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INVESTIGATING THE EFFECTS OF  
NEW LYSYL OXIDASE (LOX) AND  
LYSYL OXIDASE LIKE 2 (LOXL2)  
INHIBITORS ON CLEAR CELL RENAL  
CELL CARCINOMA INVITRO

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A thesis submitted in fulfilment of the requirements of the Manchester Metropolitan University for the degree of Master of Science (by Research).

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## ABSTRACT

Renal cell carcinoma (RCC) is a common type of cancer worldwide. It is found mainly in adults, and its incidence in males is higher than in females. RCC has different histological subtypes, the commonest (>80%) being clear cell RCC (ccRCC), which originates in the proximal convoluted tubes. ccRCC is highly resistant to chemotherapy. Even targeted therapies do not lead to complete responses in the majority of the patients. New treatment options to ccRCC patients need to be developed.

The lysyl oxidase family consists of lysyl oxidase (LOX) and LOX like enzymes (LOXL). LOX and LOXL2 have varying degrees of similarity in their structures and functions. They catalyse the crosslinking of the extra cellular matrix (ECM) proteins and promotes tumour progression and metastasis. The critical roles of LOX and LOXL2 in cancer (including ccRCC) development potentiate them as therapeutic targets.

In this project, anti-cancerous properties of novel compound-1, 4 and 5 were investigated which include studying their effects on the proliferation, viability, adhesion, migration, and apoptosis of Caki-2 cells in vitro, as well as their regulation on the expression of LOX and LOXL2 and cellular signalling pathways.

The results show that compound-1, 4 and 5 significantly reduced the expression of LOX/LOXL2 and LOX activity in Caki-2 cells. All the three compounds displayed various degrees of anti-proliferation, anti-migration, inhibition of cell-ECM adhesion, and induction of apoptosis effects. The anti-ccRCC effects of compound-4, such as hindering cell cycle progression and inducing cell apoptosis, were stronger than compound-1 and 5 in vitro. The studies on cellular signalling pathways revealed that RRM2, CDKN1B, CXCL16 and CD98HC were significantly downregulated in all three compounds treated cells in comparison to the negative control. The expressions of MMP9 and CXCL12 were downregulated in compound-4 and 5 treated cells, but upregulated in compound-1 treated cells.

In conclusion, this study showed the three novel compounds are potential inhibitors of LOX/LOXL2. They displayed variable anti-cancer activities in vitro. They may provide potential therapeutic strategies for the treatment of cancer by inhibiting LOX/LOXL2. Compound-4 might hold greater therapeutic promise for ccRCC treatment in the future.



## ABBREVIATIONS AND ACRONYMS

<b>LOX</b>	<b>LYSYL OXIDASE</b>
<b>LOXL2</b>	<b>LYSYL OXIDASE-LIKE 2</b>
<b>RCC</b>	<b>RENAL CELL CARCINOMA</b>
<b>CCRCC</b>	<b>CLEAR CELL RENAL CELL CARCINOMA</b>
<b>HIF</b>	<b>HYPOXIA INDUCIBLE FACTOR</b>
<b>VHL</b>	<b>VON-HIPPEL LANDAU</b>
<b>FH</b>	<b>FUMARATE HYDROTASE</b>
<b>BHD</b>	<b>BIRT-HOGG-DUBE</b>
<b>ARCD</b>	<b>ACQUIRED RENAL CYSTIC DISEASE</b>
<b>ESRD</b>	<b>END STAGE RENAL DISEASE</b>
<b>DM</b>	<b>DIABETES MELLITUS</b>
<b>TSGS</b>	<b>TUMOUR SUPPRESSOR GENE</b>
<b>EMT</b>	<b>EPITHELIAL MESENCHYMAL TRANSITION</b>
<b>PBRM1</b>	<b>POLYBROMO-1</b>
<b>BAP-1</b>	<b>BRC-1 ASSOCIATED PROTEIN-1</b>
<b>SETD2</b>	<b>SET DOMAIN CONTAINING 2</b>
<b>IL-2</b>	<b>INTERLEUKIN</b>
<b>IFN-A</b>	<b>INTERFERON ALPHA</b>
<b>SRCR</b>	<b>SCAVENGER RECEPTOR CYSTEINE-RICH</b>
<b>PRO LOX</b>	<b>PRO-PROTEIN</b>
<b>LTQ</b>	<b>LYSYL-TYROSYL-QUININE</b>
<b>CRL</b>	<b>CYTOKINE RECEPTOR-LIKE</b>

<b>MMTV</b>	<b>MOUSE MAMMARY TUMOUR VIRUS</b>
<b>MMPS</b>	<b>MATRIX METALLOPROTEINASES</b>
<b>ECACC</b>	<b>EUROPEAN COLLECTION OF AUTHENTIC CELL CULTURES</b>
<b>FBS</b>	<b>FOETAL BOVINE SERUM</b>
<b>CCK-8</b>	<b>CELL COUNTING KIT-8</b>
<b>DMSO</b>	<b>DIMETHYL SULFOXIDE</b>
<b>RNA</b>	<b>RIBONUCLEIC ACID</b>
<b>CDNA</b>	<b>COMPLEMENTARY DNA</b>

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# APPENDICES

**Appendix 1:** Microscopic images showing Caki-2 cell morphology at 20% confluence

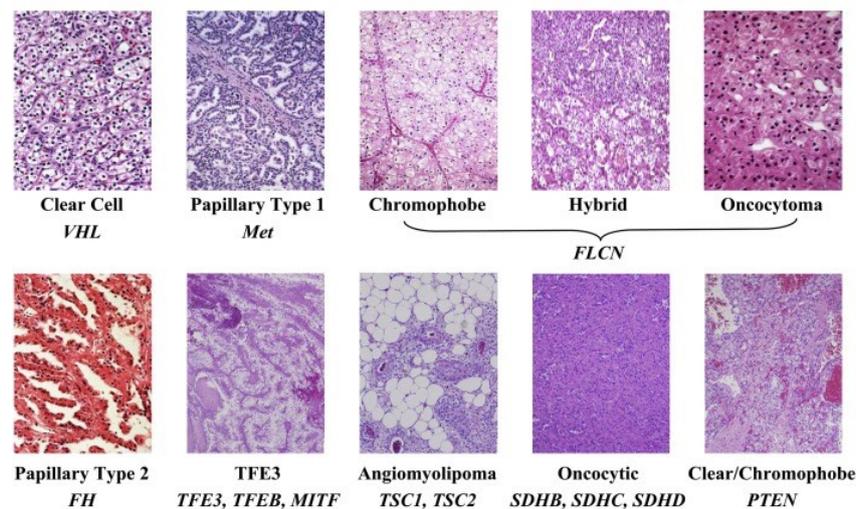
**Appendix 2:** Microscopic images showing Caki-2 cell morphology at 90% confluence



## 1. Introduction

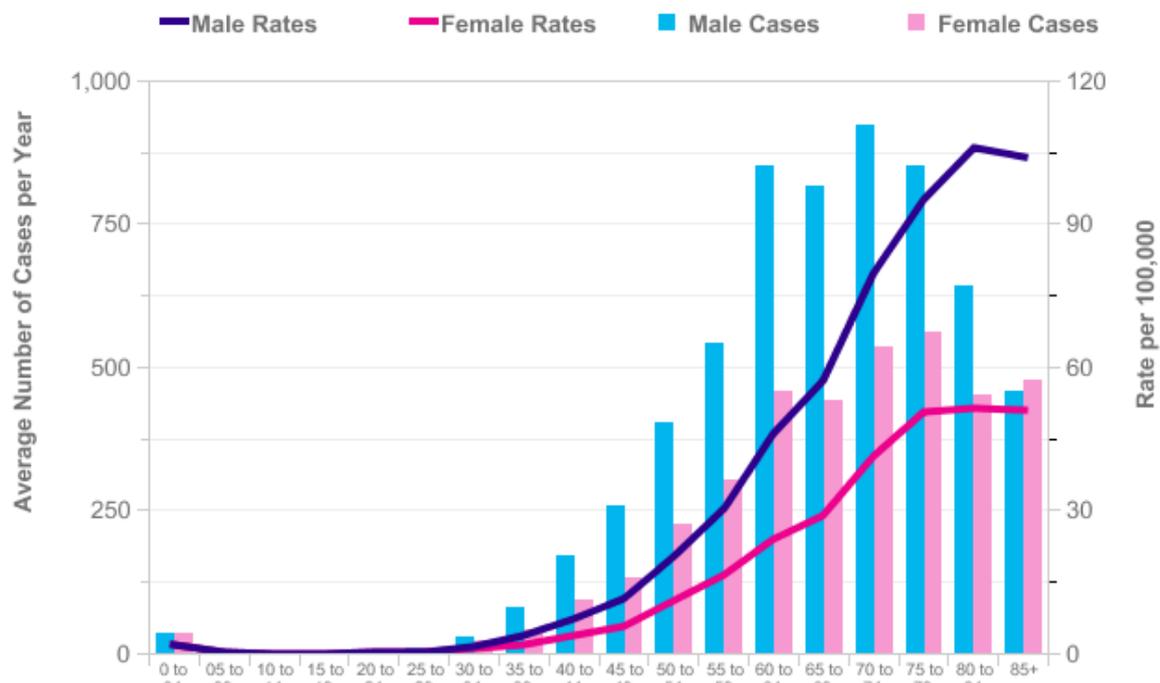
Kidney cancer is a type of malignancy that occurs in the tissue of the kidneys; Kidney cancer has an incidence of approximately 270,000 worldwide annually, and causing over 115,000 deaths every year (Ferlay *et al*, 2014). They consist of a number of histologically and genetically distinct types of cancers (Figure 1.1). Each type differs in its clinical course and responses to therapy. A patient presenting with a small cancer localized in the kidney, can be treated by surgery and can expect a 5 to 10-year 95% chance of survival. However, nearly 80% of patients presenting with advanced kidney cancer, in which the primary tumour has spread to other organs, will die within 24 months (Pascual.D and Borque, 2008).

### Human Renal Epithelial Neoplasms



**Figure 1.1:** Histological images showing human epithelial neoplasms of the different types of renal cell carcinoma (Taken from Bottaro, 2005).

There were 12,000 cases of kidney cancer in the UK alone in 2014. Approximately, 7,839 (63%) of kidney cancers occur in males and 4,684 (37%) in females, which comes to a male/female ratio of around 17:10 (Figure 1.2). The major incidence is in the sixth and seventh decades of age (Rubagotti *et al*, 2006).



