The *In-vitro* effect of Apigenin on Vascular Endothelial Growth Factor and Interleukin-8 production within Caco-2 cells.

This dissertation is being presented in the school of health sciences at Cardiff Metropolitan University toward a BSc (Hons) Biomedical Science degree.

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Declaration

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Statement 2

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>Caco2</td>
<td>Caucasian colon adenocarcinoma (human epithelial colorectal adenocarcinoma cells)</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<td>μM</td>
<td>Micromolar</td>
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Abstract

Background

It has been stated that inflammation is the ‘foundation’ of many diseases and disorders. In recent times, there has been a growing interest in the potential of using natural chemicals such as flavonoids as treatment for these sorts of diseases. Apigenin is a flavonoid that is abundant in many fruits and vegetables. Many studies have supported the claim that apigenin has the potential to be used as an anti-inflammatory agent. In this study the flavonoid was studied on its anti-inflammatory and anti-angiogenic effects in caco-2 cells. The aim of this study was to investigate the role of apigenin in reducing the process of inflammation and angiogenesis via the production of VEGF and Interleukin 8.

Methodology

Apigenin was initially observed on its cell viability effects to help establish a suitable concentration to test throughout the Elisa experiments. 12.5 and 3.125μM of the compound was examined on human epithelial colorectal adenocarcinoma cells (Caco-2) using a Quantikine Duo ELISA to establish the concentration of the inflammatory factors IL-8 and VEGF being secreted by the Caco-2 cells following 6 and 24-hour exposure periods.

Results

Cell viability results revealed that from the concentrations 25μM and above, Apigenin was cytotoxic and had a negative effect on the viability of the cells. There were mixed results with apigenin effect on the production of VEGF and IL-8 in Caco2 cells. In the quantification of VEGF, 12.5μM of Apigenin suppressed the production of VEGF at 4 hours, there was no significant effect of apigenin on VEGF production in Caco2 cells when compared to control cells at the concentration of 3.125 μM.

In the quantification of IL-8 at 24 hours, there was a calculated significant decrease on the production of IL-8 in the cells (p= 0.0130).at the 3.125μM. 12.5μM of apigenin at 24 hours, increased the production of IL-8 when compared to the control (p= 0.001).
Conclusion
The results of this study found that apigenin has anti-inflammatory and anti-angiogenic effects in caco-2 cells by reducing the expression of VEGF and IL-8. Thus, this could be applied further in therapeutic approach in patients with inflammatory diseases by combining apigenin to standard treatment to enhance efficacy. Further investigation is recommended to understand the underlying mechanism and therapeutic dosage of apigenin as it was evident in this study that apigenin has some potential to be used to treat inflammatory diseases including malignant tumours.
1. Introduction

It has been shown extensively known that inflammation plays a vital role in a range of diseases such as diabetes, cardiovascular diseases and cancer \(^1, 2\). There has been a vast amount of interest in dietary compounds as ways of treating these kinds of diseases. These compounds known as nutraceuticals have been found to be able to treat and prevent diseases \(^3, 4\). Nutraceuticals are foods or parts of food that have a healthy benefit as well as nutritional purposes \(^5\). A well-known sub group of nutraceuticals are flavonoids. Flavonoids are well known to be cancer chemo preventive agents. Various studies have found that flavonoids have anti-carcinogenic properties through a variety of mechanisms \(^6, 7, 8, 9\). These include inhibiting tumour growth in many different mechanisms such as by causing cell cycle arrest and causing apoptosis in the cancer cells \(^10\). Flavonoids have also been stated to inhibit cell proliferation as well as angiogenesis in cancer cells \(^11\).

Angiogenesis is a very important biological process. It is the process of forming new blood vessels from a pre-existing vasculature. Angiogenesis is important in both health and also in disease state \(^12\). This therefore means it is a very vital component in a number of process such as reproduction, development as well as wound healing and inflammatory diseases.

Angiogenesis is however also very significant in the growth of tumours. Cancer cells also require the angiogenesis process to grow and spread. All cells including tumour cells require oxygen and nutrients to survive and grow \(^13\).

