Emodin and Epicatechin Gallate Do Not Impair the Motility of Colorectal Cancer Cell Line SW480 cells by Inhibiting Aquaporin-5 Water Channels

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Abstract

Aquaporin water channels (AQPs) are a group of specialised water-selective transporters that plays a role in cancer cell migration. AQP-5, an AQP subtype that has a -low expression in the normal colonic epithelium is upregulated in colorectal cancer (CRC). AQP-5 plays a role in cancer metastasis, and knockdown of AQP-5 increases the sensitivity of CRC to chemotherapy, but there are no pharmacological reagents that target AQP-5. Emodin and epicatechin gallate (ECG) are traditional Chinese medicines that suppress the migration and invasion of cancer cells. This project aims to investigate if emodin and ECG reduce CRC cell migration by targeting AQP-5. The rate of migration was quantified using a circular wound closure assay and the rate of invasion was quantified through a transwell invasion assay. Emodin and ECG were tested on SW480 CRC cell line, with high expression of AQP-5. Results showed that emodin derived from rhubarb significantly blocked cell migration at a range of concentrations (≥50μM, $p<0.0001$). Emodin also reduced cell invasion at 100μM ($p<0.05$) with no significant cytotoxic effects on the SW480 cells. In contrast, ECG significantly inhibited cell migration (≥25μM, $p<0.0001$) but do not block cell invasion ($p>0.05$) in SW480 cells. The effects of emodin and ECG were conducted on AQP-5 expressing oocytes using a quantitative swelling assay. Results show that emodin and ECG do not block AQP-5 (NS, $p>0.05$). These results are the first to demonstrate that the anti-migratory effects of emodin on SW480 cell line are not through the inhibition of AQP-5.

Introduction

Aquaporin water channels. AQPs are a family of membrane water channel that maintains the water balance in response to osmotic gradients created by the active transport of solutes[1]. In 1992, the discovery of an AQP subtype, AQP-1 (previously known as CHIP28) was made through the injection of CHIP28 RNA into a Xenopus laevis oocyte, and this caused the oocyte plasma membrane to be highly permeable to water[2]. AQPs ability to transport water is dependent on its structure. AQPs are arranged in a homo-tetrameric structure. Each monomer consists of six transmembrane domains connected by five loops, with both carboxylic and amino terminals located in the cytoplasm[3]. In classical aquaporins (AQP-0, AQP-1, AQP-2, AQP-4, AQP-5, AQP-6, AQP-8), the transport selectivity in the pore of the channel is characterised by two regions of constriction. The first constriction is formed by two highly conservative Asn-Pro-Ala (NPA) motif in loops B and E, allowing a selective, bi-directional and single-file passage of water through the pore[4]. The second constriction is referred to as the ar/R selectivity filter in the extracellular end of the pore, allowing AQPs to selectively allow the entry of water molecules[5].

Colorectal cancer (CRC). To date, CRC remains the second most common cause of death in Australians of all ages, particularly in people over the age of 70[6]. In 2012-2016, it was reported that 70.1% of patients diagnosed with CRC survived 5 years after diagnosis, which is largely due to early diagnosis and improved postoperative care[6]. Surgery, colectomy or segmental resection and chemotherapy are the most common treatments in the early and late stages of CRC. However, tumour recurrence happens in 30-50% of all cases, generally presenting metastasis[7]. A study reported that dormant residual tumour cells were present in patients post-treatment[8]. After the withdrawal of chemotherapy, the remaining dormant residual cells would resume growth and are more resistant to chemotherapy[8]. It is important to note that chemotherapy damages the intestinal

mucosal lining due to its rapid turnover rate as chemotherapy treatment does not distinguish between normal and cancer cells[7, 9].

Aquaporins in Cancer. The metastatic cascade is a process where primary tumour reaches a distant organ (common sites are lungs, liver, bone and brain) and develop metastases[10]. AQPs play a role in the metastatic cascade of cancer cells which involves tumour angiogenesis, migration, invasion and proliferation[4]. The expression of AQP is upregulated in most cancer cells (Table 1).

Tumor type	Aquaporins	AQP level
Astrocytoma	AQP1, 4, ?8, 9	high
Breast cancer	AQP5	high
	AQP1	high
	AQP4	low
Cholangiocarcinoma	AQP1	low
	AQP1	high
Colorectal cancer	AQP1, 3, 5	high
	AQP8	low
Cervical cancer	AQP1, 3	high
	AQP5	high
Choroid plexus tumor	AQP1	high
Hemangioblastoma	AQP1	high
Laryngeal cancer	AQP1	high
Leukaemia	AQP5	high (CML)
Liver cancer	AQP3, 5	high
	AQP8, 9	low
Lung cancer	AQP1, 3, 5	high
	AQP1, 4	high
Meningioma	AQP4	high
Nasopharyngeal cancer	AQP1	high
Oesophageal cancer	AQP3, 5	high
Ovarian cancer	AQP1, 5, 9	high
Renal	AQP3	high
Skin, SCC	AQP3	high
Stomach cancer	AQP5	high
	AQP4	low
	AQP3	high
Thyroid cancer	AQP4	high (papillary)
	AQP3, 4	low (undifferentiated)
	AQP7	high
Tongue cancer	AQP3, 5	high (SCC)
Urinary bladder	AQP3	low

Table 1. AQP expression in human tumours. From Papadopoulos and Saadoun, 2015[11].

Cell migration. AQPs accelerate cell migration via two roles: (1) Facilitates a rapid change in cell volume and cell shape, allowing migrating cells to squeeze through the extracellular space[1]; (2) Rapid formation and retraction of cell membrane protrusion (lamellipodia) due to osmolarity changes at the leading edge facilitates cell migration (Fig. 1)[1]. Ions uptake and actin cleavage at the leading edge creates an osmotic gradient which increases the osmotic water permeability[12]. The entry of water increases local hydrostatic pressure, causing the formation of membrane protrusion, creating space for actin polymerisation which stabilises membrane protrusions, resulting in an enhanced cell migration[1, 12, 13]. An in-vivo study showed that AQP-1 expressing tumour cells have an increased lamellipodia area and increased metastatic potential and invasiveness[1, 13]. In the absence of AQPs, cell migration occurs at a slower rate because the lipid bilayer is slightly permeable to water[12].