**Figure 1.2:** The incidence of kidney cancer in the UK comparing rates of male to female cases and rates per 100, 000 (The image was taken from Kidney Cancer UK, 2018).

### 1.1 Renal cell carcinoma

Renal cell carcinoma (RCC) arises from the renal epithelial cells which form the lining of the very small tubules in the kidney. RCC accounts for about 85% of kidney cancers. A quarter of patients present with locally invasive or metastatic RCC. Median survival for patients with metastatic disease is about 13 months. The pathogenesis of RCC is not clearly understood; although it has been suggested that the disease may be related to aspects of an individual's lifestyle including smoking cigarettes, obesity, hypertension, End Stage Renal Disease and diabetes (Maher, 2013; Di Stefano *et al*, 2016).

### 1.2 Clear cell renal cell carcinoma (ccRCC)

Clear cell renal cell carcinoma (ccRCC) is the main histological subtype of RCC (Figure 1.3). It originates mainly in the proximal convoluted tubules and it is closely associated with mutations of Von-hippel Lindau (*VHL*) gene. *VHL* mutations lead to stabilization of hypoxia

inducible factors (HIF-1 $\alpha$  and HIF-2 $\alpha$ , also known as HIF1A and EPAS1) in both the sporadic and familial forms of ccRCC. (Maher, 2013; Di Stefano *et al*, 2016).

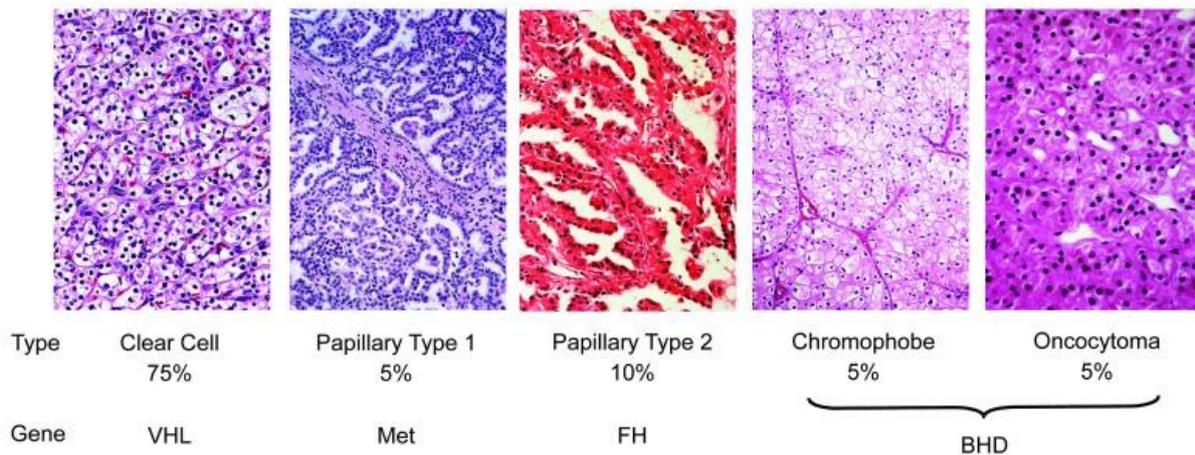
### **1.2.1 Etiology of ccRCC**

The etiological factors associated with ccRCC are gender, ethnic background, smoking, obesity and increased blood pressure.

The gender of an individual is one of the etiological factors affecting the incidence of ccRCC. It is generally reported to be higher in males compared to females. Also, an individual's ethnic background is a common factor in the development of ccRCC. The incidence is commoner in Europeans and northern American white populations compared to those of Asian countries where the incidence is lower. However, this difference in ethnic groups may be due to the variations in the frequency of diagnostic imaging, access to health care and the presence of other potential risk factors such as genetic differences. A large number of studies have established a strong link between smoking and ccRCC (Moore *et al*, 2011).

Meta-analysis of many studies supports a strong link between excess body weight as identified by measuring the body mass index (BMI) and ccRCC. Strong evidence has also shown a link between elevated blood pressure and ccRCC. The strong evidence is also greatly linked to the continuous use of antihypertensive drugs which increases the risk of ccRCC (Moore *et al*, 2011).

## Human Renal Epithelial Neoplasms



**Figure 1.3:** The percentage frequencies of the renal carcinoma subtypes. The genetic basis of cancer of the kidney. The Von-Hippel Lindau (*VHL*) gene is a tumor suppressor which has a critical role in acting as a 'gatekeeper' for the regulation of growth and differentiation of human kidney cancer cells (Kaelin and Maher, 1998). *Met* receptor tyrosine kinase is a receptor specifically for the Hepatocyte growth factor. *Met* has been suggested to be responsible for the growth, invasion and metastasis of many tumours including Papillary type 1 RCC, where the *Met* is upregulated in RCC cell lines ((Lutterbach *et al*, 2007). The Fumarate hydratase (*FH*) gene is a hereditary kidney cancer gene. The loss of function and mutations that occur in the *FH* gene have been suggested to be associated with the development of hereditary papillary RCC. Close inspection of the gene pathway suggests that it is strongly deregulated in papillary RCC compared to other RCC types (Sudarshan, Linehan and Neckers, 2007). Birt-Hogg-Dube (*BHD*) is a syndrome inherited genodermatosis which is characterized by a predisposition to RCC. It has been suggested that the loss of heterozygosity at the *BHD* locus occurs in approximately 17% of RCC. Results also suggest that *BHD* acts as a tumor suppressor gene which leads to the development of RCC when both copies of the gene become inactivated (Vocke *et al*, 2005). (The images were taken from Battaro, 2005).

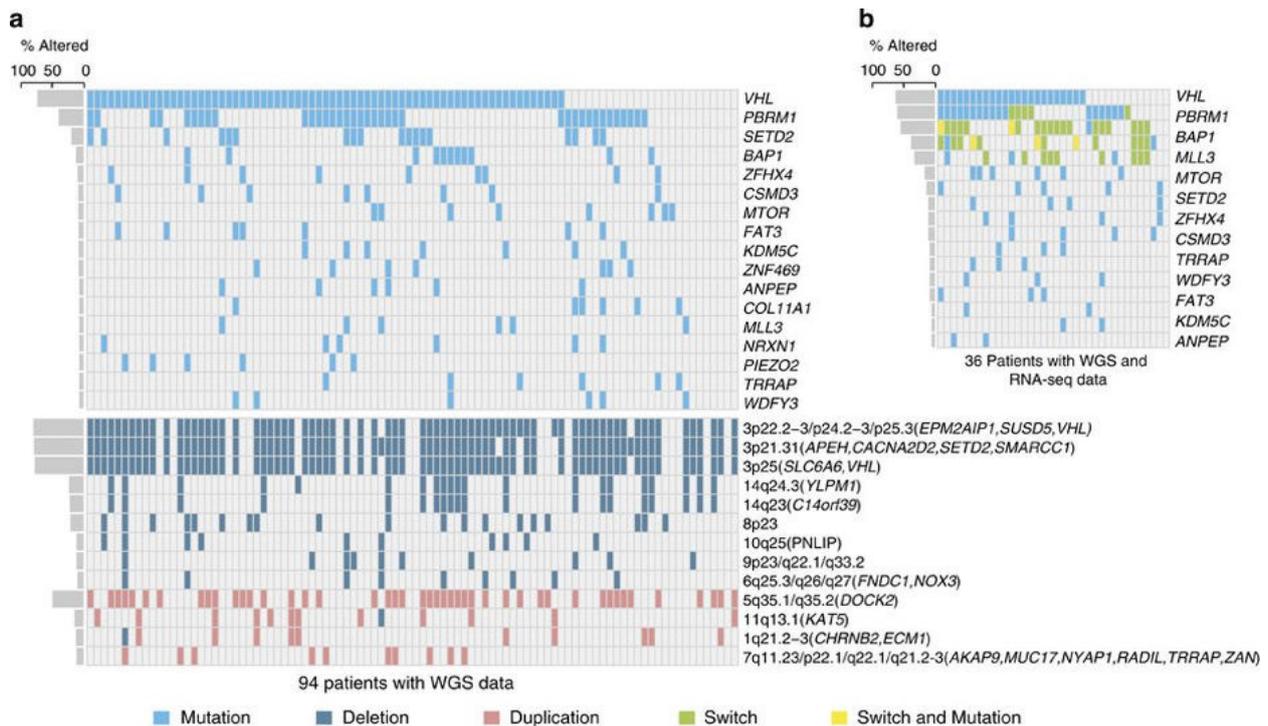
### 1.2.2 Etiology of other diseases that lead to ccRCC:

Acquired renal cystic disease (ARCD) consists of multiple hyperplastic renal cysts on each of the patient's kidneys. It is mostly found in patients with end stage renal disease (ESRD) and is seen in patients who are on a long-term haemodialysis. Therefore, ARCD is most often associated with adenomas and can eventually progress to ccRCC. The incidence of ccRCC in ARCD is much higher in the general population, which increases three to six times and in severe cases may increase twenty times. This suggests that ARCD is definitely a high risk factor for ccRCC.

Diabetes mellitus (DM) has been reported to be associated with increased risk of ccRCC with a relative risk of 1.3 in males compared to 1.7 in females (Fu *et al*, 2011).

