Determined the cytotoxic properties and mechanisms against drug resistance of an aqueous extract of *Fagonia indica* on colon cancer cells

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**Abstract:** *Fagonia indica* is a perennial plant grown in arid climates and utilised in traditional medicine as a treatment for cancer. Previous research has highlighted potential antineoplastic effects of *Fagonia indica* against breast cancer cell lines. Despite this, there has been little research demonstrating the potential of an aqueous extract of *Fagonia indica* against colon cancer. Colon cancer is a leading cause of cancer-related mortality, with drug resistance remaining a barrier to treatment. The aim of the present study was to investigate the cytotoxic mechanisms of *Fagonia indica* in colon cancer cells, including pathways related to proliferation, angiogenesis and inflammation and ABC membrane transporters. Treatment with *Fagonia indica* caused a reduction in wild-type and chemotherapy resistant colon cancer. Results indicated a role for Akt/ MAPK signalling as a mechanism of *Fagonia indica* induced cell death, alongside a reduction in the expression of VEG-F, NK-κB and ICAM-1. RT-PCR demonstrated a reduction in expression of ABCG2/ABCC4 in resistant colon cancers. Further research is required to isolate bioactive compounds of *Fagonia indica* for use against colon cancer.

**Keywords:** *Fagonia indica*, colon cancer, proliferation, angiogenesis, ABCC4, ABCG2

1. Introduction

Colon cancer is one of the most frequently diagnosed cancers and a leading cause of cancer related mortality in both males and females [1-2]. Although survival rates of colon cancer are increasing in many high-income countries, there has been an increase in incidence among a younger population of 1.4% per year [3]. Previous studies have identified several potential risk factors for colon cancer including, BMI, Westernised diets, abnormal bowel frequency and aspirin use [4-6]. Long-term inflammatory bowel diseases; such as Crohn’s and ulcerative colitis, are also a strong risk factor for bowel cancers [7]. Colon cancer is becoming increasingly prevalent in low-income countries with rapidly Westernising lifestyles, despite significant improvements in screening programmes and treatment options [8].
Current therapeutic interventions for colon cancer include a surgical resection and combination chemotherapy, including 5-fluorouracil (5-FU) and oxaliplatin [9]. Alarming drug resistance to single chemotherapy agents occurs almost universally, with resistance developing as a selection pressure via DNA mutation and metabolic alterations [10-12]. The ATP-binding cassette transporters (ABCA-ABCG) are a superfamily of efflux proteins, which transport structurally diverse substances across the cell membrane, against the concentration gradient [13-14]. The ability of transmembrane proteins to mediate transport of chemotherapeutic compounds and their metabolites has since been linked to aberrant expression of one, or many of these ABC transporters in multidrug resistant cancers (MDR) [15-17]. Studies have shown that exposure with low-dose chemotherapeutic agents for 10 days was sufficient to upregulate AGCG2 in relation to multidrug resistance phenotypes in colon cancer [18-19]. In combination therapies for colon cancer, additional compounds can confer resistance to 5-FU and oxaliplatin via the upregulation ABC transporters including ABCC4 [20]. Inhibiting the expression of ABC transporters in cell lines expressing resistance, can result in a reversal of the resistant phenotype. For example, inhibition of ABCC4 in acute myeloid leukaemia enhanced the accumulation of intracellular drug concentration by 150% [17]. Inhibiting ABCC4 in colon cancer cell lines resulted in a significant increase in intracellular accumulation and sensitivity of treatment with 5-FU [21]. Therefore, inhibition or downregulation of ABC transporters is vital in reversing multidrug resistance, enhance accumulation of intracellular drug concentration and reduce the likelihood of metastasis [21-22].

The vascular endothelial growth factor (VEGF) family have an increasingly established role as angiogenic activators and propagators of inflammation in colon cancer. 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) [23]. Overexpression of VEGF-A has been identified in several studies of human colon cancer and is correlated with an increased risk of invasion and metastasis. VEGF has been shown to directly regulate the expression of ICAM-1 (intracellular adhesion-molecule 1) via the phosphatidylinositol 3 OH-kinase (PI3K)/AKT pathway in brain microvascular endothelial cells [24]. Aberrant expression of both VEGF and ICAM-1 has been used to predict clinical outcome of patients with metastatic colon cancer, with a positive correlation between protein expression of VEGF and ICAM-1 in tissue from biopsied colon cancers compared with healthy controls [25]. The tendency of this relationship was significantly higher in tumors of increased size or with metastasis [26]. There has also been a strong correlation between VEGF-associated angiogenesis and the propagation of inflammatory states. Studies have demonstrated the capacity of VEGF to induce a pro-inflammatory phenotype via the induced expression of ICAM-1 and VCAM-1 in inflamed bowel disease (IBD) [27]. 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.

