP B-31 and NCCTG N9831. *Journal of Clinical Oncology*, 32(33):3744-3752.

Perrone, A., Masullo, M., Bassarello, C., Hamed, A. I., Belisario, M. A., Pizza, C., & Piacente, S. (2007). Sulfated triterpene derivatives from Fagonia arabica. *Journal of Natural Products*, *70*(4), 584–588.

Petronellia, A., Pannitterib, G., & Testaa, U. (2009). Triterpenoids as new promising anticancer drugs. *Anti-Cancer Drugs*, *20*(10), 880–892.

Pietta, P. G. (2000). Flavonoids as antioxidants. *Journal of Natural Products*, *63*(7), 1035–1042.

Plunkett, W., Huang, P., & Gandhi, V. (1995). Preclinical characteristics of gemcitabine. *Anti-Cancer Drugs*, *6*(6), 7–13.

Plunkett, W., Huang, P., Xu, Y. Z., Heinemann, V., Grunewald, R., & Gandhi, V. (1995). Gemcitabine: metabolism, mechanisms of action, and self-potentiation - PubMed. *Seminars in Oncology*, *4*(11), 3–10.

Poljsak, B., & Milisav, I. (2019). The Role of Antioxidants in Cancer, Friends or Foes? *Current Pharmaceutical Design*, *24*(44), 5234–5244.

Poma, P., Labbozzetta, M., D'Alessandro, N., & Notarbartolo, M. (2017). NF-κB Is a Potential Molecular Drug Target in Triple-Negative Breast Cancers. *OMICS: A Journal of Integrative Biology*, *21*(4), 225–231.

Pranteda, A., Piastra, V., Stramucci, L., Fratantonio, D., & Bossi, G. (2020). The p38 mapk signaling activation in colorectal cancer upon therapeutic treatments. *International Journal of Molecular Sciences*, *21*(8), 2773.

Principe, P. (2005). *Monetising the Pharmacological Benefits of Plants.* US Environmental protection Agency, Washington, D.C.

Proniewska-Skretek, E., & Mariak, Z. (2004). Tumor angiogensis in uveal melanoma. Role of vascular endothelial growth factor (VEGF). *Klinika Oczna*, *106*(4–5), 682–685.

Qian, Z., Wang, X., Song, Z., Zhang, H., Zhou, S., Zhao, J., & Wang, H. (2015). No Title. *BioMed Research International*, 7.

Qu, J. miao, You, J., Liu, H., Huang, Q. di, & Guo, G. long. (2013). Effect of 5-fluorouracil on the expression of ATP-binding cassette superfamily G member 2 in human colon

cancer cell SW480. *Chinese Journal of Gastrointestinal Surgery*, *16*(1), 89–92.

Qureshi, H., Asif, S., Ahmed, H., Al-Kahtani, H. A., & Hayat, K. (2016). Chemical composition and medicinal significance of Fagonia cretica: A review. *Natural Product Research*, *30*(6), 625–639.

Qureshi, R., & Raza Bhatti, G. (2008). Ethnobotany of plants used by the Thari people of Nara Desert, Pakistan. *Fitoterapia*, *79*(6), 468-73.

Qureshi, R. A., Ghurfan, M. A., Gilani, S., Yousaf, Z., Miana, G. A., & Batool, L, A. (2009). Indigenous medicinal plants used by local women in southern Himalayan regions of Pakistan. *Pakistan Journal of Botany*, *41,* 19-25.

Radisavljevic, Z., Avraham, H., & Avraham, S. (2000). Vascular endothelial growth factor up-regulates ICAM-1 expression via the phosphatidylinositol 3 OH-kinase/AKT/nitric oxide pathway and modulates migration of brain microvascular endothelial cells. *Journal of Biological Chemistry*, *275*(27), 20770–20774.

Radomska-Lesniewska, D. M., Balan, B. J., & Skopinski, P. (2017). Angiogenesis modulation by exogenous antioxidants. *Central European Journal of Immunology*, *42*(4), 370–376.

Rahman, A.-U. (1986). Isolation, structural and synthetic studies on the chemical constituents of medicinal plants of Pakistan. *Pure and Applied Chemistry*, *58*, 663-673.

Rajendran, V., & Jain, M. V. (2018). In vitro tumorigenic assay: colony forming assay for cancer stem cells. In *Methods in Molecular Biology*, *1692*, 89–95.

Ramalhete, C., Mulhovo, S., Molnar, J., & Ferreira, M. J. U. (2016). Triterpenoids from Momordica balsamina: Reversal of ABCB1-mediated multidrug resistance. *Bioorganic and Medicinal Chemistry*, *24*(21), 5061–5067.

Rampurwala, M., Wisinski, K. B., & O'Regan, R. (2016). Role of the androgen receptor in triple-negative breast cancer. *Clinical Advances in Hematology and Oncology*, *14*(3), 186–193.

Rashid, U., Rashid Khan, M., Jan, S., Bokhari, J., & Shan, N. A. (2013). Assessment of phytochemicals, antimicrobial and cytotoxic activities of extract and fractions from Fagonia olivieri. *BMC Complementary and Alternative Medicine*, *13*, 67.

Rein, M. J., Renouf, M., Cruz-Hernandez, C., Actis-Goretta, L., Thakkar, S. K., & da Silva Pinto, M. (2013). Bioavailability of bioactive food compounds: A challenging journey to bioefficacy. *British Journal of Clinical Pharmacology*, *75*(3), 588–602.