### 1.2.3 The molecular basis of ccRCC:

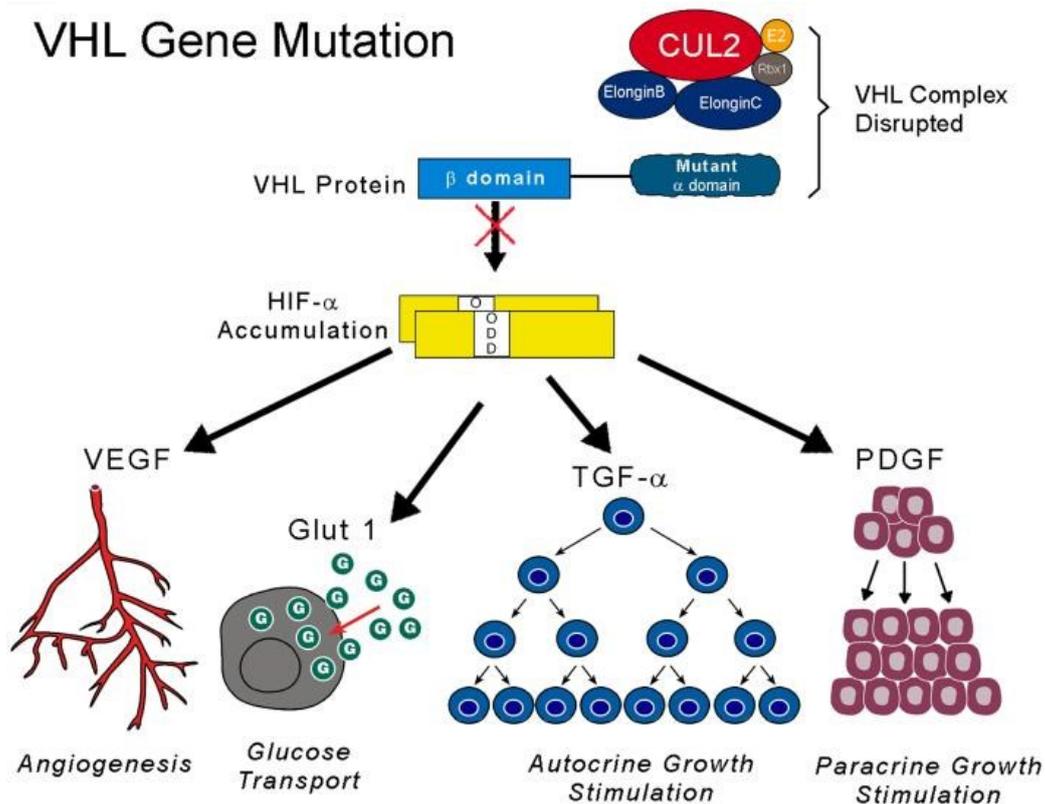
Unlike many other cancers, tumour suppressor genes (*TSGS*) such as *TP53* and *RB1* are not as frequent in ccRCC. Mutations in *TP53* and *RB1* are thought to be found in only 11% of all types of kidney cancers and *TGS* and *CDKN2A* are only mutated in 10% of the ccRCC tumours. The *VHL* gene product and its regulation of the hypoxia inducible factor (*HIF*) is closely linked to the biology of ccRCC. Establishing the link between the *VHL* gene the *VHL* syndrome of the central nervous system hemangioblastomas, pheochromocytoma/ paraganglioma and ccRCC in 1993 rapidly led to the discovery that the *VHL* mutations tightly associated with sporadic ccRCC are present in 90% of the tumours. Loss of the *VHL* gene function leads to the loss of regulation of the HIF family members including HIF1 $\alpha$ , HIF2 $\alpha$  and HIF3 $\alpha$ . Studies conducted using Xenograft showed that the restoration of the VHL expression or suppression of the down regulated HIF2 $\alpha$  leads to the impairment of these tumours (Srinivasan *et al*, 2015).



**Figure 1.4:** The Patterns of somatic variations in ccRCC samples and all the genes that are altered and lead to the formation of ccRCC communications. (Image taken from Munari *et al*, 2014).

### 1.2.3.1 VHL and HIF

The tumour suppressor gene *VHL* is located on chromosome 3p25 and encodes two functionally similar proteins (*pVHL*) of ~ approximately 28-30kDa and 19kDa, which arise from; alternative translation initiations. *pVHL* is a factor with multiple functions but acts mainly as an adaptor protein which guides proteins to their different targets many cellular processes including angiogenesis, metabolism, glucose uptake, the suppression of epithelial to mesenchymal transition (EMT), apoptosis transcription regulation, prevention of aneuploidy and secretion of components from ECM. Its best known function is to regulate ubiquitination and proteasomal degradation of HIF.



**Figure 1.5:** The molecular targets of the VHL pathway in clear cell renal carcinoma and the mutation of the VHL gene in clear cell kidney cancer (Image taken from Linehan, 2004)

Germline mutations of the *VHL* gene are spread across three exons and the most common mutations that occur are missense-mutations followed by non-sense mutations. Sporadic ccRCC has been characterized by the inactivation of the VHL gene by mutation, deletion or promoter hyper-methylation in 70% of tumours. Mutations in the *VHL* leads to HIF1 $\alpha$  and HIF2 $\alpha$  deregulation, effects on the rate of cell growth (Figure 1.5). The VHL mutant tumours that expressed both HIF1 $\alpha$  and HIF2 $\alpha$  (H2 tumours) showed over expression of the AKT/mTOR pathway, while the VHL mutant tumours which only expressed HIF2 (H2 tumours) showed a more rapid replication, and these tumours were marked by over expression of Ki-67. Therefore, ccRCC can be characterized as H1H2 or H2 types, each of which has a different rate of tumour cell proliferation. The first whole sequencing study of ccRCC confirmed that genetic heterogeneity exists in ccRCC. This emphasizes that even though the vast majority of ccRCC contain mutated VHL, it is, however likely that the majority of patients with ccRCC have a unique gene signature. Although the mutated VHL

gene alone is unable to cause sporadic ccRCC mutations that occur within the gene have been consistently considered as clear biomarkers (Baldewijns *et al*, 2010).

### **1.2.3.2 Hypoxia-driven tumorigenesis of ccRCC**

Low oxygen levels lead to the accumulation and inactivation of HIF $\alpha$ . HIF $\alpha$  forms complexes with HIF $\beta$  which leads to the inactivation of a number of genes involved in the angiogenesis process (such as VEGFA), proliferation process (TGF- $\alpha$  and EGFR), cell migration and invasion (CXCR4) and metabolic shifts towards glycolysis (Glut1), which all eventually contribute to ccRCC tumorigenesis (Figure 1.5). The activated HIF $\alpha$  pathway is seen as the key pathway that leads to ccRCC due to its ability to activate multiple oncogenic pathways and its involvement in establishing abnormal epigenome sequences (Atala, 2012).

### **1.2.4 upregulated genes in ccRCC**

In this study, some upregulated genes, which are associated with the in development and aggressiveness of ccRCC, were tested following the treatment of potential LOX-inhibitors. The changes of the gene expression profile would indicate the underlying mechanisms of the LOX-inhibitors. These genes and their association with ccRCC are outlined below.

#### **Heat shock-protein (*HSP90*) and ccRCC:**

Heat shock protein-90 encoded by *HSP90* is an essential molecular chaperone in eukaryotes. It is involved in the activation of a group of proteins referred to as “clients, and HIF is one of the client proteins. *HSP90* is regarded as a master regulator of the hypoxic responses and plays a critical role and progression of ccRCC. It has been shown that perturbing *HSP90* function promoted HIF-1 $\alpha$  and HIF-2 $\alpha$  protein degradation in ccRCC (Bohonowych *et al*, 2010; Woodford *et al*, 2016).

#### **Ribonucleotide reductase M2 subunit (*RRM2*) and ccRCC:**

*RRM2* encodes for both large and small subunits of the human ribonucleotide reductase (RNR) complex. *RRM2* in general is overexpressed in ccRCC. It enhances ccRCC tumour to become chemo-resistant and initiates cellular invasiveness. Many studies have been conducted in order to suppress the *RRM2* gene activity (Abudawood *et al*, 2013; Mark S, 2004).

**Hepatocyte growth factor receptor gene (*c-MET*) and ccRCC:**

*c-MET* proto-oncogene encodes for the cell-surface receptor tyrosine kinase. *c-Met* and its ligand, hepatocyte growth factor, have been implicated in ccRCC tumour development, invasion, migration and angiogenesis. *c-MET* overexpression is also linked with the dysregulation of the VHL /HIF signalling pathway and promotes ccRCC progression and metastasis (Harshman and Choueiri, 2013; Shuch *et al*, 2015; Lalani *et al*, 2017).

**C-X-C Motif Chemokine Ligand 12 (*CXCL12*) and ccRCC**

*CXCL12* is a constitutively expressed chemokine with very important homeostatic functions. Elevated *CXCL12* expression has been linked with several inflammatory conditions. A study has shown that both growth arrest and hypoxia are potentially important inducers of *CXCL12* expression. In ccRCC, pVHL coordinately regulates expression of metastasis-associated genes *CXCR4/CXCL12* and *MMP2/MMP9* but the exact molecular mechanism of this regulation remains unclear (Begona Santiago, 2010; Struckmann *et al*, 2008).

**Matrix metalloproteinase-9 (*MMP9*) and ccRCC**

Matrix metalloproteinase-9 (*MMP9*) is involved in the breakdown of extracellular matrix such as type IV and V collagens in normal physiological processes that occur in the human body. It involves embryonic development, reproduction, tissue remodeling, disease processes and metastasis. High expression level of *MMP9* is associated with poor prognosis in patients with ccRCC (Deepak *et al*, 2006; Niu *et al*, 2018).

**1.2.4.1 Other LOX-inhibitors**

To date, different methods have been studied to inhibit LOX and/or LOXL2 activities, for example, using LOX or LOXL2 siRNA, microRNA-26a/b and microRNA-29s, anti-LOX and anti-LOXL2 antibodies. However, to be generally applicable of these molecules, a number of intracellular and extracellular barriers still need to be overcome, such as the difficulty of delivering siRNA, miRNA and antibody into cell, low inhibitory efficacy (Osawa *et al*, 2014; Nishikawa *et al* 2015; Kurozumi *et al*, 2016; Kato *et al*, 2017; Barry-Hamilton *et al*, 2010). Therefore, small molecular inhibitors have advantages in inhibiting the activities of extracellular and intracellular LOX and LOXL2.

$\beta$ -aminopropionitrile (BAPN) is currently used as an irreversible LOX inhibitor in research. However, BAPN has variable potency and a long-term side effect including bone deformities (Nilsson et al, 2016; Tao Li et al, 2018). PXS compound is a novel haloallylamine-derived, small-molecular LOXL2 inhibitor with oral bioavailability. It has high potency and strong selectivity for LOXL2. PXS inhibited breast cancer MDA-MB-231 cells proliferation, migration and invasion in vitro and reduced primary tumour angiogenesis in athymic nude mice (Chang et al. 2017; Stangenberg et al. 2018). Another synthesized compound, (2-chloropyridin-4-yl) methanamine 20, was shown to be a mostly reversible inhibitor on LOXL2 and selective for LOXL2 over LOX (Hutchinson *et al*, 2017).