The process of angiogenesis is turned on and off depending on the state. However excessive angiogenesis can cause a variety of diseases such as psoriasis and a variety of cancers, insufficient angiogenesis can however also

\[\text{Figure 1- excessive angiogenesis vs insufficient angiogenesis on a variety of diseases.}\]
contribute to ulcers, chronic wounds and coronary artery diseases. The effects of excessive or insufficient angiogenesis in a variety of diseases is portrayed in the figure 1.^{14} The angiogenesis process is a very highly regulated biological process that is controlled by molecules that either promote it or inhibit it, a short list of activators and inhibitors of angiogenesis is shown in figure 2.\textsuperscript{15, 16} Some of these molecules that regulate the angiogenesis process are cytokines and growth factors. Cytokines are protein molecules that have an effect on interactions and communications between cells. Cytokines are predominately released at sites of infection. They are produced by t helper cells and macrophages.\textsuperscript{17, 18} Cytokines are key molecules in important immune processes such as angiogenesis. There are many cytokines and growth factors that are involved in angiogenesis. These include Interleukin 1 (IL-1), Interleukin 6 (IL-6), Interleukin 12 (IL-12), Tumour Necrotising Factor (TNF) Interleukin-8 (IL-8) and Vascular Endothelial Growth factor (VEGF).\textsuperscript{19, 20} VEGF is a major growth factor that is involved in the angiogenesis process, it is described as a potent angiogenic factor and is said to promote angiogenesis as well as vasculogenesis. This consequently means it is very useful for processes such as wound healing. However, VEGF has been found to be secreted by tumour cells and thus suggests its major contribution to tumour angiogenesis.\textsuperscript{21, 22} By regulating this growth factor, there is potential to regulate angiogenesis which can lead to novel methods in inhibiting tumour angiogenesis. This would be major breakthrough in cancer prevention and treatment.\textsuperscript{23}

The chemokine IL-8 also plays a major role in the inflammation and angiogenesis processes. It is well known to possess tumourigenic and proangiogenic properties.\textsuperscript{24, 25} IL-8 has been detected in a large number of human tumours and is often associated with poor prognosis of the disease.\textsuperscript{26, 27} Additionally, IL-8 is known as a very potent

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**Figure 2:** activators and inhibitors of angiogenesis.

<table>
<thead>
<tr>
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<th>Inhibitors</th>
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<tr>
<td>IL-8</td>
<td>Angiostatin</td>
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<tr>
<td>VEGF</td>
<td>Interferon</td>
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<td>Angiogenin</td>
<td>Interleukin</td>
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<td>Basic Fibroblast</td>
<td>Thrombospondin 1</td>
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<tr>
<td>Hepatocyte</td>
<td>Endostatin</td>
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<tr>
<td>Placental GF</td>
<td>TIMP 1</td>
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<td>Angiopoietin 1</td>
<td>2-methoxyoestradiol</td>
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pro-inflammatory cytokine. It is essential in acute inflammation as it recruits and activates neutrophils; which are the most abundant and the first white blood cells to travel to sites of infections. IL-8 also plays a key role in neutrophil degranulation. This chemokine has been associated with a variety of inflammatory diseases including obesity, it has been stated that secretion of IL-8 increases with oxidant stress, therefore causing employment of inflammation cells. The study concluded that antioxidants are effective in reducing IL-8 secretion. A flavonoid that is a potent antioxidant may prove to be effective in the regulation of IL-8.

Many studies have demonstrated that one way that flavonoids have anti-inflammatory properties are by inhibiting cytokines and growth factors and thus being a possible therapy for inflammation related diseases such as cancers. Various flavonoids such as Luteolin, Kaempferol, quercetin and apigenin appear to be modulators of pro inflammatory cytokines such as IL-1β, IL-6 and TNF-α through a variety of mechanisms.

One particular flavonoid, Apigenin (4, 5, 7, - trihydroxyflavone) is a flavone that is abundant in a large variety of fruits, vegetables and beverages including onions, oranges, red wine and chamomile tea. There is growing interest in apigenin due to its many chemo preventive properties. It has been recognized as a potent antioxidant and an anti-inflammatory agent. Apigenin is said to be anti-inflammatory by lowering oxidative stress and also by inhibiting the expressing of numerous inflammatory factors. Several studies have also reported the anti-cancer properties of apigenin through its anti-inflammatory mechanisms. A study by Yin et al reported that apigenin was the most effective inhibitors on the growth of human thyroid cancer cell lines, it was stated to inhibit the cell lines by inducing apoptosis on the tumour cells. Additionally, Ruela-de-Sousa et al concluded that apigenin is a potential chemo preventive and chemotherapeutic agent in leukaemia cell lines due to the inhibition of cell proliferation and cell cycle arrest of the tumour cells. Xu et al also found that apigenin suppressed colorectal cancer cell proliferation by inhibiting β-catenin, a protein that is associated with a variety of malignancies.