Reinboth, M., Wolffram, S., Abraham, G., Ungemach, F. R., & Cermak, R. (2010). Oral bioavailability of quercetin from different quercetin glycosides in dogs. *British Journal of Nutrition*, *104*(2), 198–203.

Repetto, G., del Peso, A., & Zurita, J. L. (2008). Neutral red uptake assay for the estimation of cell viability/ cytotoxicity. *Nature Protocols*, *3*(7), 1125–1131.

Ried, K., Eng, P., & Sali, A. (2017). Screening for circulating tumour cells allows early detection of cancer and monitoring of treatment effectiveness: An observational study. *Asian Pacific Journal of Cancer Prevention*, *18*(8), 2275–2285.

Rizhikovs, J., Zandersons, J., Dobele, G., & Paze, A. (2015). Isolation of triterpene-rich extracts from outer birch bark by hot water and alkaline pre-treatment or the appropriate choice of solvents. *Industrial Crops and Products*, *76*, 209–214.

Rizwan, Z., Zia Ud Den, N., Ameen, N. E., Hussain, A., & Jamil, A. (2020). Medicinal potency of protein and methanolic extracts of Fagonia species. *Asian Journal of Plant Science & Research*, *10*(4).

Robey, R. B., & Hay, N. (2009). Is Akt the "Warburg kinase"?-Akt-energy metabolism interactions and oncogenesis. *Seminars in Cancer Biology*, *19*(1), 25–31.

Roelands, J., Kuppen, P. J. K., Vermeulen, L., Maccalli, C., Decock, J., Wang, E., … Hendrickx, W. (2017). Immunogenomic classification of colorectal cancer and therapeutic implications. *International Journal of Molecular Sciences*, *18*(10), 2229.

Saha, R. N., Jana, M., & Pahan, K. (2007). MAPK p38 Regulates Transcriptional Activity of NF-κB in Primary Human Astrocytes via Acetylation of p65. *The Journal of Immunology*, *179*(10), 7101–7109.

Said, S. A., Al-Saadi, S. H. A., Al-Abri, A. R., Akhtar, M. S., Weli, A. M., & Al-Riyami, Q. (2014). Cytotoxic properties of some herbal plants in Oman. *Journal of Taibah University for Science, 8*(2), 71-74.

Sarikahya, N. B., Nalbantsoy, A., Top, H., Gokturk, R. S., Sumbul, H., & Kirmizigul, S. (2018). Immunomodulatory, hemolytic and cytotoxic activity potentials of triterpenoid saponins from eight Cephalaria species. *Phytomedicine*, *38*, 135–144.

Sartippour, M. R., Shao, Z. M., Heber, D., Beatty, P., Zhang, L., Liu, C., … Brooks, M. N. (2002). Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. *Journal of Nutrition*, *132*(8), 2307–2311.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M., & Yoga Latha, L. (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary and Alternative Medicines*, *8*(1), 1–10.

Saurin, W., Hofnung, M., & Dassa, E. (1999). Getting in or out: Early segregation between importers and exporters in the evolution of ATP-binding cassette (ABC) transporters. *Journal of Molecular Evolution*, *48*(1), 22–41.

Scaldaferri, F., Vetrano, S., Sans, M., Arena, V., Straface, G., Stigliano, E., … Danese, S. (2009). VEGF-A Links Angiogenesis and Inflammation in Inflammatory Bowel Disease Pathogenesis. *Gastroenterology*, *136*(2), 585-95.

Scheffler, K., Krohn, M., Dunkelmann, T., Stenzel, J., Miroux, B., Ibrahim, S., … Pahnke, J. (2012). Mitochondrial DNA polymorphisms specifically modify cerebral β-amyloid proteostasis. *Acta Neuropathologica*, *124*(2), 199–208

Schellerer, V. S., Langheinrich, M. C., Zver, V., Grützmann, R., Stürzl, M., Gefeller, O., … Merkel, S. (2019). Soluble intercellular adhesion molecule-1 is a prognostic marker in colorectal carcinoma. *International Journal of Colorectal Disease*, *34*(2), 309–317.

Schuler, M., Bossy-Wetzel, E., Goldstein, J. C., Fitzgerald, P., & Green, D. R. (2000). p53 Induces Apoptosis by Caspase Activation through Mitochondrial Cytochrome c Release. *The Journal of Biological Chemistry, 275*(10), 7337-7342.

Seidman, R., Gitelman, I., Sagi, O., Horwitz, S. B., & Wolfson, M. (2001). The role of ERK 1/2 and p38 MAP-kinase pathways in Taxol-induced apoptosis in human ovarian carcinoma cells. *Experimental Cell Research*, *268*(1), 84–92.

Shaheen, H., Qureshi, R., Akram, A., & Gulfraz, M. (2014). Inventory of medicinal flora from Thal Desert, Punjab, Pakistan. *African Journal of Traditional, Complementary, and Alternative Medicines, 11*(3), 14-20.

Shaker, K. H., Al Jubiri, S. M., Faten, A. E. H., & Abdullah, A. (2013). New Compounds

from Bassia muricata and Fagonia indica. *International Journal of Pharmaceutical Sciences Review and Research*, *23*(1), 231–236.

Shakibaei, M., Buhrmann, C., Kraehe, P., Shayan, P., Lueders, C., & Goel, A. (2014). Curcumin Chemosensitizes 5-Fluorouracil Resistant MMR-Deficient Human Colon Cancer Cells in High Density Cultures. *PLoS ONE*, *9*(1), e85397.