### **1.3 Treatment of ccRCC**

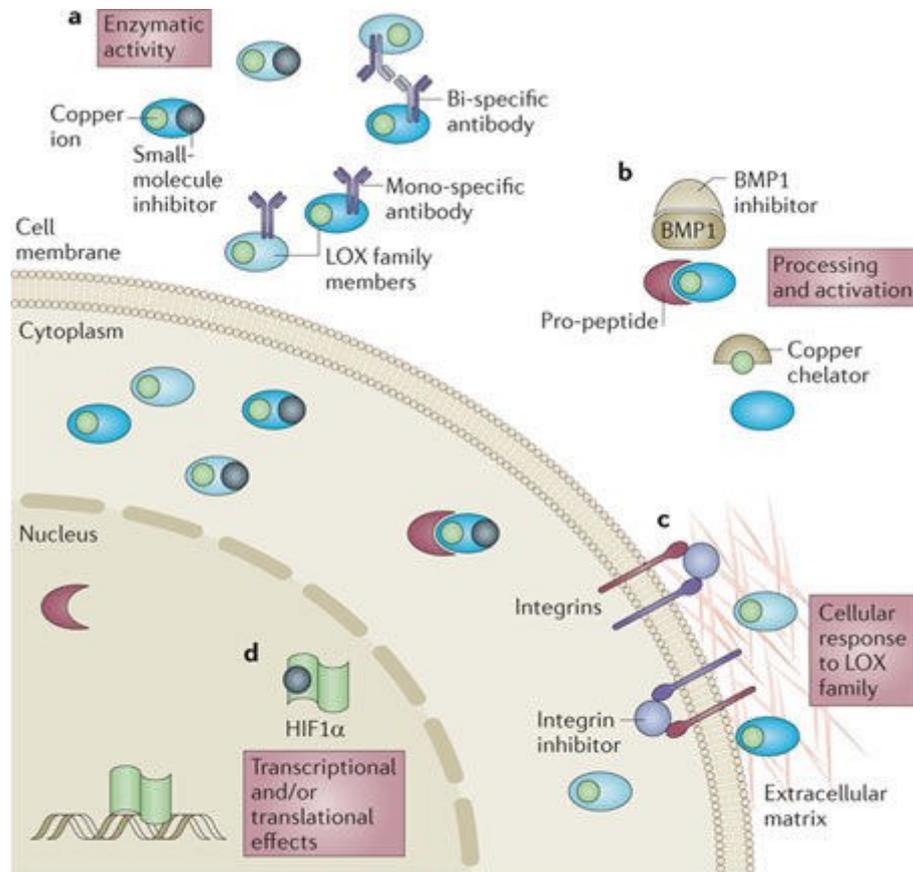
Kidney cancer including ccRCC is highly resistant to chemotherapy. The principal options for ccRCC treatment are surgery, radiation therapy, immunotherapy, molecular-targeted therapy which depend on the stage of tumor. Targeted therapy and immunotherapy are standard treatments in patients with metastatic disease (Huang *et al*, 2010)

#### **1.3.1 Targeted therapy for ccRCC**

In VHL mutated tumours, the overexpression of HIF stimulates signaling pathways involving VEGF, PDGF and TGF $\alpha$  (Section 1.2.3 and Figure 1.5). This provides a strategy in the treatment of ccRCC by blocking these pathways using targeted therapy. The tyrosine kinase inhibitors, sunitinib and Sorafenib, have been used to target VEGF and PDGF. Both drugs improved progression-free survival (PFS) in clinical trials and had been approved by the FDA for use as therapeutic agents for ccRCC in its advanced stages. Pazopanib and axitinib, two newer tyrosine kinase inhibitors, have been recently approved to treat metastatic ccRCC. Patients taking pazopanib exhibited an improvement in PFS versus those patients taking a placebo both in a first-line setting. Axitinib was examined against sorafenib as a second line agent and once again showed improvements in PFS. Two other targeted therapies, temsirolimus and everolimus, block the mTOR protein, which is involved in cancer cell proliferation. These drugs had also been approved to treat ccRCC patients by the FDA (Huang *et al*, 2010).

Unfortunately, targeted therapies do not lead to complete responses in the majority of the patients, and eventually patients become refractory to the treatments. To provide a wider

range of drug treatment options to ccRCC patients, more newly synthesized agents need to be developed.



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**Figure 1.6:** The therapeutic targeting methods followed by the LOX family. (This image was taken from Barker *et al*, 2012).

### 1.3.2 Additional newly synthesized drugs for ccRCC treatment

Nivolumab, an immune checkpoint inhibitor, was approved for advanced ccRCC in the United States in 2015 (and in Europe in 2016). Nivolumab blocks PD-1 protein located on T-cell surfaces, and thus exposes cancer cells to an immune attack. Nivolumab is now the treatment of choice for the patients who do not respond to first-line treatment (Schrödter *et al*, 2016). Recent study has shown that HIF-2 $\alpha$  antagonist, such as PT2399, may have potential to treat ccRCC. Current anti-ccRCC drugs including tyrosine kinase inhibitors, checkpoint inhibitor, interleukin (IL-2) or interferon alpha (IFN- $\alpha$ ), only slightly extend the PFS and some of these drugs can cause severe toxic side effects (Nishikawa *et al*, 2015). Therefore, developing new drugs with high PFS effects and minimized toxicities is the continuous needs for the treatment of ccRCC (Singer *et al*, 2012).

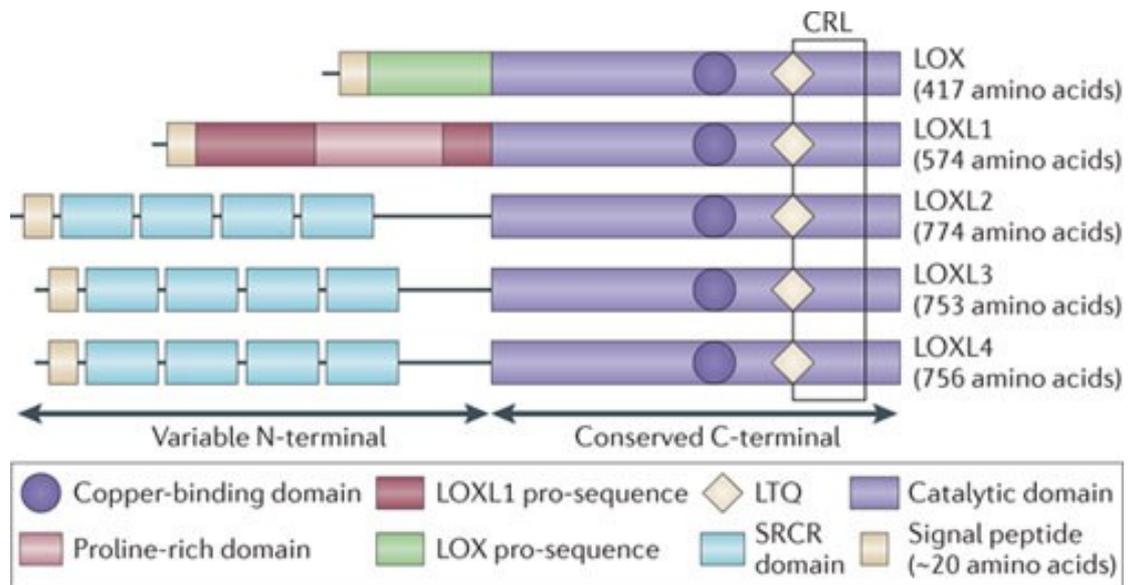
### 1.4 The characteristics of LOX and LOXL2

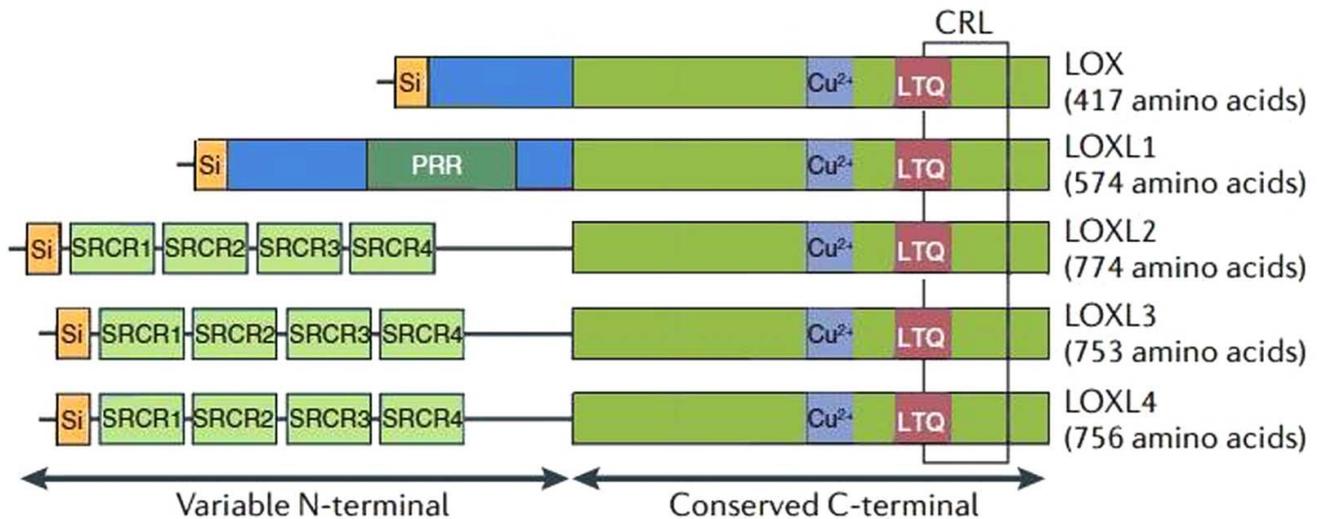
The lysyl oxidase family consists of lysyl oxidase (LOX) and four-LOX like enzymes (LOXL1-LOXL4). These enzymes are copper amine oxidases which simultaneously catalyse the crosslinking of the extra cellular matrix (ECM) proteins such as elastin and collagen. The collagen crosslinking in the ECM, caused by LOX and LOXL2 leads to increased stiffness and promotes tumor progression and metastasis in the kidney. Both LOX and LOXL2 have varying degrees of similarity to each other; however, LOX was also found in the cytoplasm and nucleus influencing proliferation and transformation from normal to malignant phenotypes (Chang *et al*, 2017). LOX expressions were found to be upregulated in ccRCC tissues compared to their normal counterparts (Fallah *et al*, 2010). Therefore, targeting LOX and LOXL2 proteins may lead to a new strategy for controlling ccRCC development.