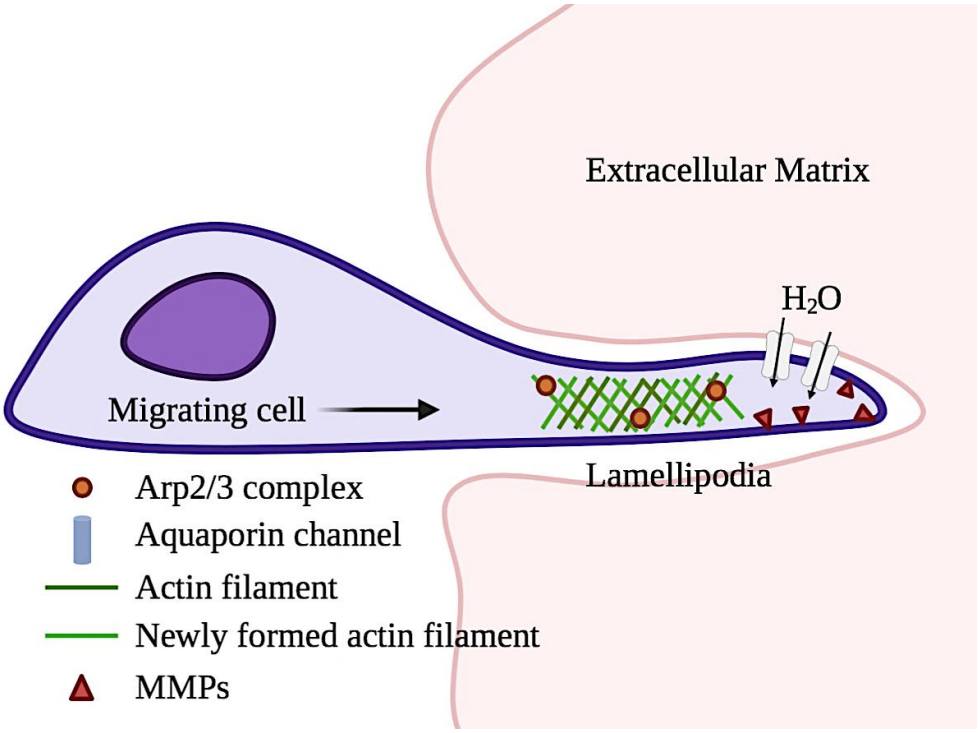


Figure 1. Factors involved in tumour cell migration and invasion: (1) Aquaporins facilitates cell migration by facilitating the formation of lamellipodia, (2) Arp2/3 complex is a regulatory protein of actin polymerisation that drives the formation of lamellipodia, (3) Matrix Metalloproteinases (MMPs) are proteolytic enzymes for matrix degradation. Adapted from Hu and Verkman, 2006; Yamaguchi and Condeelis, 2007[13, 14].

Angiogenesis. Formation of new blood vessels (angiogenesis) to supply nutrients and oxygen and remove waste is essential for tumour growth[15]. A study has shown that subcutaneous implantation of melanoma cells in AQP-1 null mice reduced tumour growth due to impaired angiogenesis with a reduced tumour microvessel proliferation and the presence of necrotic tissues around viable tumour cells[12]. Another in-vivo study showed similar results whereby AQP-1 deficiency in breast tumour resulted in reduced tumour growth due to impaired angiogenesis with fewer and smaller micro-vessels compared to wild-type mice[15].

Cell proliferation. Studies showed that AQP-3 and AQP-5 play a role in tumour proliferation[11, 16-19]. An in-vivo study by Hara-Chikuma and Verkman showed that skin tumour proliferation was impaired in AQP-3 null mice[16]. It is proposed that AQP-3 deficient mice impaired glycerol transport, which is closely related to cellular proliferation as glycerol supplementation corrected the reduced proliferation in AQP-3 null mice[11, 16]. In colon cancer, the overexpression of AQP-5 activated the Ras-MAPK pathway which enhanced the transcription of genes involved in cell proliferation[20]. Another study showed that in AQP-5 null mice, there is a reduced proliferation and metastasis of lung cancer due to a decreased activation of the MAPK/ERK signalling pathway[18]. Huang also reported similar results whereby the inhibition of AQP-5 through the administration of acetazolamide reduced the proliferation and migration of human gastric carcinoma cell line[19]. These studies show a link between AQP-3 and AQP-5 in tumour cell proliferation, but further studies are required to confirm the role of AQPs in cell proliferation.

AQP-5. AQP-5 is present in the lacrimal gland, salivary gland and sweat duct[21]. Multiple studies have reported that the overexpression of AQP-1, AQP-3 and AQP-5 in CRC is associated with increased lymphatic metastasis[17, 19, 20, 22-27]. Our interest lies in AQP-5 because they are over-expressed on colorectal carcinoma cells with a minimal expression on normal colonic surface

epithelium as opposed to AQP-3[19-22]. The extracellular-signal-regulated kinase (ERK) pathway induces epithelial-mesenchymal transition, one of the first steps in the metastatic progression of cancer cells[20, 28]. Kang et al. reported that there was a significant association of AQP-5 expression and liver metastasis in CRC through the activation of ERK pathway[20]. It was recently reported that AQP-5 knockout significantly enhanced the sensitivity of CRC cell lines HT29 and HCT116 to 5-fluorouracil (a chemotherapy drug) by enhancing cell apoptosis[29]. AQP-5 serves as an attractive drug target due to its low expression on normal colonic epithelium, thus reducing the damage to the intestinal mucosal lining[20]. As of now, there is no pharmacological reagent that targets AQP-5.

Emodin and Epicatechin Gallate. Emodin, a natural anthraquinone derivative in rhubarb decreases the progression of multiple cancer cells[30, 31]. Ok et al. reported that emodin downregulates CXCR4 expression, a key receptor involved in the metastasis, invasion and proliferation of tumour cells in prostate and lung cancer[30]. Another study reported that emodin suppresses the growth, migration and invasion of CRC cells through the inhibition of vascular endothelial growth factor receptor, a receptor that regulates endothelial migration and proliferation[31]. Another interesting candidate, ECG, abundant in tea, is found to induce CRC cell line apoptosis by inducing p53, a protein mutated in most cancer[32]. Besides, ECG is found to inhibit the migration and invasion of lung cancer cells by reversing the epithelial-mesenchymal transition[33]. We predict that emodin and ECG targeted AQP-5 as there is an overexpression of AQP-5 in the cancer cell type mentioned[4].

Hypothesis and Aims. Based on the analysis above, we hypothesised that emodin and ECG reduce the migration and invasion of CRC SW480 cell line through the inhibition of AQP-5. Moon et al. confirmed the expression of AQP-1, AQP-3 and AQP-5 in SW480 cell line through reverse transcriptase-PCR, western blot assay and in-situ hybridisation[27]. This study aimed to investigate the effects of emodin and ECG in cancer cell migration and invasion through a circular wound closure assay and transwell assay. The second aim was to investigate the effects of emodin and ECG

on AQP-5 through an oocyte swelling assay where AQP-5 complementary RNA was injected into Xenopus laevis oocytes in order to express AQP-5 water channels.

Materials and Methods

Cell line. The human colorectal adenocarcinoma cell line SW480 (ATCC CCL-228TM) was cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY, USA), 1% Gibco GlutaMAX (Life Technologies, Grand Island, NY, USA) and 100 units/ml of penicillin-streptomycin (Life Technologies), at 37°C in a humidified 5% CO_2 incubator until it reached the desired confluency.

Drug Treatment. Anthraquinone derivative emodin from frangula bark and flavonoid derivative (–)-ECG from green tea were purchased from Sigma-Aldrich. Powdered emodin was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to create 5000x, 10000x and 20000x stock solutions and ECG was dissolved in Milli-Q water to create 10000x stock solution. Stock solutions were stored in an airtight vial. Mitotic inhibitor 5-fluoro-2'-deoxyuridine (FuDR; Sigma Aldrich) was added in the working solutions (DMEM, 10% FBS, 1% Gibco GlutaMAX, penicillin-streptomycin and FuDR) to prevent cell proliferation. Emodin and ECG were diluted in FuDR-drug DMEM to achieve the final concentrations required in circular wound closure assay, transwell invasion assay, and cytotoxicity assay. DMSO (1μL/ml) was used as vehicle control. For the oocyte swelling assay, emodin and ECG stock solutions were diluted in 10x isotonic solution to achieve the final concentration.