Shangary, S., Ding, K., Qiu, S., Nikolovska-Coleska, Z., Bauer, J. A., Liu, M., … Wang, S. (2008). Reactivation of p53 by a specific MDM2 antagonist (MI-43) leads to p21-mediated cell cycle arrest and selective cell death in colon cancer. *Molecular Cancer Therapeutics*, *7*(6), 1533–1542.

Shastri, S., Chatterjee, B., & Thakur, S. S. (2019). Achievements in Cancer Research and its Therapeutics in Hundred Years. *Current Topics in Medicinal Chemistry*, *19*(17), 1545–1562.

Shaukat, A., Dostal, A., Menk, J., & Church, T. R. (2017). BMI Is a Risk Factor for Colorectal Cancer Mortality. *Digestive Diseases and Sciences*, *62*(9), 2511–2517.

Shaukat, S. S., Aziz, S., Ahmed, W., & Shahzad, A. (2012). Population structure, spatial pattern and reproductive capacity of two semi-desert undershrub Senna Holoserica and Fagonia Indica in southern Sindh, Pakistan. *Pakistan Journal of Botany, 44(*1), 1-9.

Shehab, Naglaa G., Mahdy, A., Khan, S. A., Noureddin, S. M., & Abd-Elhady, H. K. (2011). Chemical Constituents and Biological Activities of *Fagonia indica Burm* F, Study Shows. *Research Journal of Medicinal Plant*, *5*(5), 531–546.

Shehab, Naglaa Gamil, Abu-Gharbieh, E., & Bayoumi, F. A. (2015). Impact of phenolic composition on hepatoprotective and antioxidant effects of four desert medicinal plants. *BMC Complementary and Alternative Medicine*, *15*(1), 401.

Shehab, N. G., Shahiwala, A., Benouared, I., & Khan, R. (2020). Preparation and antihepatotoxicity activity of Fagonia indica extract and its solid dispersion formulation. *Pakistan Journal of Pharmaceutical Sciences*, *33*(3), 1025–1032.

Sheng, Y. N., Luo, Y. H., Liu, S. Bin, Xu, W. T., Zhang, Y., Zhang, T., … Jin, C. H. (2020). Zeaxanthin induces apoptosis via ROS-regulated MAPK and Akt signaling pathway in human gastric cancer cells. *OncoTargets and Therapy*, *13*, 10995–11006.

Shi, P., Du, W., Wang, Y., Teng, X., Chen, X., & Ye, L. (2019). Total phenolic, flavonoid content, and antioxidant activity of bulbs, leaves, and flowers made from Eleutherine bulbosa (Mill.) Urb. *Food Science and Nutrition*, *7*(1), 148–154.

Shinkaruk, S., Bayle, M., Laïn, G., & Déléris, G. (2003). Vascular Endothelial Cell Growth Factor (VEGF), an emerging target for cancer chemotherapy. *Current Medicinal Chemistry - Anti-Cancer Agents*, *3*(2), 95–117.

Shinoda, H., Ishida, S., Noda, K., Koto, T., Oguchi, Y., Okada, Y., & Ikeda, E. (2004). Reactive oxygen species upregulate the production of VEGF and MMPs in retinal glial cells | IOVS | ARVO Journals. *Investigative Opththalmology & Visual Science*, *45*(13), 745.

Shukla, K., Sonowal, H., Saxena, A., & Ramana, K. V. (2019). Didymin by suppressing NF-κB activation prevents VEGF-induced angiogenesis in vitro and in vivo. *Vascular Pharmacology*, *115*, 18–25.

Sia, D., Alsinet, C., Newell, P., & Villanueva, A. (2014). VEGF Signaling in Cancer Treatment. *Current Pharmaceutical Design*, *20*(17), 2834–2842.

Sibbering, M., & Courtney, C. A. (2016). Management of breast cancer: Basic principles.

Surgery (United Kingdom), *34*(1), 25-31.

Singh, M. P., Rai, S., Pandey, A., Singh, N. K., & Srivastava, S. (2019). Molecular subtypes of colorectal cancer: An emerging therapeutic opportunity for personalized medicine. *Genes and Diseases*, *8*(2), 133-145.

Singh Rana, P., & Saklani, P. (2017). Phytochemical screening and TLC profiling of various extracts of Reinwardtia indica. *International Journal of Pharmacognosy and Phytochemical Research*, *9*(4), 523-517.

Singletary, S. E. (2003). Rating the Risk Factors for Breast Cancer. *Annals of Surgery*, *237*(4), 474–482.

Skurk, C., Maatz, H., Kim, H. S., Yang, J., Abid, R., Aird, W. C., & Walsh, K. (2004). The Akt-regulated Forkhead Transcription Factor FOXO3a Controls Endothelial Cell Viability through Modulation of the Caspase-8 Inhibitor FLIP. *Journal of Biological Chemistry*, *279*(2), 1513–1525.

Smith, S. M., Lyu, Y. L., & Cai, L. (2014). NF-κB affects proliferation and invasiveness of breast cancer cells by regulating CD44 expression. *PLoS ONE*, *9*(9), 106966.

Smittenaar, C. R., Petersen, K. A., Stewart, K., & Moitt, N. (2016). Cancer incidence and mortality projections in the UK until 2035. *British Journal of Cancer*, *115*(9), 1147–1155.