#### 1.4.1 LOX-family structure

All the LOX-protein family members share a highly-conserved homologous region at their carboxyl-terminal (Figure 1.7). This region includes the copper binding motif, cytokine receptor-like domain and the residues for lysine tyrosyl-quinone cofactor formation. These specific regions contain all the necessary elements that are required for their enzymatic activity. However, all the LOX-family members differ in sequence mainly in their amino-terminal (N-terminal) region. Moreover, human LOX has three glycosylation sites in the N-terminal region, consisting of Asn81, Asn97 and Asn144 residues which are also required for

enzymatic activity. LOXL2 has three predicted glycosylation sites (Asn288, Asn455 and Asn644) by mass spectrometry. LOXL2 contains four scavenger receptor cysteine-rich (SRCR) domains, which have a vital role in protein-protein interactions. The fourth SRCR in LOXL2 is required for full catalytic activity. LOX is secreted as pro-proteins (proLOX) into the extracellular space, where it is hydrolyzed by metalloproteinase. Release of the N-terminal region activates LOX enzyme (Barker *et al* 2012; Iturbide *et al*, 2015).





**Figures 1.7:** The structural homology of the LOX family members which contain a carboxyl terminus that is highly conserved and comprises a copper-binding motif in addition to a lysyl-tyrosyl-quinone (LTQ) cofactor residue and a cytokine receptor-like (CRL) domain. The N-terminal pro-peptides are viable and play a vital role in the substrate specificity and function. (The images were taken from (Wu and Zhu, 2015) *et al*, 2015 and (Barker *et al*, 2011)).

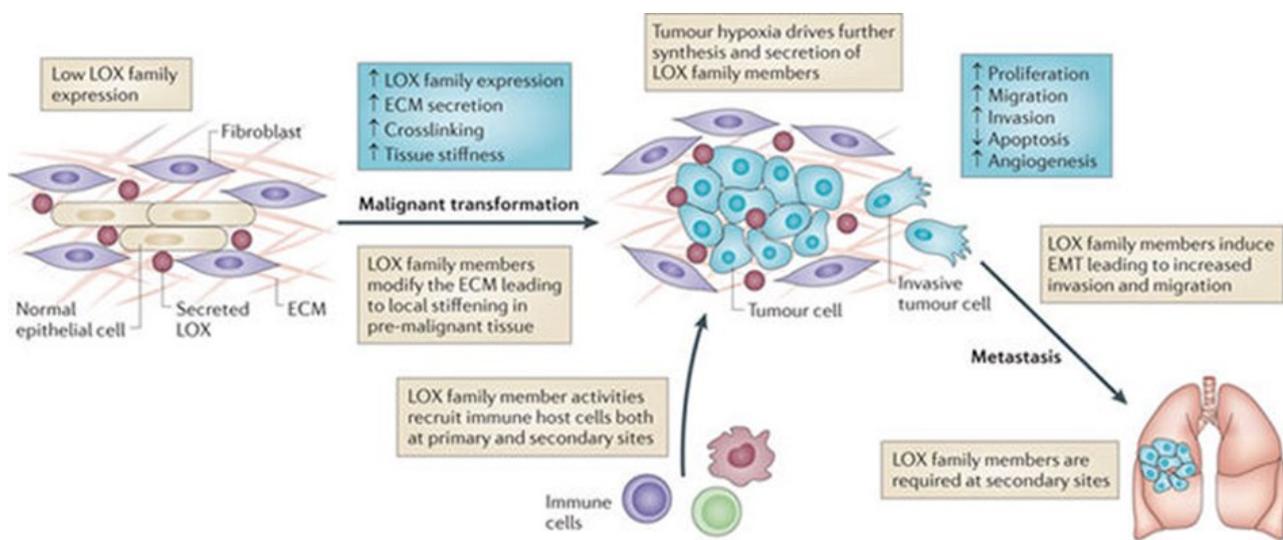
#### 1.4.2 Biological functions of LOX and LOXL2

LOX family members are involved in a variety of biological processes. The primary function of LOX and LOXL2 is the covalent crosslinking of collagens and elastin in ECM, which is required for the structural integrity of many tissues (Erlar *et al*, 2006). In addition to their roles in the ECM, LOX and LOXL2 showed other intracellular and extracellular functions (Iturbide *et al*, 2015).

LOX has shown an important role in gene transcription. LOX transcriptionally regulates several gene promoters, like collagen III, elastin, and cyclin D1 and mouse mammary tumor virus (MMTV) promoter (Oleggini and Di Donato, 2011). Overexpression of LOX correlates with chromatin de-condensation, mitotic abnormalities, cell proliferation and cell survival in COS-7 and NRK-49F cells *in vitro* (Mello *et al*, 2010).

The transfection of *ras*-transformed NIH 3T3 cells with LOX antisense construct, introduces a recognizable change in the chromatin compaction of the cells which leads to a tighter organization. It suggests the capability of LOX in having the ability to impact on the chromatin structure (Itribude *et al*, 2015).

LOXL2 has been involved in gene transcription, cell motility/migration and adhesion, angiogenesis and differentiation, demonstrating their ability to affect a wide spectrum of biological processes (Martin *et al*, 2015; Herranz *et al*, 2012; Millanes-Romero *et al*, 2013). LOXL2 participates in epithelial-mesenchymal transition (EMT), by repressing E-cadherin in a Snail1-dependent manner (Peinado *et al*, 2005). Recently study shows that LOXL2 catalytic activity is dispensable for EMT induction (Cuevas *et al*, 2014). LOXL2 function is also critical for chondrocytes differentiation by inducing epigenetic changes of TAF10 (Iftikhar *et al*, 2010). Dysregulation of LOXL2 has been linked to many diseases, including osteoarthritis, fibrosis and heart diseases (Bais and Goldring, 2017; López-Jiménez *et al*, 2017; Zou *et al*, 2017).



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**Figure 1.8:** The potential role of the LOX family members in the progression of ccRCC. The increased expression of the LOX-family in the pre-malignant tissue is greatly linked with tumor incidence due to the enhanced malignant transformation by active remodeling of the local microenvironment; this in turn leads to increased secretion and deposition of the ECM

components, crosslinking and tissue stiffness. (This image was taken from Barker *et al*, 2012).

### 1.4.3 LOX, LOXL2 and cancer development

It was found that LOXL2 was significantly upregulated in human ccRCC tumor tissues, compared with adjacent noncancerous renal tissues, and this elevated expression correlated with the pathologic stages of ccRCC. LOXL2 knockdown suppressed proliferation, migration, and invasion of ccRCC cells (Hase *et al*, 2014).

It has been suggested that LOX can be either a tumor suppressor and a tumor promotor. The tumor suppressor role was supported by over-expression of LOX inducing N1H3T3 cell transformation. Knockdown of LOX could restore transformed cells to original N1H3T3 phenotype (Lee *et al*, 2010).

More evidence validated recently backed up the fact that LOX is a metastasis promoter. This Studies have shown that LOX mRNA and protein expression is upregulated in various cancer types and especially in ccRCC. Collagen and elastin crosslinking catalyzed by LOX and LOXL2 might contribute to malignant transformation of cells ( (Barker *et al*, 2011).

In addition, LOX, may function by synergistically with matrix metalloproteinases (MMPs) in the remodeling of the ECM and in the occurrence of metastasis. The coordinated actions of LOX and MMPs generate a dynamic pro-tumorigenic microenvironment that enhances tumor progression. In addition, the transcriptional regulation of LOX and LOXL2 and dysregulation in their expression in certain genetic backgrounds also appears to play an important role in tumorigenesis (Janine *et al*, 2013 and Lingsong *et al*, 2015).).

The critical roles of LOX and LOXL2 in cancer (including ccRCC) development potentiate LOX and LOXL2 as therapeutic targets. Therefore, introducing synthesized novel drugs to target LOX/LOXL2, identifying their effects on the bioactivities of ccRCC cells are essential that could hold great therapeutic promise for ccRCC patients in the future.

## 1.5 Aim and objectives

To investigate the effects of new potential drugs, inhibitors of *LOX* and *LOXL2* that are potential drugs for treating on ccRCC cells *in vitro*.

### Objectives

- To test the levels of *LOX/LOXL2* enzymes in ccRCC cell line Caki-2 cells following the treatment of new *LOX* and *LOXL2* inhibitors (Compound 1 to 5).
- To study the effects of the compounds 1 to 5 on the proliferation, viability, cell cycle, apoptosis, cell adhesion and migration of Caki-2 cells *in vitro*.
- To study the effects of the compounds on the metabolism production of NAD(P)H of Caki-2 cells *in vitro*.
- To identify the changes in cell signaling pathways in Caki-2 cells ccRCC by observing the notch-signaling pathway following the compound treatment using western blot experiments and quantitative real time-PCR.

## 2. Methods

### 2.1 Cell line and cell culture

Human Caki-2 cell line used in this investigation was supplied by the European collection of Authenticated cell cultures (ECACC). The cell line originates from a kidney tumour in a 69-year-old Caucasian male. The Caki-2 cells are tumorigenic when tested in nude mice. The Caki-2 cell type is epithelial and the growth mode shown by the cells is adherent (Public Health England, [www.phe-culturecollections.org.uk](http://www.phe-culturecollections.org.uk), 2017).

Caki-2 cells were cultured in a complete medium consisting of RPMI-1640 (LONZA, UK) supplemented with 10% foetal bovine serum (FBS) (LONZA, UK), 10 unit/ml penicillin and 100µg/ml streptomycin (Sigma Aldrich, UK). The cells were incubated at 37°C and 5% CO<sub>2</sub> until about 80% cell confluence was achieved. Then the cells were used in subsequent.

Caki-2 cells in continuous culture mode are prone to genetic drifts and susceptible to microbial contamination. The established Caki-2 cell line is a valuable resource and its replacement is relatively expensive and time consuming. Unused cells were therefore frozen down and preserved in liquid nitrogen for long-term storage.