Fagonia indica is a small, spiny undershrub belonging to the family Zygophyllaceae, found largely in warm arid regions of most continents [28-30]. Indigenous use of Fagonia indica varies greatly between individual communities [31-36]. Typically, the aerial section of the shade-dried plant is ground into a powder and administered orally as an aqueous tea [33]. Treatment of experimentally produced subcutaneous tumors in albino rats with Fagonia indica extract, resulted in a significant increase in overall survival of both male and females [37]. Cytotoxic
properties have also been demonstrated in in vitro studies on breast (MCF-7) and colon (HCT) cancer cell lines [38]. Further research found that MCF-7 and MDA-MB-231 cells were more susceptible to cell cycle modulation, double strand DNA damage and activation of p53, p21 and downstream signaling targets upon treatment with Fagonia indica [39]. Interestingly, loss of cell viability was also attributed; in part, to p53 independent FOXO3a activation. FOXO3 has numerous modes of action, including regulation of apoptosis, DNA damage responses and cell cycle arrest [40]. Despite this, there is little understanding regarding the mechanism of Fagonia indica extract induced apoptosis in colon cancer. In the current study, the effect of Fagonia indica is determined on two p53 positive colon cancer cell lines, in order to expand on the pathways described in previous work [39]. There has been no prior research regarding the effect of Fagonia indica extract on pathways related to tumour growth and propagation, such as angiogenesis and inflammation. This study investigated the potential for Fagonia indica extract to inhibit the growth of colon cancer cells in vitro and inhibit pathways related to tumour growth and apoptosis. For the first time, the effect of Fagonia indica against chemotherapy resistant colon cancer cell lines was investigated, with a particular interest in the relationship between extract treatment and ABC transporters, ABCC4 and ABCG2.

2. Results

2.1 An aqueous extract of Fagonia indica decreases cell viability and intracellular ATP in colon cancer cells.

In order to determine whether an aqueous extract of Fagonia indica was cytotoxic against in vitro colon cancer cells; RKO and H630, cell viability and intracellular ATP levels were determined as shown in Figure 1. Extract treatment in the concentration range 0-2.5 mg/mL over 72 hours induced a significant time- and concentration-dependent reduction in cell viability (Figure 1A-B). After 72 hours, 2.5 mg/mL of extract treatment caused a 62% and 64% reduction in cell viability in RKO and H630 cell lines, respectively. Altered metabolism is a key feature of tumorigenesis, and high intracellular ATP levels are synonymous with tumour development. Intracellular ATP levels were measured in in vitro colon cancer cell lines (Figure 1C-D). Extract treatment at 1 mg/mL over 24 hours induced a significant reduction in both RKO and H630 intracellular ATP levels by 70.3% and 68.3%, respectively.
Figure 1. *Fagonia indica* extract treatment reduces *in vitro* cell viability and intracellular ATP 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, as a positive control for 24-72 hours. Cell viability was determined as a percentage of an untreated DMSO vehicle control using the NR assay. (C) H630 and (D) RKO 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 assay. ATP concentration was determined as a percentage of an untreated control. Data denoted * (p<0.05), ** (p<0.01), *** (p<0.001) and # (p<0.0001) were significantly different compared to the untreated control analysed by one-way (C-D) and two-way (A-B) 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.
2.2 Fagonia indica treatment reduces proliferation and migration of colon cancer cells.

Having established that an aqueous extract of *Fagonia indica* was able to significantly reduce cell viability, the effect of the extract on the ability of cells to proliferate and migrate was investigated. A colony formation assay was used to determine the ability of cells to reproduce from a single cell. Pre-treatment of H630 and RKO cells with *Fagonia indica* for 24 hours caused a significant reduction in colony formation from a single cell after 7 days (Figure 2). In H630 cells, pre-treatment with *Fagonia indica* for 24 hours, reduced colony formation after 7 days by 29.6% (Figure 2B). For RKO cells there was a significant reduction in colony formation by 29.5% (Figure 2D). Alongside increased proliferation, enhanced migration is a phenotype of cancer and is a key indicator of potential for metastasis. The scratch wound assay is a well-developed method to analyse cell migration *in vitro*. Treatment with *Fagonia indica* extract (1 mg/mL) did not significantly reduce wound closure of either H630 (Figure 3A-B) or RKO (Figure 3C-D) cells after 24 hours.