As previously mentioned, Inflammation is the contributing cause of numerous diseases and disorders, hence controlling it is crucial in the prevention of a wide range of diseases. Being able to control inflammation would also help find novel and innovative
treatments for these diseases\textsuperscript{38}. There is lots of evidence that show that flavonoids have shown a high potential in regulating inflammation and Apigenin is one of these flavonoids that shows the potential to be a potent anti-inflammatory compound\textsuperscript{39,40}.

The aim of this study was therefore to investigate the role of apigenin in reducing the process of inflammation and angiogenesis via the production of VEGF and Interleukin 8. This study hypothesised that Apigenin decreases the production of the growth factor VEGF and the cytokine interleukin 8 in caco-2 cells and therefore inhibits chronic inflammation. Consequently, this will also inhibit angiogenesis and as a result inhibit tumour growth in cancer cells as well.
2 Methodology and materials

2.1 Ethics
The Cardiff Metropolitan University Biomedical Sciences Ethics Panel granted the application for ethical approval.

Project Reference Number:

2.2 Tissue culture and Reagents
Heterogeneous Human epithelial colorectal adenocarcinoma cells (CaCo-2) cells were cultured using minimum essential medium containing earl salts, foetal bovine serum, 1mmol sodium pyruvate, 2 mmol glutamine, 1% non-essential amino acids and antibiotics, streptomycin and penicillin. Apigenin (A3134) was purchased from Sigma Aldrich and dissolved in dimethyl sulfoxide (DMSO), and stored at -20°C. Capture Antibody IL-8 and VEGF (Capture Ab) were purchased from R&D Systems. Detection antibodies for IL-8 and VEGF were also purchased from R&D Systems. Caco-2 cells purchased from European collection of authenticated cell culture, (Ecacc) Public Health Salisbury, UK were maintained in a 5% CO₂ humid incubator at 37°C.

2.3 Cell viability Assay
A cell viability assay was conducted to establish the optimum concentrations for the caco-2 cells to work at. Cells were plated at 10 000 cells per well and cultured overnight until 80% confluence was achieved. The cells were then treated with apigenin at concentrations of 100, 50, 25 12.5, 6.125 and 3.125 μM. Control cells were treated with DMSO only. The cells were then counted after both 24hours and 48 hours with a Tecan M200 infinite multi detection reader using I-control software/excitation of 560nm and emission of 590nm set on optimal gain. Experiments were performed in quadruplicates to allow four sets of data to be obtained for statistical analysis.

2.4 Time course
The cells were primed with API at either 4 hours or at 24 hours and then followed by a 1-hour incubation before there was addition of Interleukin 1 beta (IL-1B). IL-1B is added to cells to induce an inflammatory environment in the Caco-2 cells
2.5 Enzyme-Linked Immunosorbent Assay (ELISA) Quantification of Human VEGF and Human IL-8
The protocol of this was conducted as written by the manufacturer instructions (R &D Systems).

A 96-well Elisa plate was coated with diluted Capture Antibody and incubated overnight at room temperature.

Each well was washed with Wash Buffer three times. Plates blocked by adding Block Buffer to each well and incubated at room temperature for a minimum of 1 hour.

The samples and standards in reagent diluent were added and incubated for 2 hours at room temperature. Detection Antibody was added and diluted in reagent diluent, to each well. And then incubated for 2 hours at room temperature.

Streptavidin-HRP was added to each well, the plate was covered and then incubated for 20 minutes at room temperature. Substrate Solution was added to each well and then Incubated again for 20 minutes at room temperature. A Stop Solution was then added to each well. The optical density of each well was then determined immediately, using a microplate reader set to 450 nm.