Sokolov, A., Ashenden, S., Sahin, N., Lewis, R., Erdem, N., Ozaltan, E., … Cokol, M. (2019). Characterizing ABC-Transporter Substrate-Likeness Using a Clean-Slate Genetic Background. *Frontiers in Pharmacology*, *10*(APR), 448.

Soly, W., Zhanjie, L., Lunshan, W., & Xiaoren, Z. (2009). NF-κB signaling pathway, inflammation and colorectal cancer. *Chinese Journal of Cellular and Molecular Immunology*, 6, 327–334.

Soomro, A. L., & Jafarey, N. A. (2003). Effect of *Fagonia indica* on experimentally produced Tumours in Rats. *Journal of the Pakistan Medical Association*, *53*(6), 1.

Song, M., & Chan, A. T. (2017). Diet, Gut Microbiota, and Colorectal Cancer Prevention: a Review of Potential Mechanisms and Promising Targets for Future Research. *Current Colorectal Cancer Reports*, *13*(6), 429–439.

Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., … Borresen-Dale, A.-L. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences*, *98*(19), 10869–10874.

Stebounova, L., Ebert, S. M., Murry, L. T., Adams, C. M., & Murry, D. J. (2018). Rapid and Sensitive Quantification of Ursolic Acid and Oleanolic Acid in Human Plasma Using Ultra-performance Liquid Chromatography–Mass Spectrometry. *Journal of Chromatographic Science*, *56*(7), 644–649.

Stegh, A. H. (2012). Targeting the p53 signalling pathway in cancer therapies – The promises, challenges and perils. *Expert Opinion on Therapeutic Targets.*, *16*, 67-83.

Stochmal, A., Oleszek, W., & Kapusta, I. (2008). *Thin Layer Chromatography in Phytochemistry.* CRC Press, Boca Raton.

Stramucci, L., Pranteda, A., Stravato, A., Amoreo, C. A., Pennetti, A., Diodoro, M. G., … Bossi, G. (2019). MKK3 sustains cell proliferation and survival through p38DELTA MAPK activation in colorectal cancer. *Cell Death and Disease*, *10*(11), 1–13.

Strell, C., Lang, K., Niggemann, B., Zaenker, K. S., & Entschladen, F. (2010). Neutrophil granulocytes promote the migratory activity of MDA-MB-468 human breast carcinoma cells via ICAM-1. *Experimental Cell Research*, *316*(1).

Subramanian, M., Jones, M. F., & Lal, A. (2013). Long non-coding RNAs embedded in the Rb and p53 pathways. *Cancers*, *5*(4), 1655–1675.

Sui, H., Zhou, L.-H., Zhang, Y.-L., Huang, J.-P., Liu, X., Ji, Q., … Li, Q. (2016). Evodiamine Suppresses ABCG2 Mediated Drug Resistance by Inhibiting p50/p65 NF-κB Pathway in Colorectal Cancer. *Journal of Cellular Biochemistry*, *117*(6), 1471–1481.

Sui, X., Kong, N., Ye, L., Han, W., Zhou, J., Zhang, Q., … Pan, H. (2014). P38 and JNK MAPK pathways control the balance of apoptosis and autophagy in response to chemotherapeutic agents. *Cancer Letters*, *344*(2), 174–179.

Sultana, S., Khan, M. A., Ahmad, M., & Zafar, M. (2006). Indigenous Knowledge of Folk Herbal Medicines by the Women of District Chakwal, Pakistan. *Ethnobotanical Leaflets*, *10*, 243-253.

Sun, Y., Gu, M., Zhu, L., Liu, J., Xiong, Y., Wei, Y., & Li, F. (2016). Gemcitabine upregulates ABCG2/BCRP and modulates the intracellular pharmacokinetic profiles of bioluminescence in pancreatic cancer cells. *Anti-Cancer Drugs*, *27*(3), 183–191.

Sun, S., Gong, F., Liu, P., & Miao, Q. (2018). Metformin combined with quercetin synergistically repressed prostate cancer cells via inhibition of VEGF/PI3K/Akt signaling pathway. *Gene*, *664*, 50–57.

Sun, Y. L., Patel, A., Kumar, P., & Chen, Z. S. (2012). Role of ABC transporters in cancer chemotherapy. *Chinese Journal of Cancer*, *31(*2), 51–57.

Sung, J. J. Y., Kuipers, E. J., & El-Serag, H. B. (2009). Systematic review: The global incidence and prevalence of peptic ulcer disease. *Alimentary Pharmacology and Therapeutics*, *29*(9), 938-46.

Sunters, A., Fernández De Mattos, S., Stahl, M., Brosens, J. J., Zoumpoulidou, G., Saunders, C. A., … Lam, E. W. F. (2003). FoxO3a Transcriptional Regulation of Bim Controls Apoptosis in Paclitaxel-treated Breast Cancer Cell Lines. *Journal of Biological Chemistry*, *278*(50), 49795–49805.

Szakacs, G., Paterson, J. K., Ludwig, J. A., Booth-Genthe, C., & Gottesman, M. M. (2006). Targeting multidrug resistance in cancer. *Nature Reviews: Drug Discovery*, 5, 219-234.

Szakiel, A., Pączkowski, C., Pensec, F., & Bertsch, C. (2012). Fruit cuticular waxes as a source of biologically active triterpenoids. *Phytochemistry Reviews*, *11*(2), 263–284.