Circular Wound Closure Assay. Circular Wound Closure Assay was performed based on the methods established by Deloso and Pei[34]. SW480 cells were plated in FuDR-containing drug DMEM in a 96 well-plate; a confluent monolayer was achieved at 24 hours following plating. Circular wounds were created with a sterile p10 pipette tip attached to a sterile p200 pipette tip. Cells were washed two times with phosphate-buffered saline to remove cell debris, and 100μL of media with and without emodin or ECG or vehicle in FuDR-drug DMEM was applied to each well. Complete

wounds were imaged after 20 hours at 10x magnification with a Canon 6D camera on a Nikon inverted microscope. Microscopic images were standardised using XnCoverter software, and wound areas were quantified using Fiji software (ImageJ; version 1.52; U.S. National Institutes of Health). All experiments were repeated in duplicate wells. The percentage of wound closure was calculated as a percentage of initial wound area for the same well as a function of time.

$$\left[\frac{Area_0 - Area_{24}}{Area_0}\right] \times 100\%$$

Transwell Invasion Assay. The assay was performed using a 6.5 mm Corning® Transwell® polycarbonate membrane cell culture inserts with 8 μM pore size (cat #CLS3422; Sigma-Aldrich, St. Louis, MO). The upper surface of the insert was coated with 40μL of extracellular matrix gel from Engelbreth-Holm-Swarm murine sarcoma (Sigma-Aldrich, St. Louis, MO) that was diluted in Milli-Q water. Inserts were dehydrated in the laminar hood for 24 hours and were rehydrated for 1-2 hours in the incubator with 50μL of serum-free medium (0% FBS). SW480 cells were cultured in normal conditions until the plate reaches 40% confluency and cells were starved in reduced-serum medium (2% FBS) for 24-36 hours prior to plating in the upper chamber. Cells were detached and resuspended in full-serum medium (10% FBS). 1.5 x 10^6 of SW480 cells in 100μL was added to the upper chamber, making a total of 150μL cell suspension per transwell. 700μL of pharmacological treatment in full-serum medium (10% FBS) was added in the lower chamber that acts as a chemo-attractant for cells to invade (Fig. 2). Cells were then incubated for 24 hours at 37°C in a humidified 5% CO_2 incubator. Non-migrated cells at the top of the membrane were removed using a cotton-tipped applicator. Inserts were soaked in 70% ethanol for 10 minutes to allow cell fixation (allowing it to dry for 10-15 minutes upon removal from ethanol); followed with soaking in 0.2% crystal violet for 5-10 minutes for staining, excess crystal violet was washed in distilled water and was allowed to dry. Migrated cells at the bottom of the membrane were imaged at 10x and 20x magnification with a Canon 6D camera on a Nikon inverted microscope; cell count was performed and averaged for statistical analysis.

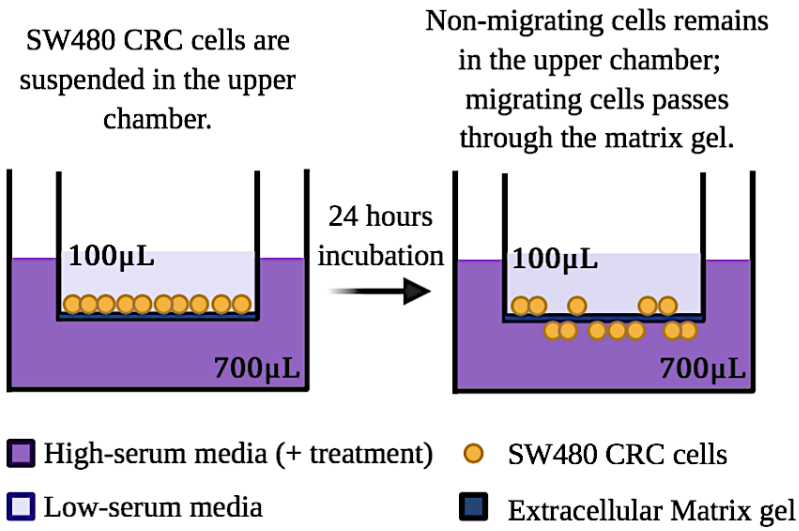


Figure 2. Illustration of Transwell Invasion Assay. Adapted from Creative Bioarray[35].

Oocyte Swelling Assay. Extraction was done by Professor Andrea Yool in accordance with the Australian Code for the care and use of animals for scientific purposes. An adult female *Xenopus laevis* frog was anaesthetised and unfertilised oocytes were extracted. Harvested oocytes were defolliculated with collagenase (type 1A, 2mg/ml; Sigma-Aldrich, St. Louis, MO) in isotonic saline (100mM NaCl, 5mM $MgCl_2$, 2mM KCl and 5mM HEPES; pH 7.6) for 1.5 hours at 18°C. Healthy oocytes were washed three times with isotonic solution before being injected with 0.5µL of wild-type AQP-5 cRNA. Injected oocytes were incubated in frog Ringer's saline (100mM NaCl, 5mM $MgCl_2$, 2mM KCl, 5mM HEPES, 0.6mM $CaCl_2$, 5% horse serum, 100 U/ml penicillin, 50 µg/ml tetracycline and 100 µg/ml streptomycin; pH 7.6) at 18°C for 48 hours to allow protein expression.

Human AQP-5 cDNA subcloned in a *Xenopus β*-globin expression plasmid was transcribed using T3 polymerase (T3 mMessage mMachine; Ambion, Austin, TX), synthesised by Saeed Nourmohammadi. cRNA was resuspended in sterile water and stored at –80°C.

Prior to experimental assay, control and AQP-5 expressing oocytes were pre-incubated in isotonic saline (without serum, antibiotic-free) for an hour. The experimental assay was conducted by Dr Pak

Hin Chow. Each oocyte acted as its own control. Each oocyte was first assayed without drug treatment and was incubated for 2 hours in isotonic saline with vehicle or drug treatments and then reassessed in a second swelling assay (Fig. 3). Swelling rates were measured in 50% hypotonic saline (50% isotonic saline + 50% diluted water). Oocytes were imaged using a grayscale camera (Cohu, San Diego, CA) attached to a dissecting microscope (Olympus SZ-PT; Olympus, Macquarie Park, Australia) at one frame per second for 30 seconds. Swelling rates were calculated as the slope values of linear regression fits of the change in volume as a function of time in hypotonic solution using GraphPad Prism.