![Figure 2: Fagonia indica treatment inhibits proliferation of colon cancer cell lines.](image-url)

(A) H630 (B) RKO (C) H630 (D) RKO

Relative colony formation

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**Figure 2:** *Fagonia indica* treatment inhibits proliferation of colon cancer cell lines. (A-B) H630 cells were seeded at a density of 200 cells per well and (C-D) RKO cells were seeded at 400 cells per well in a 24 well plate, after pre-treatment
with 1.5 mg/mL aqueous *Fagonia indica* or 1 μM Gemcitabine, as a positive control for 24 hours. After 1 week, colonies of >50 cells were stained using 0.01% crystal violet stain and counted using an EVOS microscope (A, C). (B, D) 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.

| Control | 1 mg/mL Fagonia indica |
|---------|-------------------------|
| 0 hr    |                         |
| 24 hr   |                         |

Figure 3: *Fagonia indica* treatment inhibits migration of colon cancer cell lines. (A) H630 and (C) 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. Wound closure was determined using ImageJ software and is displayed as percentage wound closure after 24 hours for H630 (B) and RKO cell lines (D). 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.

2.3 *Fagonia indica* effects p38/MAPK signalling pathways and reduces expression of VEGF-A, NF-κB and ICAM-1

P38 mitogen activated protein kinase (MAPK) signalling is activated upon stress-stimuli from the environment and is associated with inhibition of cell cycle progression and induction of apoptosis. SB203580 is a selective inhibitor of p38 MAPK and was used to inhibit *Fagonia indica*
activation of p38 signalling (1.5 mg/mL). Inhibition of p38 significantly alleviated loss in cell viability in H630 cells by 31.6% (Figure 4A) and by 31.4% in RKO cells (Figure 4B). When activated, p38 indirectly inhibits oncogene Akt. Upon activation, Akt acts as a mediator for the P13K/Akt kinase signalling pathway, leading to increased cellular metabolism and proliferation. Activating Akt signalling pathways with SC79, significantly alleviated the loss in cell viability in H630 cells by 35% (Figure 4C), this was much lower in RKO cells at 15.3% (Figure 4D).

Figure 4: Effects of p38 MAP kinase inhibition and AKT activation on Fagonia indica induced colon cancer cytotoxicity. (A) H630 cells were treated with 1.5 mg/mL of aqueous extract and 10 μM of SC79 Akt activator, or (C) 10 μM of p38 MAPK inhibitor SB203580 for 48 hours. (B) RKO cells were treated with 1.5 mg/mL of aqueous extract and 10 μM of SC79 Akt activator, or (D) 10 μM of p38 MAPK inhibitor SB203580 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) were statistically significant compared to the untreated DMSO vehicle control, analysed using one-way ANOVA with Sidaks’s multiple comparison test. Data
Angiogenesis and inflammation are key hallmarks of cancer, and both are processes which have important roles in the development, initiation and advancement of cancer. Expression of vascular endothelial growth factor receptor A (VEGF-A) mRNA was measured in RKO and H630 colon cancer cell lines using RT-PCR (Figure 5). After 6 hours of treatment with *Fagonia indica* (1 mg/mL) relative gene expression of VEGF-A was significantly reduced in H630 cells to 0.26, and in RKO cells to 0.51 (Figure 5A-B). After 24 hours, expression of VEGF-A stabilised to that of the control. Apatanib is a selective tyrosine kinase inhibitor of VEGF-R2. Inhibition of VEGF-R2 significantly alleviated the loss in *Fagonia indica* induced cellular viability by 8.1% and 19.6% in H630 and RKO cells, respectively (Figure 6A, C). NF-κB is a major transcription factor that upregulates several genes associated with inflammation and immune responses. Expression of NFκβ subunit; p65, was measured in both H630 and RKO colon cancer cells lines treated with *Fagonia indica* (1 mg/mL) at 6 and 24 hours via RT-PCR (Figure 5). Extract treatment significantly reduced relative gene expression in both H630 and RKO cells to 0.06 and 0.47, respectively (Figure 5C-D). Expression of p65 remained significantly reduced after 24 hours. Caffeic acid phenethyl ester (CAPE) is a specific inhibitor of the nuclear transcription factor NF-κB. Inhibition of NF-κB did not significantly affect cytotoxicity of *Fagonia indica* in RKO and H630 colon cancer cells (Figure 6B, D). Finally, expression of ICAM-1 was measured using RT-PCR. Relative gene expression was significantly reduced after 6 hours of treatment with *Fagonia indica* to 0.18 and 0.42 in H630 and RKO cells, respectively (Figure 6E-F). After 24 hours expression of ICAM-1 stabilised to that of the control in H630 cells, whereas expression dropped further to 0.19 in RKO cells.