Szerémy, P., Pál, Á., Méhn, D., Tóth, B., Fülöp, F., Krajcsi, P., & Herédi-Szabó, K. (2011). Comparison of 3 assay systems using a common probe substrate, calcein AM, for studying P-gp using a selected set of compounds. *Journal of Biomolecular Screening*, *16*(1), 112-119.

Tan, K. W., Li, Y., Paxton, J. W., Birch, N. P., & Scheepens, A. (2013). Identification of novel dietary phytochemicals inhibiting the efflux transporter breast cancer resistance protein (BCRP/ABCG2). *Food Chemistry*, *138*(4), 2267–2274.

Tan, K. W., Sampson, A., Osa-Andrews, B., & Iram, S. H. (2018). Calcitriol and calcipotriol modulate transport activity of ABC transporters and exhibit selective cytotoxicity in MRP1-overexpressing cells. *Drug Metabolism and Disposition*, *46*(12), 1856–1866.

Tan, S. P., Vuong, Q. V., Stathopoulos, C. E., Parks, S. E., & Roach, P. D. (2014). Optimized Aqueous Extraction of Saponins from Bitter Melon for Production of a Saponin-Enriched Bitter Melon Powder. *Journal of Food Science*, *79*(7), E1372–E1381.

Tang, S. M., Deng, X. T., Zhou, J., Li, Q. P., Ge, X. X., & Miao, L. (2020). Pharmacological basis and new insights of quercetin action in respect to its anti-cancer effects. *Biomedicine and Pharmacotherapy*, *121*, 109604.

Tanira, M. O. M., Ali, B. H., Bashir, A. K., Wasfi, I. A., & Chandranath, I. (1996). Evaluation of the relaxant activity of some United Arab Emirates plants on intestinal smooth muscle. *Journal of Pharmacy and Pharmacology, 48*(5), 545-550.

Taralkar, S. V., & Chattopadhyay, S. (2012). A HPLC Method for Determination of Ursolic Acid and Betulinic Acids from their Methanolic Extracts of Vitex Negundo Linn. *Journal of Analytical & Bioanalytical Techniques*, *03*(03), 1–6.

Thanki, K., Nicholls, M. E., Gajjar, A., Senagore, A. J., Qiu, S., Szabo, C., … Chao, C. (2017). Consensus Molecular Subtypes of Colorectal Cancer and their Clinical Implications. *International Biological and Biomedical Journal*, *3*(3), 105–111.

Thilakarathna, S. H., & Vasantha Rupasinghe, H. P. (2013). Flavonoid bioavailability and attempts for bioavailability enhancement. *Nutrients*, *5*(9), 3367–3387.

Thomasset, S., Berry, D. P., Cai, H., West, K., Marczylo, T. H., Marsden, D., … Gescher, A. J. (2009). Pilot study of oral anthocyanins for colorectal cancer chemoprevention. *Cancer Prevention Research*, *2*(7), 625–633.

Thornton, T. M., & Rincon, M. (2009). Non-classical p38 map kinase functions: Cell cycle checkpoints and survival. *International Journal of Biological Sciences*, *5(*1), 44–52.

Thrumurthy, S. G., Thrumurthy, S. S. D., Gilbert, C. E., Ross, P., & Haji, A. (2016). Colorectal adenocarcinoma: Risks, prevention and diagnosis. *BMJ (Online)*, *354*.

Tiberghien, F., & Loor, F. (1996). Ranking of P-glycoprotein substrates and inhibitors by a calcein-AM fluorometry screening assay. *Anti-Cancer Drugs*, *7*(5), 568–578.

Timmer, M., Werner, J.-M., Röhn, G., Ortmann, M., Blau, T., Cramer, C., … Goldbrunner, R. (2017). Discordance and Conversion Rates of Progesterone-, Estrogen-, and HER2/neu-Receptor Status in Primary Breast Cancer and Brain Metastasis Mainly Triggered by Hormone Therapy. *Anticancer Research*, *37*(9), 4859–4865.

Trachootham, D., Alexandre, J., & Huang, P. (2009). Targeting cancer cells by ROS-mediated mechanisms: A radical therapeutic approach? *Nature Reviews Drug Discovery*, *8*(7), 579–591.

Triulzi, T., Regondi, V., De Cecco, L., Cappelletti, M. R., Di Modica, M., Paolini, B., … Tagliabue, E. (2018). Early immune modulation by single-agent trastuzumab as a marker of trastuzumab benefit. *British Journal of Cancer*, *119*(12), 1487–1494.

Truong, D. H., Nguyen, D. H., Ta, N. T. A., Bui, A. V., Do, T. H., & Nguyen, H. C. (2019). Evaluation of the use of different solvents for phytochemical constituents, antioxidants, and in vitro anti-inflammatory activities of severinia buxifolia. *Journal of Food Quality*, *2019*, 1-9.

Tsukamoto, M., Sato, S., Satake, K., Miyake, M., & Nakagawa, H. (2017). Quantitative evaluation of drug resistance profile of cells expressing wild-type or genetic polymorphic variants of the human ABC transporter ABCC4. *International Journal of Molecular Sciences*, *18*(7), 1435.

Tsukamoto, M., Yamashita, M., Nishi, T., & Nakagawa, H. (2019). A Human ABC Transporter ABCC4 Gene SNP Reduces ABCC4-Dependent Drug Resistance. *Cells*, *8*(1), 39.

Tubbs, A., & Nussenzweig, A. (2017). Endogenous DNA Damage as a Source of Genomic Instability in Cancer. *Cell*, *168*(4), 644–656.