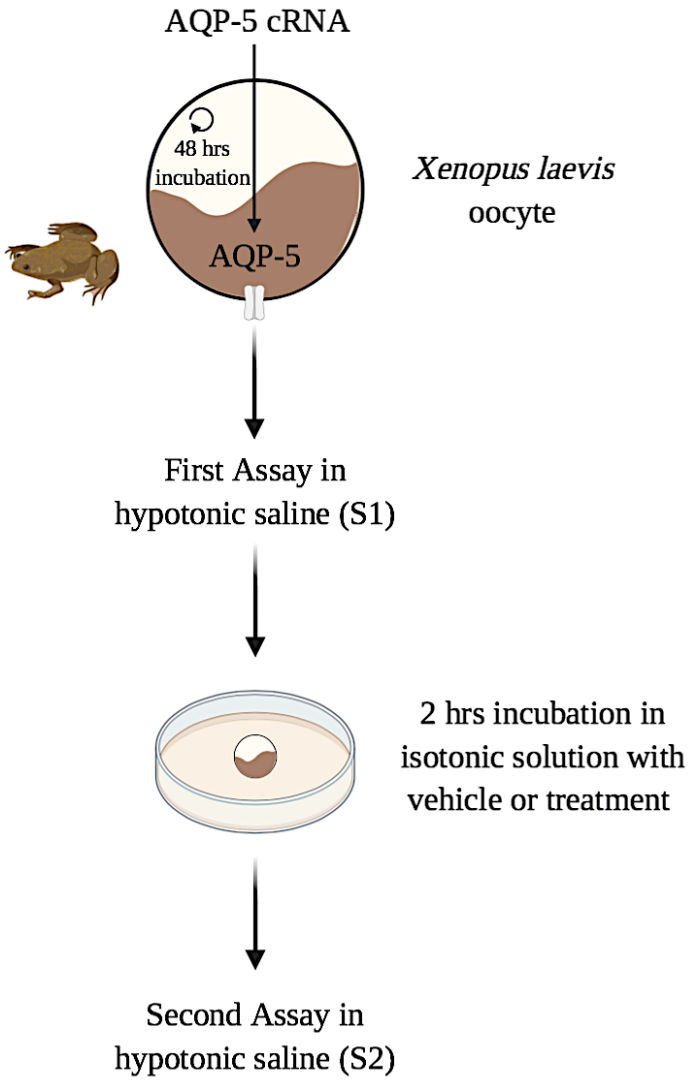


Figure 3. Illustration of Oocyte Swelling Assay. Adapted from Bianchi and Driscoll, 2006[36].

Cytotoxicity Assay. Cell viability was quantified using an alamarBlue assay, following the manufacturer's guidelines (Life Technologies). Cells were plated at $1x10^4$ cells/ml in 96-well plates, in the same FuDR-drug DMEM used in the circular wound closure assay. Treatments were applied at 24 hours after plating, and cells were incubated further for 24 hours. Cells were then treated with 10% alamarBlue solution for 1-2 hours. Fluorescence signal levels were measured with a FLUOstar Optima microplate reader (BMG Labtech, Victoria, Australia) for control and treatment groups. No-cell control was included to confirm low background fluorescence.

Statistical Analyses. XnConverter and ImageJ software were used to process microscopic images generated from the experiments. Statistical analyses were performed using GraphPad Prism 8.4.3 software. Statistical differences were evaluated using one-way ANOVA with Dunnett's multiple comparisons, Kruskal-Wallis with Dunn's multiple comparisons, paired t-test, simple linear regression and logarithm and normality tests as indicated in the figures. Symbols in the figures show $P>0.05$ (NS); $P<0.05$ (*); $P<0.001$ (***) or $P<0.0001$ (****).

Emodin and ECG block wound closure in SW480 CRC cell line. Circular wound closure assays were carried out to investigate the effects of emodin and ECG on AQP-5 expressing SW480 cell line. Emodin (at 25 to 150μM) and ECG (at 25 to 150μM) significantly impaired wound closure compared to vehicle control, at 51% (n=8) wound closure (Fig. 4). Besides that, emodin showed a dose-dependent response, where increased dosage had a more significant effect on wound closure. Emodin at 50μM had a 26% ($p<0.0001$) wound closure whereas ECG at 25μM had a 35% ($p<0.0001$) wound closure. A visual representation in fig. 5 shows the outline of circular wounds at 0 hours and 20 hours after incubation.

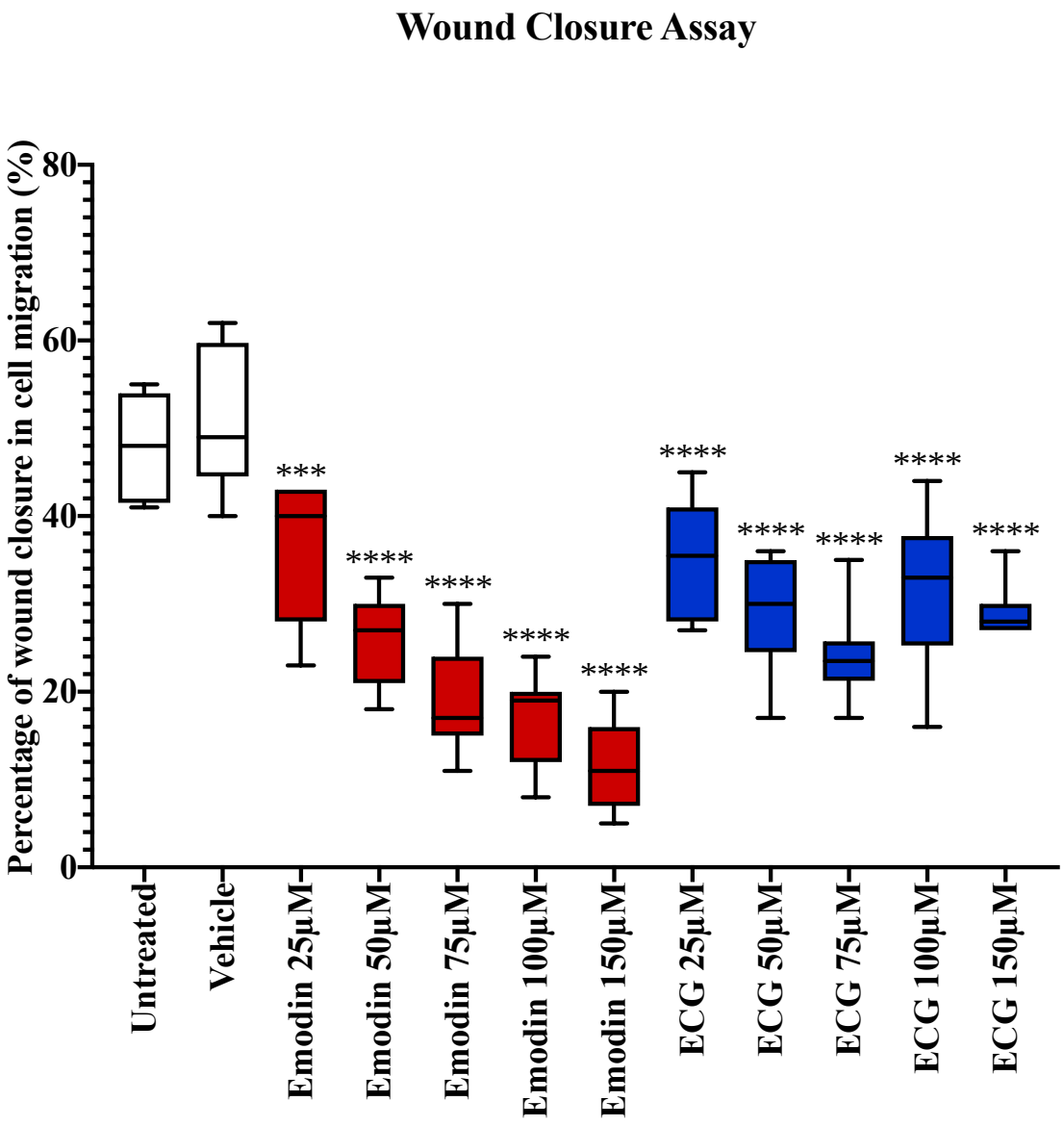


Figure 4. Wound closure assay showed a blocking effect of emodin and ECG on SW480 Colorectal Cancer cell line. Emodin and ECG at all dosage significantly reduced cell migration. Emodin showed a dose-response blocking effect. All treatments were n=8. Data were standardised for results to vehicle group. The error bar shows the min and max values; box indicates 50% of data; median is denoted by the horizontal line. ***: P<0.0005, ****: P<0.0001 (one-way ANOVA with Dunnett's).