2.6 Statistical Analysis
Graphs of data were expressed as a mean of independent triplicates ± standard deviation. Data Analysis was conducted using the Microsoft Excel and Graph Pad Prism programs. The one-way ANOVA test was used to find significant difference amongst the means. A Multiple comparison test was used as a follow up to calculate the comparisons between the different concentration groups to find significances between them. Values of $P > 0.05$ were considered to be of significance.
3. Results

3.1. Apigenin Inhibit Caco-2 cells at Concentrations over 25μM.

The effect of Apigenin on caco-2 cells was studied over a time period of 24 hour and 48 hours. The Caco-2 cell line was treated with a variety of Apigenin concentrations, these concentrations were 100, 50, 25, 12.5, 6.25 and 3.125μM. Figure 1 and Figure show the results of the concentration on the viability of the Caco2 cells over 24 hours and 48 hours respectively.

Firstly, Apigenin show cytotoxic effects at 100, 50 and 25 μM over the 24 hr time periods. Each bar is a mean of four replicates which is then plotted against viability using a cells only as the control. A one way Anova test found Significance between the means was p > 0.001. This is indicated in the figure 1.

Over the 48-hour time period, Apigenin also showed cytotoxic effects at 100, 50, 25 and a slight effect at 12.5 μM. A One-way Anova ad tukey’s multiple comparison test found that the Significance between the means was calculated as p> 0.001. This is shown in Figure 2.
Effect of different apigenin concentration on viability of caco-2 cells at 24 hours and 48 hours

**Figure 3** - Cell viability assay at 24 hours one way Anova and Tukey’s multiple comparison test used to prove significance.

**Figure 4** - Cell viability assay at 48 hours. One way ANOVA and Tukey’s multiple comparison tests to prove significance.
Due to the results of the cell viability assay, the concentrations of 12.5 and 3.125 μM were chosen as the concentrations to study the effect of Apigenin on inflammation in the Enzyme-Linked Immunosorbent Assay (ELISA). Interleukin 1 beta IL-1B was used to induce inflammation in the caco-2 cells. The cells were then treated with Apigenin at concentrations of either 12.5 or 3.125 μM. Cytokines were measured to see the inflammatory effect of Apigenin. Interleukin 8 (IL-8) and Vascular Endothelial Growth Factor (VEGF) were the cytokines used to see this effect.

3.2. Quantification of VEGF using ELISA

IL-1B is used in Elisa as it is a cytokine that initiates an inflammation environment in cells ⁴¹.

3.2.1 12.5μM of Apigenin decreases the concentration of VEGF at 4 hours in Caco-2 cells.

At the concentration of 12.5μM, Apigenin decreased the concentration of VEGF in the Caco-2 cells at 4 hours. As indicated in the figure 2, the concentration of VEGF decreased when compared to the IL-1B and cells only. There was a significant between the IL-1B group and the IL-1B with 12.5 μM of Apigenin added. Significance was calculated as <0.001. IL-1B stimulates the production of VEGF and consequently induces an inflammatory environment. When 12.5μM was added to the IL-1B, the Concentration of VEGF decreased significantly in Caco-2 cells.

3.2.2 12.5 μM of Apigenin increases the concentration of VEGF at 24 hours in Caco-2 cells.

At 24 hours, there was an opposite result, when compared to just the IL-1B added to the Caco-2 cells, the Caco-2 cells with IL-1B and 12.5 μM of Apigenin added, there was an increase of VEGF. However a multiple comparison test found that there wasn’t a significant difference between the two groups (p< 0.9805). Further experiments are hence needed for a conclusion to be drawn.
Figure 5. VEGF concentration in caco-2 cells treated with 12.5μM and either in the presence or absence of IL-1B over 4 hours and 24 hours. ANOVA and Tukey’s multiple comparison test demonstrated a significant different between control cells treated with IL-1B and cells treated with IL-1B and 12.5μM of apigenin at 4hours. (p=0.002) results are shown as ±SD of the means. (*) =0.05 (**) = 0.005 and (***) = 0.001
3.2.3 3.125 μM of Apigenin decreases the concentration of VEGF at 4 hours in Caco-2 cells.

At the concentration of 3.125 μM, Apigenin decreased the concentration of VEGF in the Caco-2 cells at 24 hours. As indicated in the figure 4, the concentration of VEGF decreased when compared to the IL-1B and cells only. However further statistical analysis found there was not a significant difference between the IL-1B group and the IL-1B with 12.5 μM of Apigenin added. Significance was calculated as <0.6207. IL-1B stimulates the production of VEGF and consequently induces an inflammatory environment. When 3.125 μM of Apigenin was added to the IL-1B, the VEGF decreased insignificantly in the Caco-2 cells.