Ullah, M. F. (2008). Cancer Multidrug Resistance: A Major Impediment to Effective Chemotherapy. *Asian Pacific Journal of Cancer Prevention*, *9*, 1–6.

Ullah, S., Rashid Khan, M., Ali Shah, N., Afzal Shah, S., Majid, M., & Asad Farooq, M. (2014). Ethnomedicinal plant use value in the Lakki Marwat District of Pakistan. *Journal of Ethnopharmacology*, *158*, 412–422.

Usta, J., Kreydiyyeh, S., Knio, K., Barnabe, P., Bou-Moughlabay, Y., & Dagher, S. (2009). Linalool decreases HepG2 viability by inhibiting mitochondrial complexes I and II, increasing reactive oxygen species and decreasing ATP and GSH levels. *Chemico-Biological Interactions*, *180*(1), 39–46.

Van Blarigan, E. L., Fuchs, C. S., Niedzwiecki, D., Zhang, S., Saltz, L. B., Mayer, R. J., … Meyerhardt, J. A. (2018). Association of survival with adherence to the American cancer society nutrition and physical activity guidelines for cancer survivors after colon cancer diagnosis the calgb 89803/alliance trial. *JAMA Oncology*, *4*(6), 783–790.

van der Deen, M., de Vries, E. G. E., Timens, W., Scheper, R. J., Timmer-Bosscha, H., & Postma, D. S. (2005). ATP-binding cassette (ABC) transporters in normal and pathological lung. *Respiratory Research*, *6*(5), 1–16.

Van Der Jeught, K., Xu, H. C., Li, Y. J., Lu, X. Bin, & Ji, G. (2018). Drug resistance and new therapies in colorectal cancer. *World Journal of Gastroenterology*, *24*(34), 3834–3848

Van Middendorp, J. J., Sanchez, G. M., & Burridge, A. L. (2010). The Edwin Smith papyrus: A clinical reappraisal of the oldest known document on spinal injuries. *European Spine Journal*, *19*(11), 1815–1823.

Van Noorden, C. J. F. (2001). Editorial: The history of Z-VAD-FMK, a tool for understanding the significance of caspase inhibition. *Acta Histochemica*, *103*(3), 241–251.

Van Tonder, A., Joubert, A. M., & Cromarty, A. D. (2015). Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. *BMC Research Notes*, *8*(1), 47.

Van Vulpen, J. K., Velthuis, M. J., Bisschop, C. N. S., Travier, N., Van Den Buijs, B. J. W., Backx, F. J. G., … May, A. M. (2016). Effects of an exercise program in colon cancer patients undergoing chemotherapy. *Medicine and Science in Sports and Exercise*, *48*(5), 767–775.

Vasil, I., & Constabel, F. (1989). *Phytochemicals in Plant Cell Cultures* (Vol. 5). San Diego: Academic Press Inc.

Vasiliou, V., Vasiliou, K., & Nebert, D. W. (2009). Human ATP-binding cassette (ABC) transporter family. *Human Genomics*, *3*(3), 281–290.

Waheed, A., Barker, J., Barton, S. J., Owen, C. P., Ahmed, S., & Carew, M. A. (2012). A novel steroidal saponin glycoside from Fagonia indica induces cell-selective apoptosis or necrosis in cancer cells. *European Journal of Pharmaceutical Sciences*, *47*(2), 464–473.

Wakeel, A., Jan, S. A., Ullah, I., Shinwari, Z. K., & Xu, M. (2019). Solvent polarity mediates phytochemical yield and antioxidant capacity of Isatis tinctoria. *PeerJ*, *7*(10), e7857

Walton, E. L. (2016). The dual role of ROS, antioxidants and autophagy in cancer. *Biomedical Journal*, *39*(2), 89–92.

Wang, J., Li, J., Cao, N., Li, Z., Han, J., & Li, L. (2018). Resveratrol, an activator of SIRT1, induces protective autophagy in non-small-cell lung cancer via inhibiting Akt/mTOR and activating p38-MAPK. *OncoTargets and Therapy*, *11*, 7777–7786.

Wang, L., & Bernards, R. (2018). Taking advantage of drug resistance, a new approach in the war on cancer. *Medical Frontiers*, *12*(4), 490–495.

Wang, R. C., Chen, X., Parissenti, A. M., Joy, A. A., Tuszynski, J., Brindley, D. N., & Wang, Z. (2017). Sensitivity of docetaxel-resistant MCF-7 breast cancer cells to microtubule-destabilizing agents including vinca alkaloids and colchicine-site binding agents. *PLoS ONE*, *12*(8), e0182400/

Wang, X., Zhang, F., Yang, L., Mei, Y., Long, H., Zhang, X., … Su, X. (2011). Ursolic acid inhibits proliferation and induces apoptosis of cancer cells in vitro and in vivo. *Journal of Biomedicine & Biotechnology*, *2011*, 419343.

Wang, X., Zhang, H., & Chen, X. (2019). Drug resistance and combating drug resistance in cancer. *Cancer Drug Resistance*, *2*(2), 141–160.

Wang, W., Kandimalla, R., Huang, H., Zhu, L., Li, Y., Gao, F., … Wang, X. (2019). Molecular subtyping of colorectal cancer: Recent progress, new challenges and emerging opportunities. *Seminars in Cancer Biology*, 55, 37–52.