**Figure 5:** *Fagonia indica* extract treatment reduces expression of VEGF, NFκβ and ICAM-1 in colon cancer cell lines. Gene expression of VEGF-A was determined on (A) H630 and (B) RKO colon cancer cells. Gene expression of NFκβ was determined on (C) H630 and (D) RKO colon cancer cells. Gene expression of ICAM-1 was determined on (E) H630.
and (F) RKO colon cancer cells. Pellets were collected and mRNA extracted after 6 and 24 hours of treatment with 1mg/mL *Fagonia indica*. Values were normalised to housekeeping genes actin and YHWAZ and displayed as relative gene expression to an untreated 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 Dunnett’s multiple comparison test. Data denoted as NS were not significant (p>0.05) All data is representative of at least three independent experiments performed in triplicate and presented as mean ± SEM.

![Bar charts](Figure 6: Effects of VEGF-A and NF-κB inhibition and on *Fagonia indica* induced colon cancer cytotoxicity. (A) H630 and (C) RKO colon cancer cells were treated with 1.5 mg/mL of aqueous extract and 1 μM of VEGF-A inhibitor Apatanib for 48 hours. (B) H630 and (D) RKO colon cancer cells were treated with 1.5 mg/mL of aqueous extract and 1 μM of NF-κB inhibitor CAPE 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) were statistically significant compared to the untreated DMSO vehicle control, analysed using one-way ANOVA with Sidaks’s multiple comparison test. Data denoted as NS was not significant. All data points are representative of three independent experiments, performed in triplicate and are expressed as mean ± SEM.)
2.4 Fagonia indica reduces cell viability in chemotherapy resistant colon cancers

Resistance to single chemotherapeutic agents occurs almost universally and drug tolerance in cancer is widely documented. Resistance of H630 and RKO cell lines was established in supplementary figure 1. *Fagonia indica* induced a time and concentration-dependent reduction in cell viability by 57.7% and 63.2% in tomudex resistant RKO (RKO-TDX) and gemcitabine resistant H630 (H630-GM) cell lines after 2.5 mg/mL of treatment for 72 hours. (Figure 7A-B) This was much lower in 5-fluorouracil resistant H630 (H630-5FU) cells, in which there was only a 34.3% reduction in cell viability after 72 hours (Figure 7C). To determine the ability of *Fagonia indica* to re-sensitize resistant cell lines to chemotherapy, H630 and RKO resistant cell lines were pre-treated with *Fagonia indica*, reduced tomudex induced cell viability cells by 27.8% (Figure 7D). Similarly, pre-treatment of H630-GM cells reduced cell viability in gemcitabine treated cells by 33.7% (Figure 7E).

![Figure 7](https://example.com/figure7.png)

**Figure 7**: *Fagonia indica* extract treatment reduces *in vitro* cell viability of gemcitabine, 5-fluorouracil and tomudex resistant colon cancer cell lines. (A) Tomudex resistant (2μM) RKO, (B) gemcitabine resistant (1μM) H630 (B) and 5-
fluorouracil (10 μM) resistant H630 cells were treated with 0-2.5 mg/mL *Fagonia indica* extract for 24-72 hours. Cell viability was determined as a percentage of an untreated DMSO vehicle control using MTT assay. (D) Tomudex resistant RKO and (E) gemcitabine resistant H630 cells were pre-treated with 1 mg/mL aqueous *Fagonia indica* extract for 24 hours prior to treatment with gemcitabine/ 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), ** (p<0.01), *** (p<0.001) and # (p<0.0001) were significant compared to the untreated control analysed by one-way (D-E) and (A-C) 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.

2.5 *Fagonia indica* reduces expression of ABC transporters in chemotherapy resistant colon cancers.

As overexpression of ABC transporters has been implicated in the acquisition of resistance phenotypes in several cancer types, therapeutic inhibitors of ABC transporters have become of interest as a potential mechanism to reverse multidrug resistance. In the current study difference in mRNA expression of ABCC4 and ABCG2 genes were established between wild-type and chemotherapy resistant clones of H630 and RKO colon cancers (Figure 8). In H630 cells, both ABCG2 and ABCC4 were over-expressed in H630-GM resistant cells by 5.9 and 7.7 times of the H630-WT control (Figure 8A, E). ABCG2 and ABCC4 were also significantly overexpressed in H630-5FU resistant cells, by 5.7 and 12.4 times of the control (Figure 8E, G). Having established overexpression of ABCG2 and ABCC4 in H630-GM resistant cells, expression of resistant cell lines post treatment with *Fagonia indica* was determined. After 6 hours of *Fagonia indica* treatment, mRNA expression of ABCG2 and ABCC4 was significantly reduced to 0.32 and 0.24, respectively. This reduction was maintained after 24 hours of treatment (Figure 8B, D). Similarly, *Fagonia indica* reduced expression of AGCG2 and ABCC4 in H630-5FU resistant cells to 0.24 and 0.18 of the untreated control after 6 hours. This reduction was maintained after 24 hours of treatment.