3.2.4 3.125 μM of Apigenin increases the concentration of VEGF at 24 hours in Caco-2 cells.

Furthermore At 24 hours, there was also an opposite result, when compared to just the IL-1B added to the Caco-2 cells, the Caco-2 cells with IL-1B and 3.125μM of Apigenin added, there was an increase of VEGF. However a multiple comparison test found that there wasn’t a significant difference between the two groups (p<0.1431). Further experiments are thus needed for a conclusion to be drawn.
Figure 6. VEGF concentration in caco-2 cells treated with 12.5 μM and either in the presence or absence of IL-1B over 4 hours and 24 hours. Anova and Tukey’s multiple comparison test did not find significant difference between control cells treated with IL-1B and cells treated with IL-1B and 3.125 μM of apigenin at either 4 hours or 24 hours.

3.3 Quantification of IL-8 using Elisa.

3.3.1 12.5μM of Apigenin increases Interleukin 8 concentration at 4 hours in Caco-2 cells.

At the concentration of 12.5 μM, Apigenin decreased the concentration of IL-8 in the Caco-2 cells at 4 hours. As can be seen in figure 4, the concentration of IL-8 increased slightly when compared to the IL-1B and cells only. There was not a significant between the IL-1B group and the IL-1B with 12.5 μM of Apigenin added. Significance was calculated as <0.9999. In summary, when 12.5μM was added to the IL-1B, the Concentration of Il-8 increased slightly albeit insignificantly in the Caco-2 cells.
3.3.2 12.5μM of Apigenin increases Interleukin 8 concentration at 24 hours in Caco-2 cells

Additionally, there was also an increase of IL-8 when 12.5μM of Apigenin was compared to the cells only with IL-1B added over the 24-hour period. This can also be seen in figure 5. The difference between the two groups was found to be significant using statistics analysis. Significance was calculated as p> 0.001.

![Quantification of IL-8 with 12.5 μM of Apigenin](chart)

**Figure 7.** VEGF concentration in caco-2 cells treated with 12.5μM and either in the presence or absence of IL-1B over 4 hours and 24 hours. Anova and Tukey’s multiple comparison test found significant difference between control cells treated with IL-1B and cells treated with IL-1B and 12.5μM of apigenin only at 24 hours (p= 0.0001).
3.3.3 3.125μM of Apigenin decreases Interleukin 8 concentration at 4 hours in Caco-2 cells.

3.125 μM of Apigenin decreased the concentration of IL-8 at 4 hours when compared with the cells and IL-1B added. This can be seen in figure 6. There however was not a significant difference between the two groups. Significance was calculated to be p <0.4032. Further studies are therefore needed for a conclusion to be drawn.

3.3.4 3.125 μM of Apigenin decreases Interleukin 8 concentration at 24 hours in Caco-2 cells

Moreover, 3.125μM of Apigenin decreased the concentration of IL-8 at 24 hours when compared with the cells and IL-1B added. This is also indicated clearly on Figure 6. This increases was calculated to be significantly different using statistical analysis. P values was calculated to be p= 0.0130. Thus, confidence is shown in in this result.

**Figure 8.** IL-8 concentration in caco-2 cells treated with 3.12μM and either in the presence or absence of IL-1B over 4 hours and 24 hours. Anova and Tukey’s multiple comparison test found significant difference between control cells treated with IL-1B and cells treated with IL-1B and 3.125 μM of apigenin only at 24 hours (p= 0.0130)
Discussion

Inflammation and Angiogenesis are very vital factors in a number of processes, including wound healing and tumour growth. By finding a way to control these processes, tumour growth can be inhibited, resulting in cancer prevention. There is sufficient evidence that shows that consumption of diets rich in fruit and vegetables due to their abundance of flavonoids such as apigenin, this is associated with decreased risks in inflammatory diseases such as varieties of malignant tumours.

In this study, the effect of the flavonoid apigenin on inflammation was studied via the production of two angiogenic factors, VEGF and the chemokine IL-8. The aim of the study was to see what effect the flavonoid apigenin had on the production of VEGF and IL-8.