Wang, W., Zhao, C., Jou, D., Lü, J., Zhang, C., Lin, L., & Lin, J. (2013). Ursolic acid inhibits the growth of colon cancer-initiating cells by targeting STAT3. *Anticancer Research*, *33*(10), 4279–4284.

Wang, Y., Gamage, J., & Zhang, Z. (2011). Separation of taxanes from Taxus canadensis using dynamic pressurized liquid extraction. *Biotechnology and Bioprocess Engineering*, *16*(4), 769–776.

Wang, Y. K., Zhu, Y. L., Qiu, F. M., Zhang, T., Chen, Z. G., Zheng, S., & Huang, J. (2010). Activation of Akt and MAPK pathways enhances the tumorigenicity of CD133+ primary colon cancer cells. *Carcinogenesis*, *31*(8), 1376–1380.

Waters, J. C., Chen, R. H., Murray, A. W., & Salmon, E. D. (1998). Localization of Mad2 to kinetochores depends on microtubule attachment, not tension. *Journal of Cell Biology*, *141*(5), 1181–1191.

Weaver, B. A. (2014). How Taxol/paclitaxel kills cancer cells. *Molecular Biology of the Cell*, *25*(8), 2677–2681.

Weber, C. C., Kressmann, S., Fricker, G., & Müller, W. E. (2004). Modulation of P-glycoprotein function by St John's Wort extract and its major constituents. *Pharmacopsychiatry*, *37*(6), 292–298.

Weir, N. M., Selvendiran, K., Kutala, V. K., Tong, L., Vishwanath, S., Rajaram, M., … Periannan, &. (2007). Curcumin induces G2/M arrest and apoptosis in cisplatin-resistant human ovarian cancer cells by modulating akt and p38 mAPK. *Cancer Biology & Therapy, 6*(2), 178-84.

Weis, S. M., & Cheresh, D. A. (2011). Tumor angiogenesis: Molecular pathways and therapeutic targets. *Nature Medicine*, *17*(11), 1359–1370.

Wen, W., Lu, J., Zhang, K., & Chen, S. (2008). Grape Seed Extract Inhibits Angiogenesis via Suppression of the Vascular Endothelial Growth Factor Receptor Signaling Pathway. *Cancer Prevention Research, 1*(7), 554-61.

Weyermann, J., Lochmann, D., & Zimmer, A. (2005). A practical note on the use of cytotoxicity assays. *International Journal of Pharmaceutics*, *288*(2), 369–376.

WHO. (2002). The world health report 2002: reducing risks, promoting healthy life. *WorldHealth Organisation, Geneva, Switzerland (2002)*, 170-172. Retrieved from WHO.

Wilkens, S. (2015). Structure and mechanism of ABC transporters. *F1000Prime Reports*, *7*, 14.

Wink, M., Ashour, M. L., & El-Readi, M. Z. (2012). Secondary metabolites from plants inhibiting ABC transporters and reversing resistance of cancer cells and microbes to cytotoxic and antimicrobial agents. *Frontiers in Microbiology*, *3*, 130.

Wolff, A. C., Elizabeth Hale Hammond, M., Allison, K. H., Harvey, B. E., Mangu, P. B., Bartlett, J. M., … Jude, S. (2018). Human Epidermal Growth Factor Receptor 2 in Breast Cancer. *Arch Pathol Lab Med*, *142*, 1364–1382.

Wood, C. D., Thornton, T. M., Sabio, G., Davis, R. A., & Rincon, M. (2009). Nuclear localization of p38 MAPK in response to DNA damage. *International Journal of Biological Sciences*, *5*(5), 428–437.

Wu, C. P., Calcagno, A. M., Hladky, S. B., Ambudkar, S. V., & Barrand, M. A. (2005). Modulatory effects of plant phenols on human multidrug-resistance proteins 1, 4 and 5 (ABCC1, 4 and 5). *FEBS Journal*, *272*(18), 4725–4740.

Wu, Q., Deng, J., Fan, D., Duan, Z., Zhu, C., Fu, R., & Wang, S. (2018). Ginsenoside Rh4 induces apoptosis and autophagic cell death through activation of the ROS/JNK/p53 pathway in colorectal cancer cells. *Biochemical Pharmacology*, *148*, 64–74.

Xia, C., Meng, Q., Liu, L. Z., Rojanasakul, Y., Wang, X. R., & Jiang, B. H. (2007). Reactive oxygen species regulate angiogenesis and tumor growth through vascular endothelial growth factor. *Cancer Research*, *67*(22), 10823–10830.

Xia, M., Knezevic, D., & Vassilev, L. T. (2011). P21 does not protect cancer cells from apoptosis induced by nongenotoxic p53 activation. *Oncogene*, *30*(3), 346–355.

Xie, Y. H., Chen, Y. X., & Fang, J. Y. (2020). Comprehensive review of targeted therapy for colorectal cancer. *Signal Transduction and Targeted Therapy*, *5*(1), 22.

Xie, Z.-Y., Lv, K., Xiong, Y., & Guo, W.-H. (2014). ABCG2-Meditated Multidrug Resistance and Tumor-Initiating Capacity of Side Population Cells from Colon Cancer. *Oncology Research and Treatment*, *37*(11), 666–672.

Xu, J., Zhang, S., Wang, R., Wu, X., Zeng, L., & Fu, Z. (2017). Knockdown of PRDX2 sensitizes colon cancer cells to 5-FU by suppressing the PI3K/AKT signaling pathway. *Bioscience Reports*, *37*(3), BSR20160447.