![Figure 8: *Fagonia indica* extract treatment reduces expression of ABCC4 and ABCG2 in chemotherapy resistant H630 colon cancer.](image)

Gene expression of ABCG2 was determined on (A) wild-type H630 and gemcitabine resistant H630 clones, (C) wild-type H630 and 5-fluorouracil resistant H630 clones to determine resistance mechanisms. Gene expression of ABCG2 was also determined after treatment with 1mg/mL *Fagonia indica* for 6 and 24 hours, in gemcitabine resistant (B) and 5-fluorouracil resistant (D) H630 clones. Gene expression of ABCC4 was also determined on (E) wild-type H630 and gemcitabine resistant H630 clones, (G) wild-type H630 and 5-fluorouracil resistant H630 clones to determine resistance mechanisms. Gene expression of ABCC4 was also determined after treatment with 1mg/mL *Fagonia indica* for 6 and 24 hours, in gemcitabine resistant (F) and 5-fluorouracil resistant (H) H630 clones.
Changes in mRNA expression in ABCG2 and ABCC4 between RKO-WT and RKO-TDX resistant cells was established. Interestingly, expression of ABCG2 was not significantly affected in RKO-TDX cells, whereas ABCC4 expression was significantly increased to 3.85 times of the control (Figure 9A, C). After treatment with *Fagonia indica* for 24 hours, expression of ABCG2 was significantly increased in RKO-TDX cells to 3.31 times the untreated control (Figure 9B), whereas expression of ABCC4 was reduced at both 6- and 24-hours post treatment (Figure 9D).

**Figure 9:** *Fagonia indica* extract treatment reduces expression of ABCC4 and ABCG2 in chemotherapy resistant RKO colon cancer. Gene expression of ABCG2 was determined on (A) wild-type RKO and tomudex resistant RKO clones. Gene expression of ABCG2 was also determined after treatment with 1mg/mL *Fagonia indica* for 6 and 24 hours, in tomudex resistant RKO clones H630 (B). Gene expression of ABCC4 was determined on (C) wild-type RKO and tomudex resistant RKO clones. Gene expression of ABCC4 was also determined after treatment with 1mg/mL *Fagonia indica* for 6 and 24 hours, in tomudex resistant RKO clones H630 (D). 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), ** (p<0.01) and *** (p<0.001) were significant compared to a 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.
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. Discussion

Fagonia indica has been utilised for decades in folk medicine as a purgative and topical treatment for cancer [38]. Previous research determined that an aqueous extract of Fagonia indica was able to induce apoptosis in breast cancer cell lines, MCF7 and MDA-MB-231 [39]. In the current study, an aqueous extract of Fagonia indica displayed significant cytotoxic activity against both RKO and H630 colon cancer cell lines. No previous study has examined the effect of an aqueous extract of Fagonia indica against colon cancer cell lines. Other research has demonstrated that ethyl-acetate and butanol extractions of Fagonia indica were able to cause significant reductions in cell viability in HCT colon cancer cell line [35]. Previously, isolated triterpenes; Indicacin and Fagonici, were extracted from an ethanolic extract and displayed up to 51.4% growth inhibition in H-29 colon cancer cells [38]. These findings are supplemented by the identification of a novel steroidal saponin glycoside from Fagonia indica which was cytotoxic against both breast and colon cancer cell lines [41]. Measuring intracellular ATP provides a reliable depiction of cellular metabolism, which also correlates to cellular viability [42]. In the current study, Fagonia indica treatment significantly reduced metabolic production of ATP. We also investigated the effect of Fagonia indica on the proliferation, clonogenic capacity and migration of colon cancer cells. Fagonia indica treatment significantly reduced the proliferative capacity of single cells over 7 days, indicating a pro-longed effect of treatment on the proliferation of colon cancer cell lines [42]. Previous studies have indicated a role of Akt activation and signalling in tumorigenic pathways, including evasion and migration [43-44]. Further research is required to determine the relationship between Fagonia indica treatment and Akt phosphorylation as a mechanism of action.