To begin with, the viability of the Caco-2 cells was tested over a variety of Apigenin concentrations, a cell viability assay was performed to understand how varying concentrations affect the cells. It is also crucial in finding what concentration of the compound is toxic to the cells. In this current study, concentrations of 100, 50, 25, 6.25 and 3.125 μM were chosen in half down dilutions. Results show that over a 24-hour period, Apigenin concentrations of 100, 50 and 25 μM had a cytotoxic effect on the Caco-2 cells. Over the 48-hour period, there was also a slight cytotoxic effect at 12.5Μm, however this was not found to be significant when compared with 6.25μM and 3.125 μM.

Previous studies on the effect of apigenin on Caco-2 cells support the results from this study. Yang et al. found that at concentrations over 20μM, Apigenin had a cytotoxic effect as the viability of the Caco-2 cells was very low when compared to the Caco-2 cells without the treatment of Apigenin. Apigenin at concentrations over 40 μM has been reported to be very active in reducing cell viability in other cancer cell lines. Horinaka et al. reported that apigenin had a cytotoxic effect on jurkat cell lines, a leukaemia cell line. Apigenin was said to be cytotoxic to these cells by inducing DR5 expression through p53-independent regulation which induces apoptosis in the tumour cells. Jayasooriya et al. also stated that apigenin induces an inhibition of cell growth and attenuates telomerase activity in human leukaemia cells. Another mechanism
in which Apigenin is said to be cytotoxic to cancer cell lines is by causing cell cycle arrest in the G2/M phase 49.

In other cell lines, Zhu et al found that at concentrations of up to 20 μM, apigenin did not induce a significant effect on T-24 bladder cells. However, at concentrations of 40 and 80 μM, apigenin reduced cell viability by inducing apoptosis in the T24 bladder cells by up to 22.2% 50. This was supported by Shi et al in 2015, who also found that Apigenin reduced the viability of the T-24 cell lines at higher concentrations > 40 51.

Consequently, 12.5 and 3.125 μM were chosen as the concentrations of Apigenin to be used carrying out an ELISA technique. IL-8 and VEGF were the two factors used to see the effect of apigenin on inflammation via production. These two factors were chosen for the ELISA because of their properties and functions. VEGF is a growth factor that is active in the process of angiogenesis whilst IL-8 is a very active chemokine in the innate immune system and as previously mentioned is also a promotor of angiogenesis 19, 20, 22, 23.

In the quantification of VEGF ELISA, the effect of apigenin on production of the growth factor were analysed, the two concentrations of 12.5 and 3.125 μM were analysed over different period of time. Both concentrations were analysed at 4 hours and 24 hours.

There were mixed results over the 4-hour period and the 24-hour period. 12.5μM of Apigenin significantly suppressed the production of VEGF at 4 hours (p= 0.002) whilst there was a higher production of VEGF at 24 hours. However, this result was not calculated to be significant. 3.125μM of apigenin had no significant effect on the production of VEGF in Caco-2 cells at 4 hours and at 24 hours.

Previous studies have found that apigenin reduces the production of VEGF in other tumour cell lines. It was reported by Osada et al that apigenin reduced the production of VEGF in human umbilical artery endothelial cells whilst Liu et al also found that apigenin inhibited the production of VEGF in human lung cancer cell lines resulting in the possibly inhibitory effects of angiogenesis, consequently preventing tumour growth 52.

This however seems to be dependent on the concentration of the apigenin, in the previous studies, significant effect of apigenin on VEGF is frequently seen at
concentrations from 10μM to 25 μM, any higher and it might start to have an effect on the viability of the cells \textsuperscript{53,54,55}. This suggests that apigenin may contain an anti-cancer agent, but has a dose dependent response. It is worth mentioning that in natural sources of apigenin there are no traces at these concentrations.

This supports the findings from this study as at 3.125μM, there was no significant effect on the production of VEGF at 4 hours and at 24 hours when compared to the cells only control. However, at 12.5μM there was a significant effect on the production of VEGF at 4 hours when compared to the cells only control and there was a slight increase but not a significant effect on the production of VEGF at 24 hours when compared to the cells only. Apigenin is said to inhibit VEGF by targeting the receptors on the HIF-1 pathway. Apigenin inhibits VEGF transcriptional activation through the HIF-1 binding site, and specifically by decreasing HIF-1α via the PI3K/AKT/p70S6K1 and HDM2/p53 pathways \textsuperscript{56}. Due to the contradictory results at both 4 hours and 24 hours, it would be interesting to see the effect of apigenin on the production of VEGF over a longer period of time for example at 36 hours or at 48 hours.