### 2.2 Compound preparation

Potential anti-LOX inhibitors (compound-1,2,3,4,5) were originally synthesised and kindly provided by Dr Alan Jones (School of Pharmacy, University of Birmingham, UK). The formula and molecular weights (Mr) of these compounds are provided in **Table 2.1**:

**Table 2.1:** the chemical formulas, extract mass and molecular weight of the five compounds:

Compound type	Chemical formula	Extract mass	Molecular weight
Compound-1	C <sub>5</sub> H <sub>8</sub> H <sub>4</sub> OS	208.04	208.24
Compound-2	C <sub>15</sub> H <sub>12</sub> N <sub>4</sub> O <sub>2</sub> S	312.07	312.35
Compound-3	C <sub>15</sub> H <sub>19</sub> N <sub>4</sub> O <sub>2</sub> S	310.03	310.33
Compound-4	C <sub>17</sub> H <sub>14</sub> N <sub>4</sub> O <sub>2</sub> S	338.08	338.39
Compound-5	C <sub>15</sub> H <sub>10</sub> N <sub>4</sub> O <sub>2</sub> S	310.05	310.33

Unfortunately, proliferation experiments on compound-2 and 3 showed the Caki-2 cell growth was greater than the negative control following the treatment with these two compounds, which clearly demonstrates that these two compounds did not inhibit Caki-2 cells growth. Therefore, compound-2 and 3 were excluded from subsequent studies. Caki-2 cells in all subsequent experiments were treated with compound-1, 4 and 5 respectively at concentrations of 10, 50 or 200 µg/ml and incubated in the same conditions. The negative control used in each of the experiments was dimethyl sulfoxide (DMSO) (0.5%, V/V) was added to negative control cells in lieu of the test compound-1, 4, or 5.

### **2.3 Cell morphology evaluation**

The morphologies of Caki-2 cells treated with or without compound-1, 4 and 5 were analysed using an inverted microscopy (Zeiss Primo Vert, Thermo Fisher Scientific). Representative cell images were using (ZEN software).

### **2.4 Proliferation assay**

A cell counting kit-8 (CCK-8) (Dojindo molecular techniques.INC.UK) was used to analyse the Caki-2 cell proliferation following the treatment with compound-1, 4 and 5. The cell counting kit-8 (CCK-8) uses a sensitive colorimetric assay to determine the viability of cells in proliferation and cytotoxicity assays. Its reagents contain highly water soluble tetrazolium salt, WST-8, which is reduced by dehydrogenase activities in the cells to an orange coloured formazan dye. This type of dye is soluble in the tissue culture media. Therefore, the amount of formazan dye generated by the activities of the dehydrogenases in cells is directly proportional to the number of living cells (Dojindo, <http://www.dojindo.eu.com>, 2018).

The Caki-2 cells were seeded in 96-well plates at a concentration of  $1.5-3 \times 10^5$  cell/ml, and 100 µl per well. The cells were treated with compound- 1, 4 and 5 at different concentrations of 10, 50 or 200 µg/ml for 24, 48 and 72 hours. The negative control cells were described in Section 2.2. At the end of compound-treatment, the cells were cultured with 10 µl of the CCK-8 solution per well and incubated for 60 minutes at 37°C with 5% CO<sub>2</sub>. The absorbance for the compound treated and untreated Caki-2 cells in each well was measured using a Multi-scan GO microplate spectrometer (Thermo Fisher Scientific, UK) at 450nm.

## 2.5 Trypan blue exclusion assay

The viability Caki-2 cell were determined using the Trypan blue dye-exclusion test. The Caki-2 cells at a concentration of  $3 \times 10^5$  cell/ml seeded into 24-well plates were treated with compound-1, 4 and 5 at concentrations of 10, 50 and 200 $\mu$ l/ml for 24, 48 and 72 hours respectively. Negative control cells as described in Section 2.2 were incubated for the same periods.

Following incubation, the cells in the 24-well plates were trypsinised. A total volume of 15 $\mu$ l of cell suspension was equally mixed with 15 $\mu$ l of Trypan blue dye (Sigma) before pipetting into a haemocytometer. A light microscope was then used to obtain a total cell count and a dead cell count (stained cells) in each of the chambers in the haemocytometer. Then the percentage of viable cells was calculated using the equation below:

$$\text{Viability (\%)} = \frac{\text{Total number of cells} - \text{Total number of dead cells}}{\text{Total number of cells}} \times 100$$

## 2.6 Lysyl oxidase activity assay

Caki-2 cells were treated with Compound-1, 4 and 5 at concentrations of 10, 50 and 200  $\mu$ l/ml for 24, 48 and 72 hours respectively and the negative control cells were treated with DMSO (Section 2.2). Then 50  $\mu$ l of each of the treated Caki-2 cell medium was pipetted into a black-walled 96-well plate (Sigma Aldrich, UK) along with 50 $\mu$ l of LOX-activity assay reaction mixture (Abcam, UK). The 96-well plate was sealed with aluminium foil to protect the mixture from the light and incubated at 37°C for 30 minutes. The Synergy™ HT Multi-Detection Microplate Reader (BioTek, UK) was used to analyse the lysyl oxidase enzyme activity by measuring the fluorescence produced (Ex/Em= 540/590 nm).

## 2.7 RNA extraction method

Ribonucleic acid (RNA) extraction was carried out using a total RNA extraction kit and protocol (EZ-RNAl) (BI, Biological Industries, Israel) Compound-1, 4 and 5 treated Caki-2 cells and negative control cells were harvested after culture for 48 hours. The cell pellet were homogenised in 0.5ml of denaturation solution and incubated for 5 minutes at room temperature. Then 0.4ml of phenol was added to each sample, followed by 0.09ml of 1-Bromo-3-Chloropropane and the mixture was left at room temperature for 10 minutes after being vigorously shaken. The mixtures were then centrifuged at 12,000g for 15 minutes at 4°C. The RNA in the upper aqueous phase were extracted and placed in separate tubes with 0.5ml per tube of isopropanol. The sample were then incubated at room temperature for 10 minutes followed by centrifugation at 12,000 x g for 8 minutes and the supernatant was discarded. The remaining RNA pellet was washed thoroughly using 75% ethanol and once again centrifuged at 7,500g for 5 minutes at 4°C and then dissolved in RNase-free water. Finally, a nanodrop 2000 machine (Thermo-Scientific) was used in order to quantify the RNA.

## 2.8 cDNA synthesis

After the RNA extraction cDNA was prepared using a double strand cDNA synthesis kit (ImProm-II™ Reverse transcription system, Promega, UK). The reagents for each reaction included 4µl of 25nM MgCl<sub>2</sub>, 4µl of ImProm-II 5X reaction buffer, 1µl of reverse transcriptase, 0.5µl of Ribonuclease inhibitor, 0.5µl of Oligo (dT)<sub>15</sub>, 4.5µl of nuclease-free water and 1µg of RNA a complete final volume of 20µl. The mixtures were incubated in a Q-cycler II (Quanta Biotech, UK) at 70°C for 5 minutes, 25°C for 5 minutes, 42°C for 1 hour and 70°C once again for 15 minutes. The final step included storing the cDNA sample at -20°C.

A SensiFAST™ cDNA synthesis kit (BIOLOINE, UK) was also used to synthesise the cDNA. The manufacturer's protocol was followed which included mixing specific amounts of each of the reagents to obtain the desired master mix. The reagents for each reaction included 1µg RNA, 4µl 5X TransAmp buffer, 1µl reverse transcriptase, and DNase/RNase free-water to a final volume of 20µl. The reagents were mixed gently by pipetting. The following program was applied by using the (Q-cycler II) thermal cycler, 25 °C for 10 min (primer annealing), 42 °C for 15 min (reverse transcription), an optional Step included 48 °C for 15 min (for highly-

structured RNA), 85 °C for 5 min (inactivation) and finally 4 °C in hold. The reaction product were then stored at -20 °C until required.

## **2.9 Real time polymerase chain reaction (PCR)**

Real time PCR was performed using the prepared cDNA samples to test the expressions of *LOX* and *LOXL2* at mRNA levels following treatment with the three compounds. Eleven genes related to cell signalling pathways including *CASP9*, *HSP90*, *CD98HC* (Table 2.2) were also examined by real time PCR. The values of gene expression in the compound treated cells were compared to those in the negative control. The two house-keeping genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and actin beta (*ACTB*) were used as controls for normalisation of gene expression. The primers used in this investigation are included in Table 2.2 below:

**Table 2.2: The primers used in Real time PCR gene expression test** (all primers were purchased from Thermo Fisher Scientific).

<b>Gene</b>	<b>Forward primers (5' to 3')</b>	<b>Reverse primers (5' to 3')</b>
<b>LOXL2</b>	<u><b>AGGATGTCGGTGTGGTGTG</b></u>	<u><b>TTGCGGTAGGTTGAGAGGAT</b></u>
<b>LOX</b>	<u><b>GAATAACTGCCGAAACTCAAAG</b></u>	<u><b>GTGTAAAAGCCAATCTCCTGTC</b></u>
<b>GAPDH</b>	<u><b>AGCCACATCGCTCAGACAC</b></u>	<u><b>GCCAATACGACCAAATCC</b></u>
<b>ACTB</b>	<u><b>CCAACCGCGAGAAGATGA</b></u>	<u><b>CCAGAGGCGTACAGGGATAG</b></u>
<b>CAPS9</b>	<u><b>AAGCCCAAGCTCTTTTTCATC-</b></u>	<u><b>ACTCGTCTTCAGGGGAAGTG</b></u>
<b>HSP90</b>	<u><b>GGGCAACACCTCTACAAGGA</b></u>	<u><b>CTTGGGTCTGGGTTTCCTC</b></u>
<b>RRM2</b>	<u><b>CGCTCCACTATGCTCTCC</b></u>	<u><b>GCTAAATCGCTCCACCAAG</b></u>
<b>CDKN1B</b>	<u><b>TTTGACTTGCATGAAGAGAAGC</b></u>	<u><b>AGCTGTCTCTGAAAGGGACATT</b></u>
<b>MET</b>	<u><b>GGCATTGAAACTTGAAACTCATATAC</b></u>	<u><b>ACTGGGTGAAATCTTGG</b></u>
<b>MMP9</b>	<u><b>GAACCAATCTCACCGACAGG</b></u>	<u><b>GCCACCCGAGTGTAACCATA</b></u>
<b>CD98HC</b>	<u><b>CAACTACCGGGGTGAGAACT</b></u>	<u><b>AGCCAAAACCTCCAGAGCATC</b></u>
<b>VMP</b>	<u><b>GCACACCTTTCTGCTTTATCTG</b></u>	<u><b>CACCTGAGAGGCGAGCTG</b></u>
<b>CD26</b>	<u><b>GCACGGCAACACATTGAA</b></u>	<u><b>TGAGGTTCTGAAGGCCTAAATC</b></u>
<b>CXCL12</b>	<u><b>ATGCCGATTCTTCGAAAGC</b></u>	<u><b>CCACCTGCACAGCTCAGA</b></u>
<b>CXCL16</b>	<u><b>GCCCTTTCCTATGTGCTGTG</b></u>	<u><b>CAGGTATATAATGAACCGGCAGAT</b></u>

The real time PCR reactions included 12.5µl of SYBR® green (Promega), 1.5µl of the forward and reverse primers, 8µl of nuclease-free water and 3µl of cDNA to make a total volume of 25µl for each sample. PCR was carried out in a thermocycler (Stratagene MX3000). The samples were incubated at 94°C for 10 minutes followed by 45 amplification cycles of 94°C for 30 seconds, 59°C for 30 seconds and 72°C for 30 seconds. Finally, the samples were incubated at 72°C for a further 10 minutes of elongation to ensure that the maximum amount of dsDNA WAS fully extended. Ct values were recorded.