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 [45-46]. P38 MAPK signalling pathways are activated in response to double strand DNA damage, leading to transcription of cell repair and apoptotic genes, such as p21 [47]. This pathway has been used successfully as a molecular target in the development of other chemotherapeutic agents such as paclitaxel and novel treatment MTBT [47-48]. Inhibiting p38 activation with competitive inhibitor SB203580 led to a significant reduction in extract-induced loss of cell viability in H630 cell lines. P38 activation can lead to downstream signalling which indirectly inhibits Akt. Akt is an established oncogene, which upon activation can promote tumorigenic cell behaviour common in colon cancer [49]. In the current study, Akt activation by SC79 confounded the cytotoxic effect of Fagonia indica in both cell lines. Other studies have demonstrated the potential of a Rosemary plant extract to inhibit the activation and phosphorylation of Akt in vitro [50]. It is possible that Akt is a target of Fagonia indica treatment, this provides a wider picture of p53 activation and extract induced apoptosis in Fagonia indica.

Vascular endothelial growth factor (VEGF), is a signal protein secreted by cells and associated largely with the formation of new vascularisation, tumour proliferation, growth and metastasis [51]. 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 aqueous Fagonia indica
extract, led to a significant reduction in mRNA expression of VEGF-A in both RKO and H630 cancer cell lines. Inhibition of VEGF-A with Apatanib, partially abrogated the cytotoxic effects of *Fagonia indica*. 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 [24]. ICAM-1 has been associated with several tumour types due its ability to modulate inflammation and regulate vascular permeability. In the current study, ICAM-1 mRNA was reduced to a similar extent as VEGF expression in both cell lines. In RKO cancer cells, this reduced 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 NF-κB dependent manor [52-53]. In other research, inhibition of NF-κB subunits; NF-κB2 and RelA, resulted in a marked decrease in ICAM1 expression with a corresponding reduction in smooth muscle proliferation and Akt phosphorylation [54]. In the present study, expression of NF-κB subunit p65 was reduced in both cell lines after treatment with *Fagonia indica*. However, inhibiting NF-κB activation using CAPE did not significantly affect cellular cytotoxicity, suggesting that NF-κB signalling is not a key mechanism of *Fagonia indica* but may be a downstream or off-target effect of treatment.

ABCG2 and ABCC4 have been previously implicated in the acquisition of multi-drug resistance of colon cancer cells to chemotherapy, as such, inhibition of membrane transporters remains an effective mechanism of reversing resistance in these cell types [55-56]. In the present study, populations of colon cancer cells demonstrating resistance to chemotherapeutic agents tomudex, gemcitabine and 5-fluorouracil were isolated and expanded. Gemcitabine and 5-fluorouracil resistant H630 colon cancer cells had significantly increased mRNA expression of ABCG2 and ABCC4, compared to the wild-type control. Resistance to 5-fluorouracil is well documented in the literature and has been correlated previously with an over expression of ABCG2 [57]. Resistant H630 clones were treated with *Fagonia indica* extract, resulting in a significant reduction in both ABCC4 and ABCG2. Previous studies have demonstrated an ability of plant-derived molecules to inhibit ABCG2 [58]. Other studies found that plant extract of *Evodia rutaecarpa* suppressed ABCG2 mediated drug resistance in colon cancer cell lines, via the inhibition of NF-κB signalling pathways [59]. Silencing ABCC4 transport has also been shown to be a successful method of drug reversal [60-61]. 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 cells, following similar patterns to resistant H630 cell lines. It is important to establish in future work the mechanisms behind extract induced loss in gene expression [61].

We have shown for the first time that an aqueous extract of *Fagonia indica* induces cell death and metabolic disruption in two phenotypically distinct colon cancer cell lines. There is a possibility that *Fagonia indica* treatment utilises Akt/MAPK signalling pathways. In addition, extract treatment reduces VEGF, ICAM-1 and NF-κB expression which may be attributed to angiogenic and inflammatory processes. *Fagonia indica* was also able to induce cell death in chemotherapy resistant colon cancer cell lines and this was associated with down-regulation of ABC transporters, ABCG2 and ABCC4. This provides a novel mechanism by which an aqueous extract of *Fagonia indica*; which is used frequently as a treatment in traditional Pakistani communities,
can cause cytotoxicity in colon cancer cells. However, the molecular composition of the bioactive compounds of the *Fagonia indica* remains relatively unknown.

4. Materials and Methods

**Cell culture**

Wild-type (WT) and resistant RKO and H630 human colon cancer epithelial cells (produced by Professor W Wang, Wolverhampton University) were cultured in high glucose DMEM (Sigma, UK) with 2nM L-glutamine supplemented with 10% FCS (Sigma, UK) and 1% penicillin/streptomycin (50U/ml) (Sigma, UK) and incubated at 37°C with 5% CO₂. Resistant clones were produced by repeated exposure of the drug in a dose-dependent manner, detailed in Table 1.

| Colon cancer cell lines | Resistant chemotherapy | Resistant concentration | Chemotherapy Manufacturer |
|-------------------------|------------------------|-------------------------|---------------------------|
| H630-GM                 | Gemcitabine            | 10 μM                   | Santa Cruz Biotechnology  |
| H630-5FU                | 5-fluorouracil         | 10 μM                   | Sigma, UK                 |
| RKO-TDX                 | Tomudex                | 2 μM                    | Santa Cruz Biotechnology  |

*Table 1*. Conditions of drug resistant colon cancer cell lines.