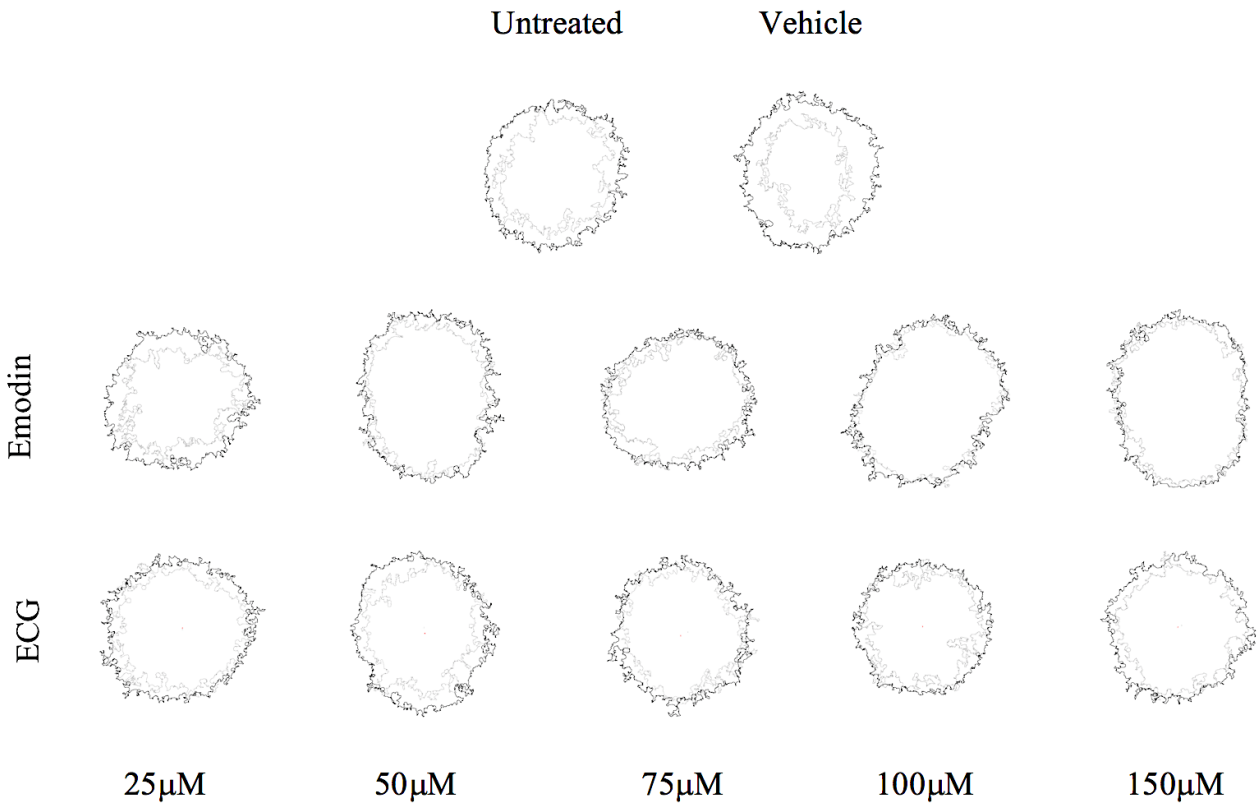


Figure 5. Outlines of circular wounds at 0hr (black line) and 20hr (grey line) of incubation.

Emodin, but not ECG, inhibited CRC invasiveness. Cell migration involves a three-dimensional penetration of cells through the extracellular matrix (ECM). The effects of emodin and ECG were investigated through a transwell invasion assay that quantifies the number of cells that pass through an ECM gel towards a chemo-attractant, visualised by staining of cell nuclei with crystal violet.

Treatment with emodin at 100μM reduced SW480 cell invasion by 86.5% (P<0.05) compared to vehicle at 24 hours post-treatment; while the lower dosage, 50μM had no significant effect (P>0.05) (Fig. 6). This suggests that emodin affected a motility mechanism in vitro. Treatment with ECG at 50μM and 100μM did not significantly reduce cell invasion (P>0.05), suggesting that ECG did not target AQP-5 (Fig. 6).