Xu, Y., Xu, C., & Price, B. D. (2012). Mechanistic links between ATM and histone methylation codes during DNA repair. *Progress in Molecular Biology and Translational Science*, *110*, 263-88.

Yan, X. J., Gong, L. H., Zheng, F. Y., Cheng, K. J., Chen, Z. S., & Shi, Z. (2014). Triterpenoids as reversal agents for anticancer drug resistance treatment. *Drug Discovery Today*, *19*(4), 482–488.

Yang, L., Garcia Canaveras, J. C., Chen, Z., Wang, L., Liang, L., Jang, C., … Rabinowitz, J. D. (2020). Serine Catabolism Feeds NADH when Respiration Is Impaired. *Cell Metabolism*, *31*(4), 809-821.e6.

Yang, S. Y., Miah, A., Sales, K. M., Fuller, B., Seifalian, A. M., & Winslet, M. (2011). Inhibition of the p38 MAPK pathway sensitises human colon cancer cells to 5-fluorouracil treatment. *International Journal of Oncology*, *38*(6), 1695–1702.

Yeh, C. C., Shih, L. J., Chang, J. L., Tsuei, Y. W., Wu, C. C., Hsiao, C. W., … Kao, Y. H. (2019). Synchronous vascular endothelial growth factor protein profiles in both tissue and serum identify metastasis and poor survival in colorectal cancer. *Scientific Reports*, *9*(1), 1–12.

Yersal, O., & Barutca, S. (2014). Biological subtypes of breast cancer: Prognostic and therapeutic implications. *World Journal of Clinical Oncology*, *5*(3), 412–424.

Yin, M. C., Lin, M. C., Mong, M. C., & Lin, C. Y. (2012). Bioavailability, distribution, and antioxidative effects of selected triterpenes in mice. *Journal of Agricultural and Food Chemistry*, *60*(31), 7697–7701.

Yin, M., Ren, X., Zhang, X., Luo, Y., Wang, G., Huang, K., … Zhang, B. (2015). Selective killing of lung cancer cells by miRNA-506 molecule through inhibiting NF-κB p65 to evoke reactive oxygen species generation and p53 activation. *Oncogene*, *34*(6), 691–703.

Yoon, C. H., Kim, M. J., Park, M. T., Byun, J. Y., Choi, Y. H., Yoo, H. S., … Lee, S. J. (2009). Activation of p38 Mitogen-Activated protein kinase is required for death receptor-independent caspase-8 activation and cell death in response to sphingosine. *Molecular Cancer Research*, *7*(3), 361–370.

Yu, Jiachuan, Luo, Y., & Wen, Q. (2019). Nalbuphine suppresses breast cancer stem-like properties and epithelial-mesenchymal transition via the AKT-NFκB signaling pathway. *Journal of Experimental & Clinical Cancer Research*, *38*(1), 197.

Yu, Jie, Ge, J., Heuveling, J., Schneider, E., & Yang, M. (2015). Structural basis for substrate specificity of an amino acid ABC transporter. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(16), 5243–5248.

Zaborsky, N., Gassner, F. J., Hopner, J. P., Schubert, M., Hebenstreit, D., Stark, R., . . . Egle, A. (2018). Exome sequencing of the TCL1 mouse model for CLL reveals genetic heterogeneity and dynamics during disease development. *Leukemia*, *33*(4), 957-68.

Zaidieh, T., Smith, J. R., Ball, K. E., & An, Q. (2019). ROS as a novel indicator to predict anticancer drug efficacy. *BMC Cancer*, *19*(1), 1224.

Zhan, S., Paik, A., Onyeabor, F., Ding, B., Prabhu, S., & Wang, J. (2020). Oral bioavailability evaluation of celastrol-encapsulated silk fibroin nanoparticles using an optimized LC-MS/MS method. *Molecules*, *25*(15), 3422.

Zhang, Q. W., Lin, L. G., & Ye, W. C. (2018). Techniques for extraction and isolation of natural products: A comprehensive review. *Chinese Medicine (United Kingdom)*, *13*, 20.

Zhang, X., Wang, X., Wu, T., Li, B., Liu, T., Wang, R., … Shao, C. (2015). Isoliensinine induces apoptosis in triple-negative human breast cancer cells through ROS generation and p38 MAPK/JNK activation. *Scientific Reports*, *5*(1), 1–13.

Zhang, S., Yang, X., & Morris, M. E. (2004). Flavonoids are inhibitors of breast cancer resistance protein (ABCG2)-mediated transport. *Molecular Pharmacology*, *65*(5), 1208–1216.

Zhang, Y. H., Wu, Q., Xiao, X. Y., Li, D. W., & Wang, X. P. (2010). Silencing MRP4 by small interfering RNA reverses acquired DDP resistance of gastric cancer cell. *Cancer Letters*, *291*(1), 76–82.

Zhao, S., Wang, L., Liu, L., Liang, Y., Sun, Y., & Wu, J. (2014). Both the mevalonate and the non-mevalonate pathways are involved in ginsenoside biosynthesis. *Plant Cell Reports*, *33*(3), 393–400.

Zhu, Z., Qian, Z., Yan, Z., Zhao, C., Wang, H., & Ying, G. (2013). A phase I pharmacokinetic study of ursolic acid nanoliposomes in healthy volunteers and patients with advanced solid tumors. *International Journal of Nanomedicine*, *8*, 129–136.