**Fagonia indica** preparation

An aqueous extract of *Fagonia indica* was produced to replicate traditional methods of consumption, following a previous described protocol [39]. The extract was filtered with filter paper (Fisher Scientific), before being subjected to a liquid-liquid partition with 3 x equal volumes of culture-grade hexane (Fisher). The aqueous phase was collected and freeze-dried under vacuum and stored at 4°C.

**Cell viability – neutral red assay**

Cell viability was determined using the Neutral red colorimetric assay [62]. In brief, cells were seeded in flat-bottomed 96-well plates (Costar) at a density of 1 x10⁴ cells/per well and left to adhere overnight. Cells were treated with plant extract (0-2.5 mg/mL) or gemcitabine (1 μM) for 24-72 hours, before addition of neutral red reagent (40 µg/mL) (Acros organics) and incubation for 2 hours. Neutral red was solubilised with neutral red de-staining solution (50% ethanol, 49% dH20 and 1% glacial acetic acid). Optical intensity was measured at an absorbance of 540 nm using a Thermoscientific Multiskan GO microplate reader.

**Cell viability – MTT assay**

Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [63]. In brief, cells were seeded in a flat-bottomed 96-well plate
(Costar) at a density of 1 x 10^4 cells/per well and left to adhere overnight. Cells were treated with inhibitor (Table 2), plant extract (0-2.5 mg/mL), or gemcitabine (1-10 μM) for 24-72 hours, prior to addition of 0.5 mg/mL MTT (Acros organics) and incubation for 2 hours. Cells were lysed and formazan product solubilised with 100 μL DMSO. Optical density was measured at an absorbance of 570 nm using a Thermoscientific Multiskan GO microplate reader.

| Inhibitor/Activator          | Target                    | Concentration | Manufacturer | Reference |
|------------------------------|---------------------------|---------------|--------------|-----------|
| Apatanib                     | VEG-FA                    | 1 μM          | Sigma        | [64-65]   |
| SC-79                        | Akt activator             | 10 μM         | Tocris       | [66]      |
| SB203580                     | Akt and p38 MAP kinase    | 10 μM         | Tocris       | [67]      |
| Caffeic acid phenethyl ester (CAPE) | NF-κB               | 1 μM          | Tocris       | [68]      |

**Table 2. Inhibitors/activators used in this study**

**Luminescent ATP assay**

Intracellular ATP was measured using the Luminescent ATP Detection Assay Kit (ab113849), following the manufacturers protocol. In brief, cells were seeded into sterile white bottom 96-well plates (Thermo Scientific) at a density of 2 x 10^4 cells/per well and left to adhere overnight. Cells were treated with 1 mg/mL plant extract or low-glucose DMEM for 24 hours and luminescence was measured on the Orion II luminescent microplate reader. Intracellular ATP levels were measured as a percentage of the control.

**Scratch wound assay**

Cellular migration of cells was assessed by wound scratch assay. Briefly, cells were seeded in 12-well plates (Corning) at a density of 2 x 10^5 cells/per well and left to adhere serum-depleted overnight. Straight scratches were introduced into each monolayer using a sterile 20 μL pipette tip, before washing with HBSS. Cells were treated with 1 mg/mL plant extract and antiproliferative drug 5 μg/mL mitomycin (Sigma, UK) for 24 hours. Images were taken on an EVOS microscope and wound closure was analysed with ImageJ software.

**Colony formation assay (CFA)**

The capacity for a single cell to proliferate was determined using a colony formation assay [69]. In brief, cells were treated for 24 hours with 2 mg/mL of *Fagonia indica*, 1 μM gemcitabine positive control or DMSO vehicle control. Cells were seeded into 24 well plates at a density of 200-400 cells/mL and incubated for 7 days. The colonies were fixed with 100% methanol (Fisher) 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).