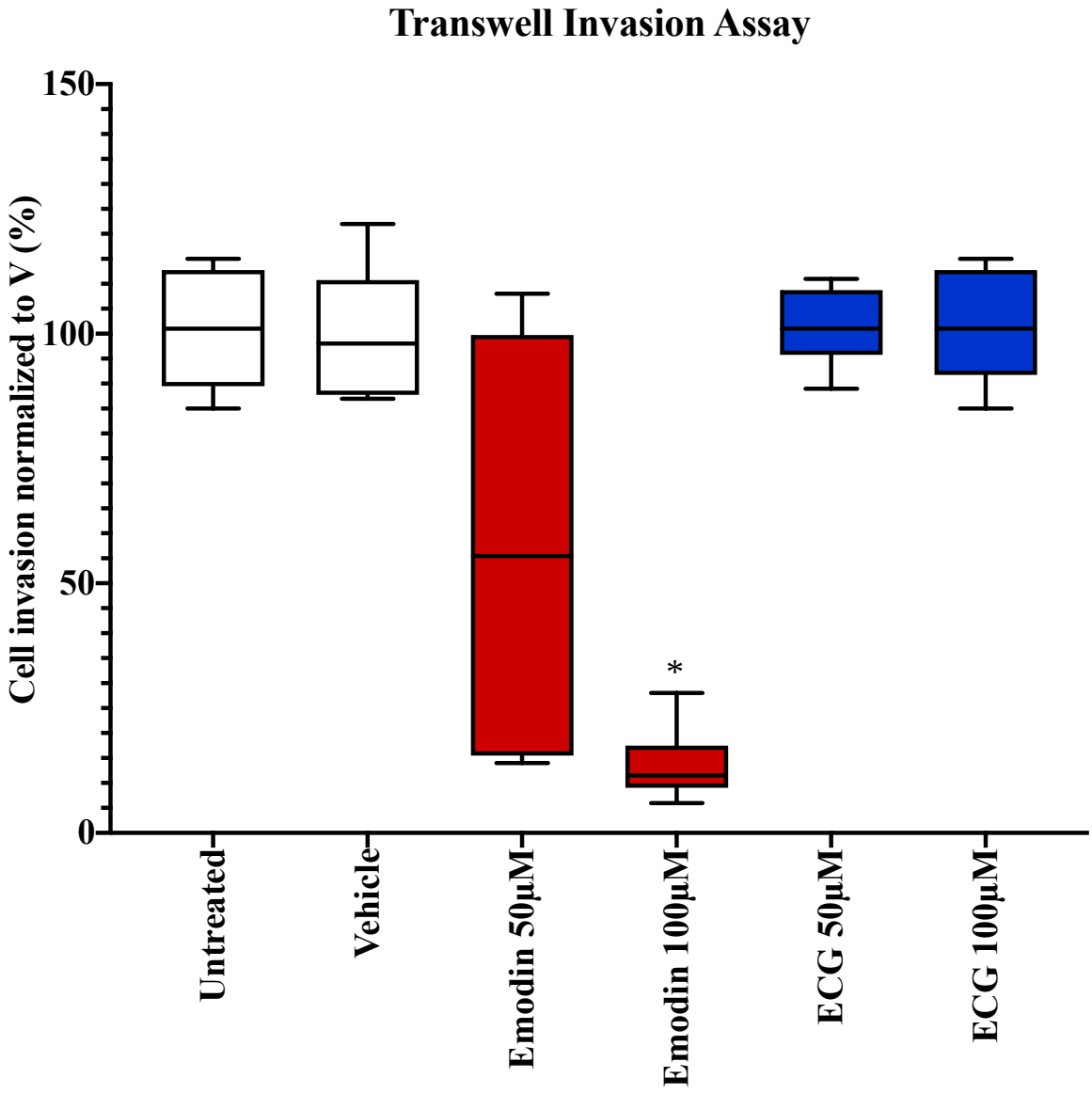
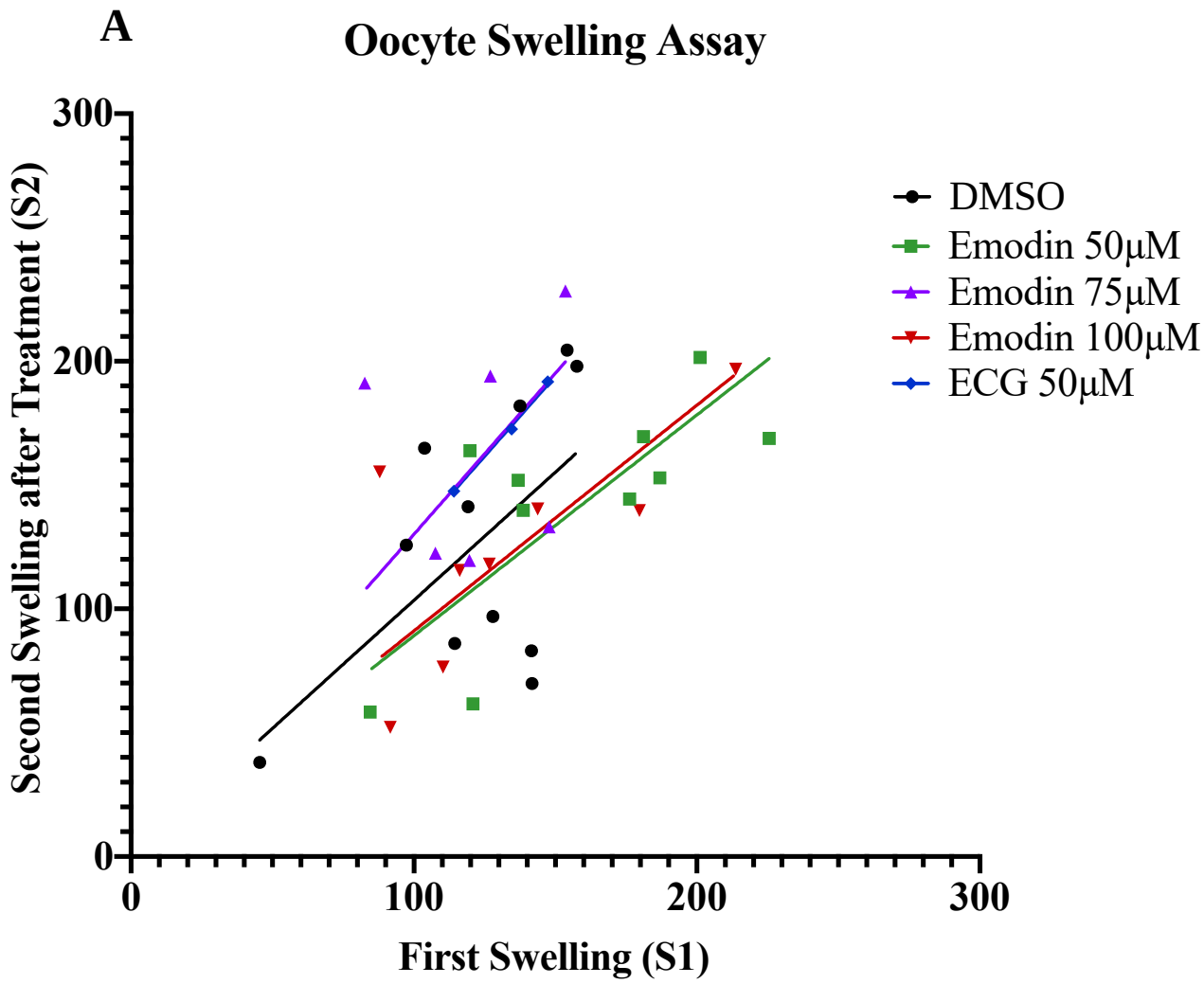


Figure 6. Transwell invasion assay showed that Emodin at 100μM significantly reduced cell invasion. Data were normalised to vehicle. The error bar shows the full range of the data; box indicates 50% of data; median is denoted by the horizontal line. *: P<0.05 (Kruskal-Wallis with Dunn's).

Emodin and ECG did not inhibit AQP-5 osmotic water permeability. Oocyte swelling assays were conducted to investigate if emodin and ECG blocked the migration and invasion of SW480 CRC cell line through the inhibition AQP-5. The osmotic water permeability of AQP-5 expressing oocytes was first assessed (S1) in 50% hypotonic solution and transferred into isotonic saline with vehicle or drug treatments (emodin 50μM, 75μM and 100μM; ECG 50μM) for 2 hours. After incubation (S2), swelling rates of oocytes were tested again with 50% hypotonic solution (Fig. 7A). The results from paired t-tests showed that there were no significant differences (NS, $p>0.05$) between the first and second swelling within each treatment groups, indicating that emodin and ECG do not have any effects on AQP-5 (Fig. 7B).