Ct values were then used to calculate the fold change of selected gene expression following the compound treatment. The equations used are presented below:

$$\Delta Ct_{\text{Target or housekeeping gene}} = Ct_{\text{treated sample}} - Ct_{\text{negative control sample}}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{Target gene}} - \Delta Ct_{\text{Housekeeping gene}}$$

$$\text{Fold change in gene expression} = 2^{-\Delta\Delta Ct}$$

## 2.10 Detection of NAD(P)H-Glo™ production

The NAD(P)H-Glo™ kit (Promega) was used following the manufacture's protocol to detect the production of NAD(P)H by Caki-2 cells.

Caki-2 cells were treated with compound-1, 4 and 5 at a concentration of 200µg/ml and incubated for 24 hours at 37°C and 5% CO<sub>2</sub>. The samples were then placed into a 96 white illuminometer-compatible well plate along with the negative controls. A volume of 50µl of NAD(P)H-Glo™ detection reagent (Promega) was added to each well and the plate was incubated for 60 minutes at room temperature before the luminescence was recorded using Synergy™ HT Multi-Detection Microplate reader.

## 2.11 Cell cycle flow cytometry assay

Caki-2 cells (6 X 10<sup>5</sup> cells/ml) were seeded into a 6-well plate, and treated with 50µg/ml or 200µg/ml of each compound for 24 hours. The negative control was set as previously described (Section 2.2). Following the treatment, the cells were trypsinised and centrifuged at 300g for 5 minutes at 4°C.

The cell pellet was re-suspended in 500µl of PBS and fixed by suspending in 3ml of ice cold 75% ethanol. The cells were then stored overnight at -20°C. The samples were centrifuged at 500g for 10 minutes at 4°C and re-suspended in 500µl of PBS along with 25µl of propodeum iodide (PI) and 20µl of 10mg/ml RNase (Sigma). The samples were then centrifuged at 5 minutes at 4°C incubated in the dark for 30 minutes at 37°C. Cell pellets were re-suspended in 500µl of FACS-binding solution, then analysed via flow cytometry (BD FACs Calibre).

### **2.12 Annexin V apoptotic detection using fluorescent microscopy assay**

Caki-2 cells treated with compound-1, 4 and 5 at concentrations of 10, 50 or 200µg/ml for 24 hours. The negative controls were applied (see Section 2.2). Cells were trypsinised, centrifuged and re-suspended in 500µl of binding buffer (BD Biosciences, UK). 5µl of PI and 5µl of Annexin V-FITC (BD Biosciences) were added to the cells, which were then incubated for 5 minutes in complete darkness at room temperature. 10µl of cell suspension along with 30µl of Vectashield DAPI H-1200 were mixed and added onto a glass slide and covered by a glass cover slip. The cells are then observed under a using a dual filter which is were set for FITC and PI. Cells that had bound Annexin V-FITC displayed green fluorescent staining in the plasma membrane, whereas cells that had lost membrane integrity show red fluorescent staining in nuclei. Five microscopic field were scanned for counting the number of stained/apoptotic cells and total number of cells.

### **2.13 Cell adhesion assay**

Caki-2 cells ( $2 \times 10^4$  cells/ml) treated with the three compounds (1,4 and 5) at concentrations of 10, 50 and 200 µg/ml respectively for 48 hours before being seeded in triplicates on ECM gel-coated (Sigma) 96-well plates. The plates were then incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> for 3 hours. The plates were then rinsed gently three times with PBS in order to remove any unattached cells. Attached cells in each well were fixed with 100% methanol for 5 minutes and then stained with 0.5% crystal violet solution for 20 minutes. After three washes with PBS, crystal violet was eluted with 2% SDS extraction buffer for 30 minutes and the plate was placed on an orbital shaker at room temperature. The optical densities of the Caki-2 cells were measured using a Multi-scan™ GO microplate reader (LabSystems) at 620 nm.

### **2.14 Cell migration assay**

Caki-2 cells at a concentration of  $2 \times 10^5$  cells/ml in complete medium were seeded in a 24-well plate (1ml/well) and incubated in a humidified 5% CO<sub>2</sub> atmosphere, at 37 °C overnight. Cells in each well were subsequently wounded with a sterile pipette tip to produce uniform and straight edged cuts. The wounded cell monolayer was washed three times with PBS to remove cellular debris and dislodged cells. Cells were incubated for further 24 hours in complete medium containing 200 µg/ml compound-1, 4, 5 respectively. Negative control was described in Section 2.2. After 24 hours incubation, the medium was removed from each well. Cells were rinsed three times with PBS, fixed in ethanol for 5 minutes, and allowed to air-dry. The cells were stained with 0.1% methylene blue for 2 minutes and washed abundantly with distilled water. The plates were left for air-dry. Pictures of five random areas of each wound were taken using phase contrast microscopy at 40X magnification. The migration of the cells into the cell denuded area was quantified by measuring the distance of migrated cells in each field of view.

### **2.15 Statistical analysis**

All experimental assays were repeated at least three times, and the results are expressed as mean  $\pm$ SD. The quantitative experiments were analysed using Student's T-test.  $p < 0.05$  were considered significant.

### 3. Results

**Table 3.1 Results summary. Significant effects of each compound on Caki-2 cellular processes *in vitro* are presented as '+', while insignificant effects are presented as '-', untested are presented as 'u'. <sup>a</sup>, cell viability at 48 and 72 hours following compounds treatment; <sup>b</sup>, 72 hours cell proliferation; <sup>c</sup>, 48 hours cell migration;**

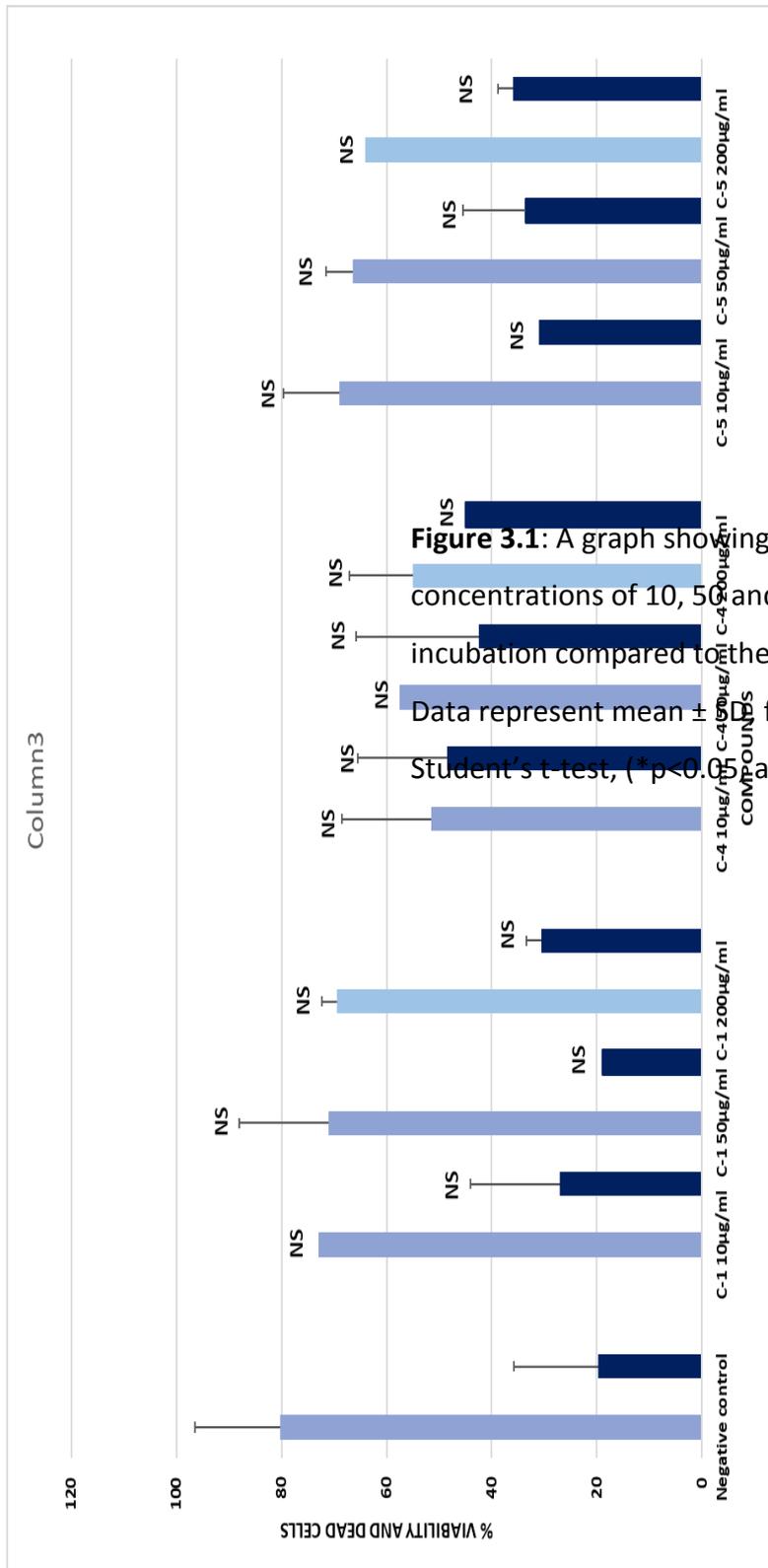
	compound-1 (µg/ml)			compound-4 (µg/ml)			compound-5 (µg/ml)		
	10	50	200	10	50	200	10	50	200
<b>Reduce cell viability <sup>a</sup></b>	+	+	+	+	+	+	+	+	+
<b>Inhibit cell proliferation <sup>b</sup></b>	+	+	+	+	+	+	+	+	+
<b>Inhibit LOX activity</b>	-	-	-	-	+	+	-	+	+
<b>Decrease NAD(P)H production</b>	u	u	+	u	u	+	u	u	+
<b>Cell cycle arrest at G1 phase</b>	u	+	+	u	+	+	u	+	+
<b>Reduce cell adhesion (ECM)</b>	-	+	+	+	+	+	-	-	-
<b>Inhibit cell migration <sup>c</sup></b>	u	u	-	u	u	+	u	u	+
<b>Induce cell apoptosis</b>	u	+	+	u	+	+	u	+	+

### 3.1 The effects of compound-1, 4 and 5 on the viability of Caki-2 cells

Figure 3.1 The percentage of viable and dead Caki-2 cells following 24, 48 and 72 hours treatment with compound-1,4 and 5 at a concentration of 10,50, and 200µg/ml are shown in figure 3.1 to 3.3. In general, the viabilities of the Caki-2 cells did not show significant changes at 24 hours' treatment with compound-1, 4 and 5 compared to the viability of the Caki-2 cells in the negative control. The average percentage of viability of the compounds treated Caki-2 cells were between 50-75%, while the negative control cells were 80% (figure 3.1).