The effect of apigenin on the production of the chemokine IL-8 was also determined using an Elisa. The two concentrations of 12.5 and 3.125μM were also analysed over different period of time. 12.5μM of apigenin had no significant effect on the production of IL-8 in Caco-2 cells when compared to the cells only control at 4 hours, however at 24 hours, apigenin increased the production of IL-8 when compared to the control (p=0.001).

3.125μM of apigenin had a different effect on the production of IL-8, at 4 hours there was a slight decrease on concentration of apigenin when compared to the control however statistical analysis did not deem this to be significant enough, at 24 hours, there was a calculated significant decrease on the production of IL-8 in the cells (p=0.0130). These results are not in correlation with previous studies, Farkas et al concluded that apigenin inhibited IL-8 production in IPEC-J2 cells as well in Caco-2 cells but this response was found to be dose dependent. 25 μM of apigenin was the concentration found to be the most effective compared to this study where the most effective concentration was found to be 3.125μM \textsuperscript{57}. This suggests that apigenin induced a dose and time response as it only inhibited the expression of IL-8 at 3.125 μM and only at 24 hours.
In other cell lines, apigenin has been said to have a negative effect on the production of IL-8, Wang et al found that apigenin inhibit the expression of IL-8 in DEHP-stimulated human umbilical vein endothelial cells. The study suggested that apigenin could therefore be used as therapeutic means for the treatment of inflammatory diseases such as DEHP-associated allergic disorders 58.

As it was with the VEGF, apigenin seems to induce a dose dependent response on the expression of IL-8, various studies have reported an inhibition on the expression of IL-8 but only at specific concentrations as seen in this study. In a recent study, Nicholas et al found that apigenin did not induce an inhibitory effect on the production of IL-8 at concentrations below 10μM, significant effect was seen at concentrations above 10μM. The study concluded that apigenin inhibited the release of inflammatory cytokines namely IL-8, the mechanism in which apigenin has been said to inhibit IL-8 expression is by inhibiting the NF-κB pathway, it is said to occur by suppressing the p65 phosphorylation 59, 60, 61.

The NF-κB pathway has been considered as the vital pathway for anti-inflammation studies 62, 63. If it can be proven that apigenin reduces inflammation via this pathway, it could be an innovative therapy in controlling chronic inflammation. There is some evidence in this study that apigenin may induce the inhibition of the pro-inflammatory factors VEGF and IL-8. Further research is therefore recommended on the flavonoid apigenin as there is some evidence it inhibits both inflammation and angiogenesis. In the further studies, additional tests could be implemented which would give a greater understanding of how the flavonoid induces its effect. Tests such as a scratch assay would assist in find out more about the effect of apigenin on angiogenesis as it would allow the effects of apigenin on wound healing to be understood. Molecular biology could be implemented in understanding the underlying mechanism in which apigenin inhibits the inflammatory factors. For example western blotting could be used as it can allow more understanding of the signal pathways and which proteins are involved in the inhibition of both angiogenesis and inflammation 64. Understanding of the mechanisms in which this inhibition occurs would therefore allow anti-inflammatory and chemo preventive drugs to be developed.
In conclusion, the results of this study found that apigenin has anti-inflammatory and anti-angiogenic effects in caco-2 cells by reducing the expression of VEGF and IL-8. Thus, this could be very useful in further studies in therapeutic approach in patients with inflammatory diseases such as various cancers by combining apigenin to standard treatment to enhance effectiveness. Moreover, further investigation is recommended to understand the underlying mechanism and the therapeutic dosage of apigenin as it was evident in this study that apigenin has a dose effect response and so supplementary research on which dose works best is recommended. It is very clear apigenin has some potential to be used to treat inflammatory diseases including malignant tumours.

In summary, there is some evidence that apigenin can inhibit the expression of both the growth factor VEGF and the cytokine IL-8. Further research is however recommended both in the Caco-2 cell line as well as other cell lines.
References