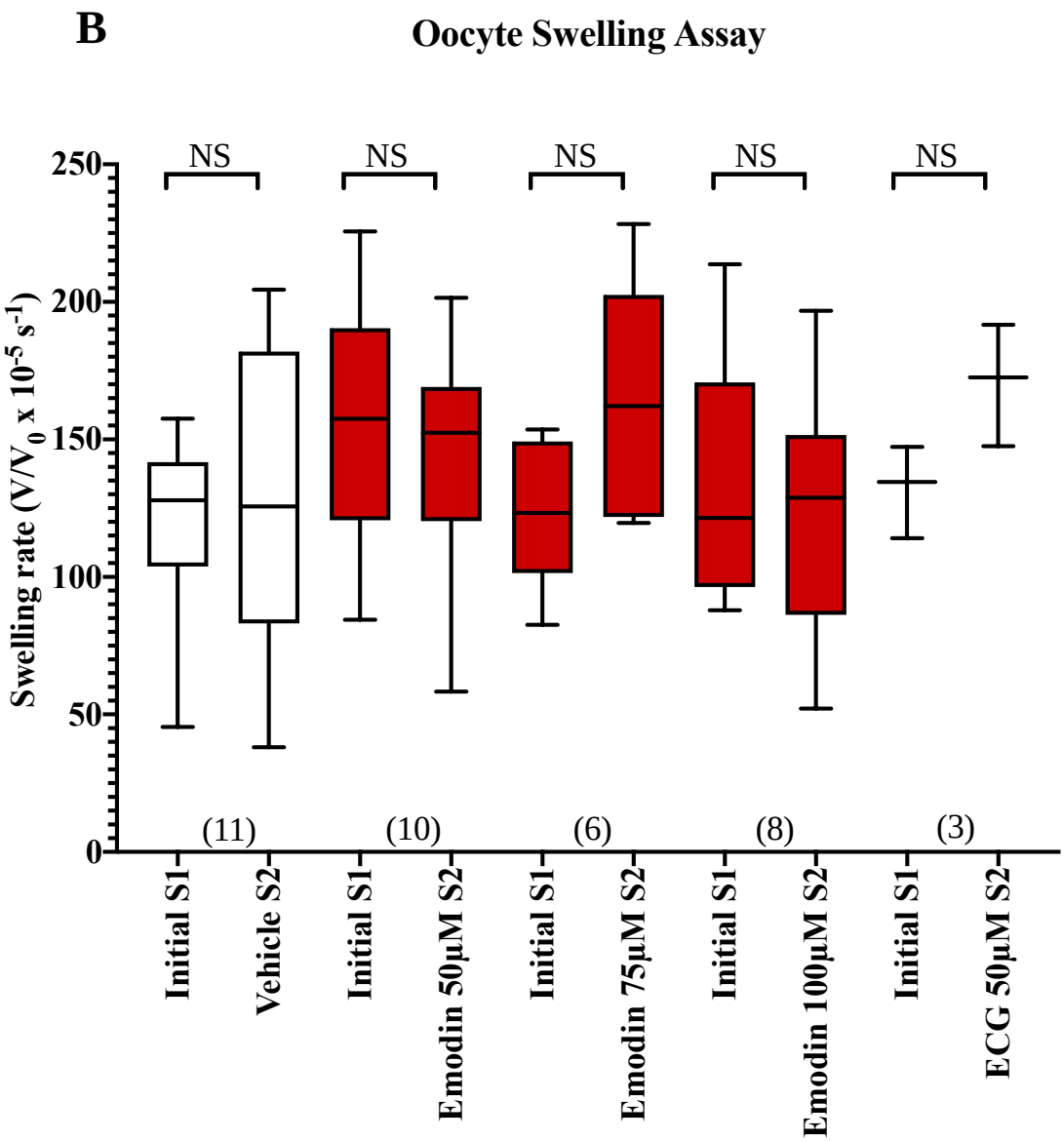


Figure 7. Osmotic water permeability of AQP-5 expressing oocytes. (A) Linear regression fits of responses of the first swelling (S1) vs second swelling (S2). (B) Linear regression fits of responses were used to calculate slope values which indicated the swelling rate. Swelling rates of AQP-5 expressing oocytes were measured before (S1) and after (S2) 2 hours of incubation with vehicle or drug treatments. No treatment groups showed responses that were different from vehicle. n values are above the x-axis. The error bar shows the full range of the data; box indicates 50% of data; median is denoted by the horizontal line. NS: P>0.05 (Simple linear regression and Paired t-test).

Emodin and ECG are not toxic to SW480 CRC cell line. Cytotoxicity assays measured the metabolic activity of the cells. There were no significant differences between vehicle and treatment groups (emodin and ECG at 50μM, 75μM, 100μM, 150μM and 200μM) (NS, P>0.05) (Fig. 8). Results were normalised to the metabolic activity of vehicle group, and indicated that the effects of emodin and ECG in SW480 cell line observed were not due to cell death. Emodin and ECG at high concentrations did not have any effects on the metabolic activity of cells, suggesting that the drugs were not toxic to SW480 cell line. There was a slight increase in metabolic activity when ECG was applied, but results were not significant (P>0.05).

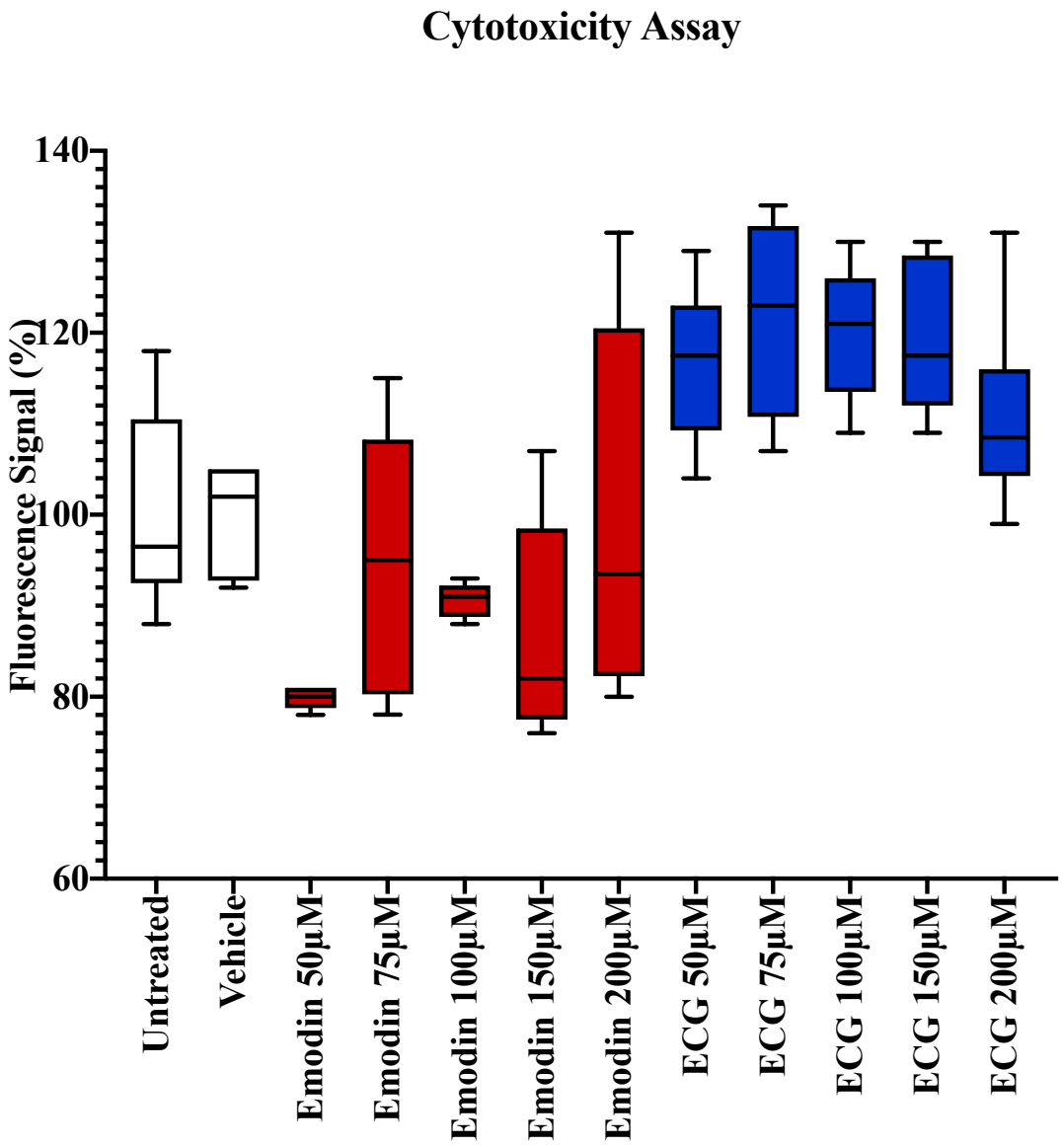


Figure 8. Absence of dose-dependent cytotoxic effects of emodin and ECG at a range of concentrations in SW480 cell line. There were no significant differences between vehicle

and treatment groups (Kruskal-Wallis with Dunn's). All treatments were n=6. Data were normalised to vehicle. The error bar shows the full range of the data; box indicates 50% of data; median is denoted by the horizontal line.