**RNA extraction and quantitative real-time PCR**

RNA was extracted using the Isolate RNA mini kit (Bioline), as per the manufacturer’s instructions. To elute the isolated RNA sample, 30-80 µL of RNase-free water was added to the Spin Column membrane, before a final spin at 6000 x g for 1 min into a 1.5 mL DNA elution tube (Sigma, UK). RNA concentration was measured using the NanoDrop™1000 spectrophotometer and stored at -80 ºC. 500 ng of RNA was reversed transcribed using the Precision nanoScript ™2 Reverse Transcription kit (Primerdesign), to produce cDNA. 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. Prior to PCR set up a ‘master mix’ was prepared containing 10 µL of PrecisionPLUS 2x qPCR mastermix with SYBR green (Primerdesign), 3 µL of RNase/DNase-free water and 1 µL of both forward and reverse primers (Table 3). For each independent reaction, 5 µL of cDNA and 15 µL of master mix were added to each well in triplicate, 5 µL of DNase-free water was used as a control. Gene expression levels were determined using the PikoReal™ 96 (Thermo Scientific) and analysed using the PikoReal™ Software 2.2. Conditions of the PikoReal™ thermal cycler were as follows, 10 min at 95 ºC, 15 sec at 95 ºC and 1 min at 60 ºC (40 cycles), and cooling of 40 ºC for 10 sec. All PCR set-up was conducted under sterile conditions using a PCR workstation (UVP).

| Primers used in qPCR | Manufacturer | Primer sequence |
|----------------------|--------------|-----------------|
| Actin                | Invitrogen   | F: CTGGAACGGTGAAGGTGACA  
 |                      |              | R: AAGGGACTTCCIGTAACATGCA |
| YWHAZ                | Invitrogen   | F: CCGCCAGGACAAACCAGTAT  
 |                      |              | R: CCGCCAGGACAAACCAGTAT |
| VEGF-A               | Invitrogen   | F: CTACCTCCACCATGCCAAGT  
 |                      |              | R: GCAGTAGCTGGCTTGATAGA |
| NFκB- P65            | Invitrogen   | F: CCTGCTTCTGCTCTAGGAG  
 |                      |              | R: TAAGGAGAAGCATTAACTTCTT |
| ICAM-1               | Invitrogen   | F: GACTCCAATGTCAGGCTT  
 |                      |              | R: TAGTGTCCTCAAGATTCG |
| ABCC4                | Invitrogen   | F: TGTGGCCTTTGAACACACG  
 |                      |              | R: CAGGCACACTGAACGGTATA |
| ABCG2                | Invitrogen   | F: CAGGAGGCTTGGGATACTT  
 |                      |              | R: TATAGGAGCTTGGGATT |

Table 3. RT-PCR primer sequences used in this study.

**Statistics**

Statistical analysis of data was conducted using GraphPad Prism. For comparisons of two groups a student t test was used. For comparisons of more than two groups with one independent
variable, a one-way Anova with multiple comparisons was used and for two independent variables a two-way Anova was used with multiple comparisons. Sidak’s and Dunnett’s post-hoc tests were used for multiple comparisons. A value of $p<0.05$ was considered significant.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1.

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Abbreviations

- ABC
- Akt
- ATP
- ABCC4
- ABCG2
- ANOVA
- BMI
- CAPE
- DMSO
- H630-WT
- H630-GM
- H630-5FU
- ICAM-1
- MAPK
- MCF-7
- MTT
- mRNA
- NF-κB
- NR
- PCR
- RKO-WT
- RKO-TDX
- RT-qPCR
- SEM
- ATP Binding Cassette
- Protein kinase B
- Adenosine triphosphate
- ABC C subfamily member 4/Multidrug resistance protein 4
- ABC G subfamily member 2/Breast cancer resistance protein
- Analysis of variance
- Body mass index
- Caffeic acid phenethyl acid
- Dimethyl sulfoxide
- Wild type H630 cells
- Gemcitabine resistant H630 cells
- 5-fluorouracil resistant H630 cells
- Intracellular adhesion molecule 1
- Mitogen activated protein kinase
- Michigan cancer foundation 7 cell line
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- messenger RNA
- Nuclear factor kappa beta
- Neutral red
- Polymerase chain reaction
- Wild type RKO cells
- Tomudex resistant RKO cells
- Reverse transcription quantitative PCR
- Standard error of the mean
SYBR green • N’,N’-dimethyl-N-[4-[(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl][-N-propylpropane-1,3-diamine
VCAM-1 • Vascular cellular adhesion molecule-1
VEGF-A • Vascular endothelial growth factor A
VEGFR-2 • Vascular endothelial growth factor receptor A
5-FU • 5-fluorouracil

Appendix A

Supplementary Figure 1: Treatment of wild-type and chemotherapy resistant colon cancer cells with gemcitabine, 5-fluorouracil and gemcitabine. (A) H630-GM and H630-WT cells were treated with 1 μM gemcitabine, (B) H630-5FU and H630-WT cells were treated with 10 μM 5-fluorouracil and (C) RKO-TDX and RKO-WT cells were treated with 2 μM 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

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