Discussion

Metastasis accounts for 90% of cancer deaths, but there is no treatment available for it[37]. ~50% of CRC patients display micro-metastasis post-treatment, particularly developing liver metastases, which could be the main reason why CRC remains the second most common cause of death in Australia[6, 38]. Current cancer treatments (chemotherapy and radiotherapy) target cancer proliferation and there are no treatments targeting cancer metastases[4]. AQP facilitates the transport of water molecules across the plasma membrane that causes the formation of lamellipodia at the leading edge facilitates cell migration[1]. A summary by Papadopoulos and Saadoun confirmed that CRC displays a high expression of AQP 1, 3 and 5 (Table 1)[11]. Expression of AQP-1, 3 and 5 are present across seven CRC cell lines[27]. Although current treatment is effective in treating CRC, tumour recurrence generally presenting metastasis remains a huge problem. Currently, there are no treatment targeting AQP-5, which is involved in the migration of cancer cells. Traditional Chinese medicine has proved to be highly effective in tumour treatments and are a rich source for new drug discovery[39]. Previous pharmaceutical studies have shown that emodin and ECG inhibit the migration and invasion in several cancer cells, but the exact target has not been identified[30, 31, 33]. Hence, this study aimed to determine the effects of emodin and ECG on AQP-5.

This study showed that emodin significantly suppressed cancer cell migration and invasion, whereas ECG only reduced cancer cell migration. This indicated that ECG does not target the chemotaxis mechanisms in cancer cell migration because they are not able to pass the ECM, a process essential for cancer metastasis as it is the initial step of cell migration. Cordero-Herrera et al. found that ECG induces SW480 cell line apoptosis through the induction of p53, but cytotoxicity assays from our study showed that ECG did not cause cell death in SW480 cell line[32]. As of now, not many studies have investigated the effects of ECG in the SW480 cell line and studies to identify the mechanism that ECG target is required. Emodin at a concentration high enough to block cell migration and

invasion did not have cytotoxic effects on CRC, showing effects were not due to cell death. Previous studies reported that emodin has anti-proliferative effects in cancer cells, so we applied mitotic inhibitor to stop the proliferation of SW480 cells, ensuring that we are only measuring the migration rate[30, 31]. Emodin also shows a dose-response effect, where increased concentrations have a more significant effect on cell migration. Hence, we concluded that the anti-migration and anti-invasion effects of emodin are attributed to the inhibition of a migrative mechanism.

We hypothesised that the effects of emodin on SW480 CRC cell migration were through the inhibition of AQP-5 water channels, but results from the oocyte swelling assays disproved this idea. Other factors that might be a target for pharmacological blockers (actin polymerisation, cell adhesion and matrix degradation) are involved in cell migration and invasion[40]. The ability of emodin to target both cell migration and invasion suggested that it might play a role in one of these cell migration components. Actin polymerisation drives the formation of lamellipodia in cancer cells, which is dependent on the generation of barbed ends of actin filaments at the leading edge (fig. 1)[41]. In colorectal carcinogenesis, Arp2/3 complex, one of the key regulatory proteins of actin polymerisation, is involved in cancer migration and invasion and is controlled by tumour-stromal interaction[40]. A study has shown that emodin inhibited the actin polymerisation in mouse leukemia cell line[42]. More recently, Liu et al. found that Arp2/3 complex inhibitor inhibited the accumulation of AQP-2 in rat kidneys and Lewis lung carcinoma cells in-vitro[43]. Emodin might be an Arp2/3 complex inhibitor which in turn inhibits the accumulation of AQP-5 at the leading edge of migrating cells.

Matrix metalloproteinases (MMPs) are proteolytic enzymes involved in matrix degradation. MMPs facilitate cancer cell migration and invasion by degrading the ECM and basement membrane of cells, playing a role in the metastases of cancer cells[44]. Multiple studies have shown that emodin significantly reduced the secretion of MMP-2, MMP-7 and MMP-9 in breast cancer, tongue cancer, colon cancer and CRC cell lines[44-47]. The MAPK/ERK signalling pathway is involved in the

regulation of MMPs. Previously, Kang et al. showed that the activation of AQP-5 in CRC tissue and cell line is associated with the activation of the ERK pathway[20]. Zhang et al. also reported similar findings where AQP-5 facilitates lung cancer proliferation and migration through the activation of the MAPK/ERK pathway[18]. Taking all these observations into consideration, it is possible that emodin is targeting the MAPK/ERK pathway or the Arp2/3 complex, which might have an indirect connection with the expression of AQP-5 in the SW480 cell line.

Although our results showed that emodin at 50-200μM did not decrease the metabolic activity of cells, other studies have highlighted that emodin at 20-80μM shows nephrotoxicity and hepatotoxicity through the induction of apoptosis when administered in the human kidney cell line[48, 49]. In contrast, Lee et al. found that emodin prevents liver damage in rats by downregulating the production of cytokines that activates pro-inflammatory cascades[50]. Sun et al. reported that emodin at 10-80μM has no significant effects on the cell viability of breast cancer cell line at 24 hours, and further administration of emodin at 40mg/kg in breast cancer-induced mouse for 8 weeks showed that there were no effects on liver and kidney function[44]. Emodin displays varying cytotoxic effects in different cell lines and more research is needed to investigate why this occurs. Emodin does have poor pharmacokinetics in that it rapidly undergoes glucuronide metabolism, but co-administration of tetrahydroxy-stilbene glucoside can slow the metabolism of emodin in rats[51]. Further experiments on the effectiveness of emodin dosage with co-administration of metabolic inhibitors would be needed to ensure that emodin is safe for pharmaceutical use and human consumption.

Limitations. In this study, only SW480 CRC cell line with high expression of AQP-5 was used to examine to effects of emodin and ECG. Additional CRC cell lines should be used to investigate if the effects of emodin and ECG on SW480 cell line can be observed in other CRC cell lines. Furthermore, due to time constraints, only one AQP channel was investigated in my studies with oocytes, though AQP-1 and 3 are also present in the SW480 cell line. Investigating the possible inhibitory effects of

emodin on other classes of AQPs present in the SW480 cell line would allow us to define or rule out the concept that emodin acts by blocking AQPs. Furthermore, because this study was conducted in-vitro, it may not translate to in-vivo, since the in-vivo model involves whole body mechanisms and our assays are limited to the effects on single cells.

Conclusion. To summarise, my study has shown a link between emodin and cancer cell metastasis and invasion. Results here show that emodin reduces cancer cell migration and invasion in-vitro, but not by blocking AQP-5. Emodin may serve as an adjunct to current cancer therapies but future research should explore the mechanisms of target of emodin in CRC and investigate the cross-talk between AQP-5 and the MAPK/ERK pathway and the Arp2/3 complex which would provide us insights on the target that emodin works on.

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