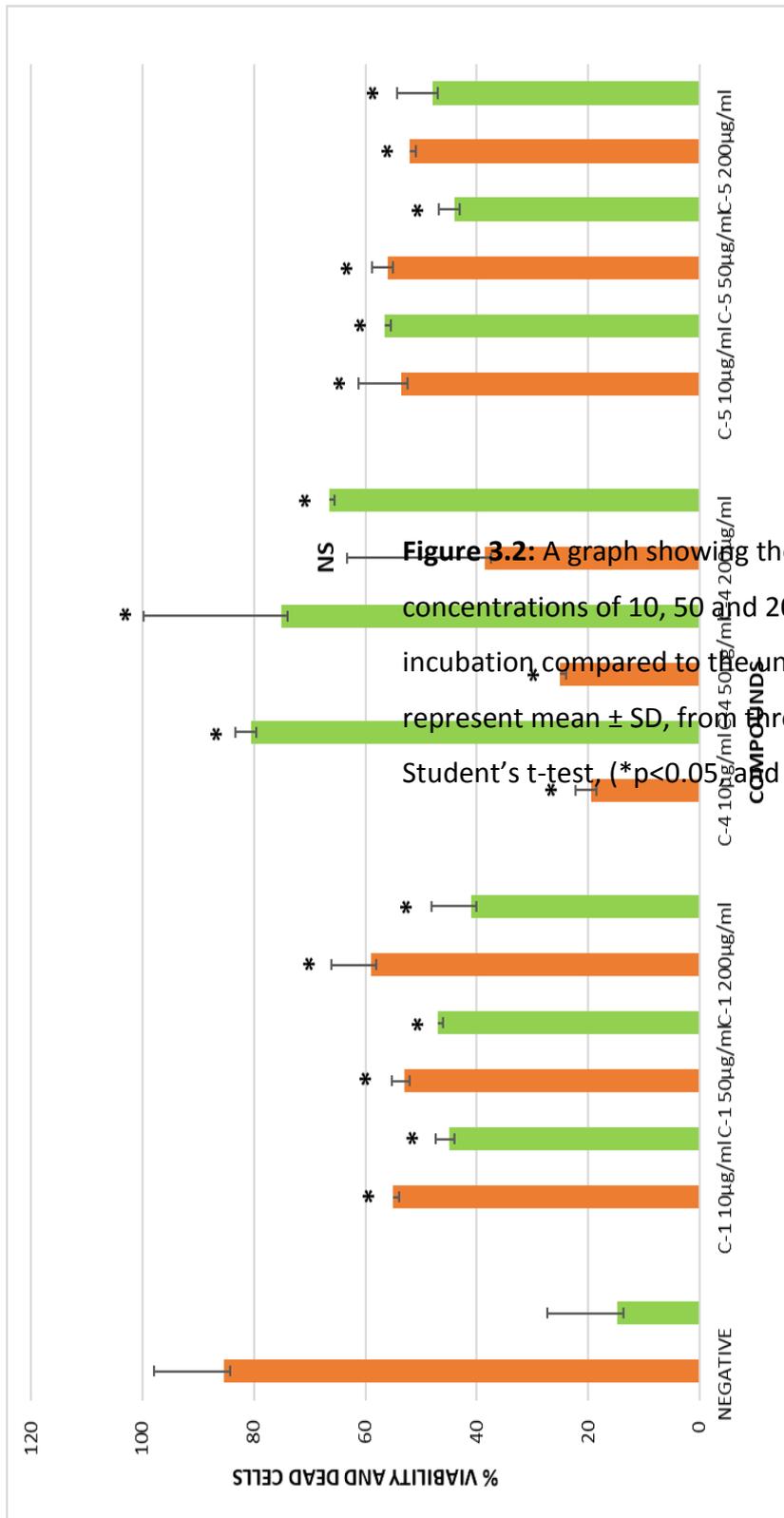
Following 48 hours treatment with compound-1, 4 and 5, the viability of the Caki-2 cells did show significant decrease when compared to the viability of negative control cells ( $p < 0.05$ ). The average percentage of viability of the compounds treated Caki-2 cells were between 19-59%, while the viability of negative control cells was 80% (Figure 3.2).

Following 72 hours treatment with compound-1, 4 and 5, the viability of the Caki-2 cells were significantly decreased when compared to the viability of negative control cells ( $p < 0.05$ ). The average percentage of viability of the compounds treated cells were between 29-55%, while the viability of negative control cells was 82% (Figure 3.3)

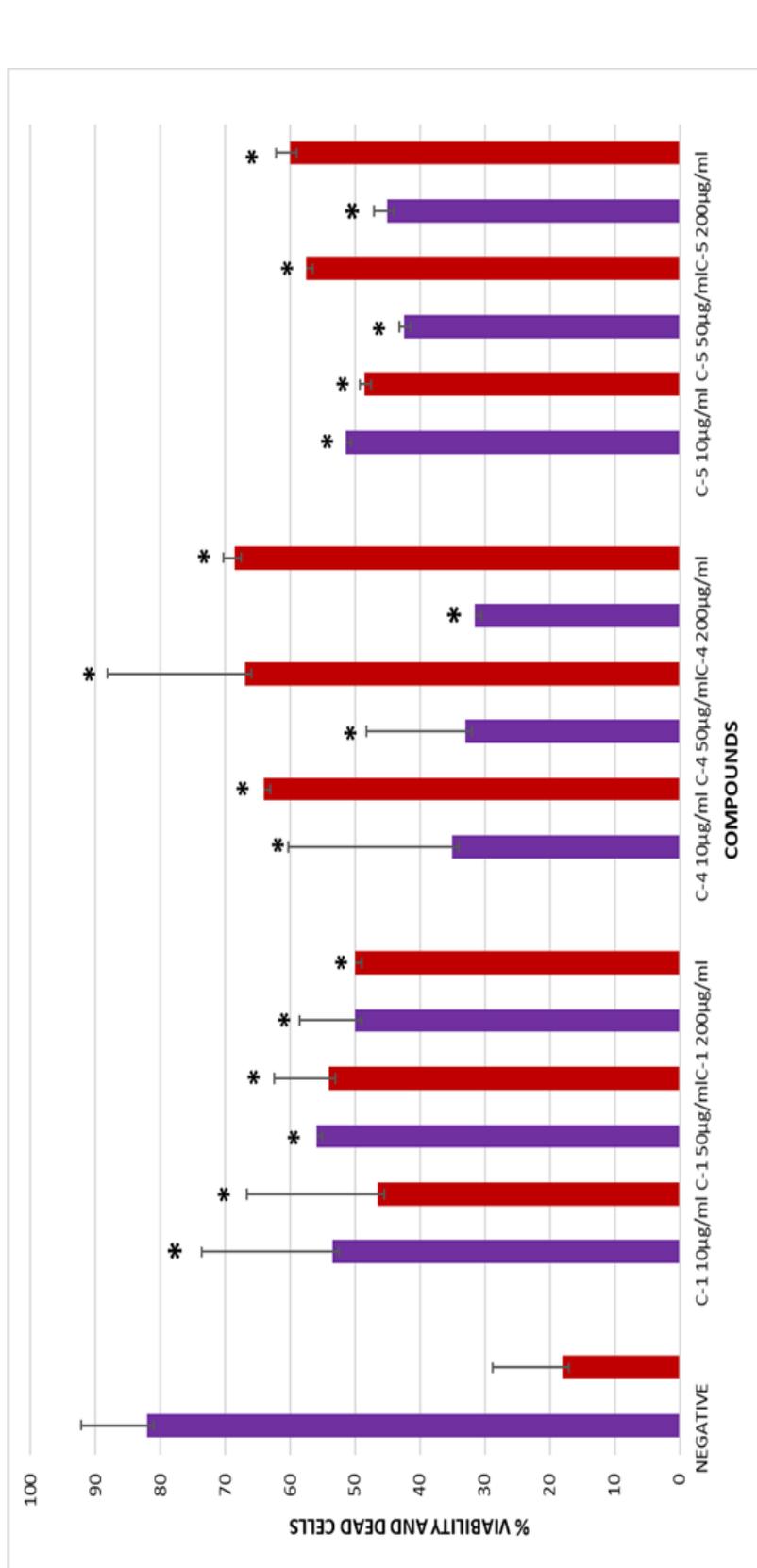


**Figure 3.1:** A graph showing the effect of compound-1, compound-4 and compound-5 concentrations of 10, 50 and 200µg/ml) on the viability of the Caki-2 cells after 24h incubation compared to the untreated negative control in a Trypan blue assay. Data represent mean ± SD from three measurements in three separate experiments. Student's t test, (\*p<0.05 and NS, not significant).

■ = % Viability  
■ = % Dead cells



**Figure 3.2:** A graph showing the effect of compound-1, compound-4 and compound-5 concentrations of 10, 50 and 200µg/ml on the viability of the Caki-2 cells after 24 hours incubation, compared to the untreated negative control in a Trypan blue assay. The bars represent mean ± SD, from three measurements in three separate experiments. Student's t-test, (\*p<0.05 and NS, not significant).



**■ = % Viability**  
**■ = % Dead cells**

ct of compound-1, compound-4 and co  
 ml) on the viability of the Caki-2 cells a  
 control in a Trypan blue exclusion assa  
 ee separate experiments (n = 3). Stud

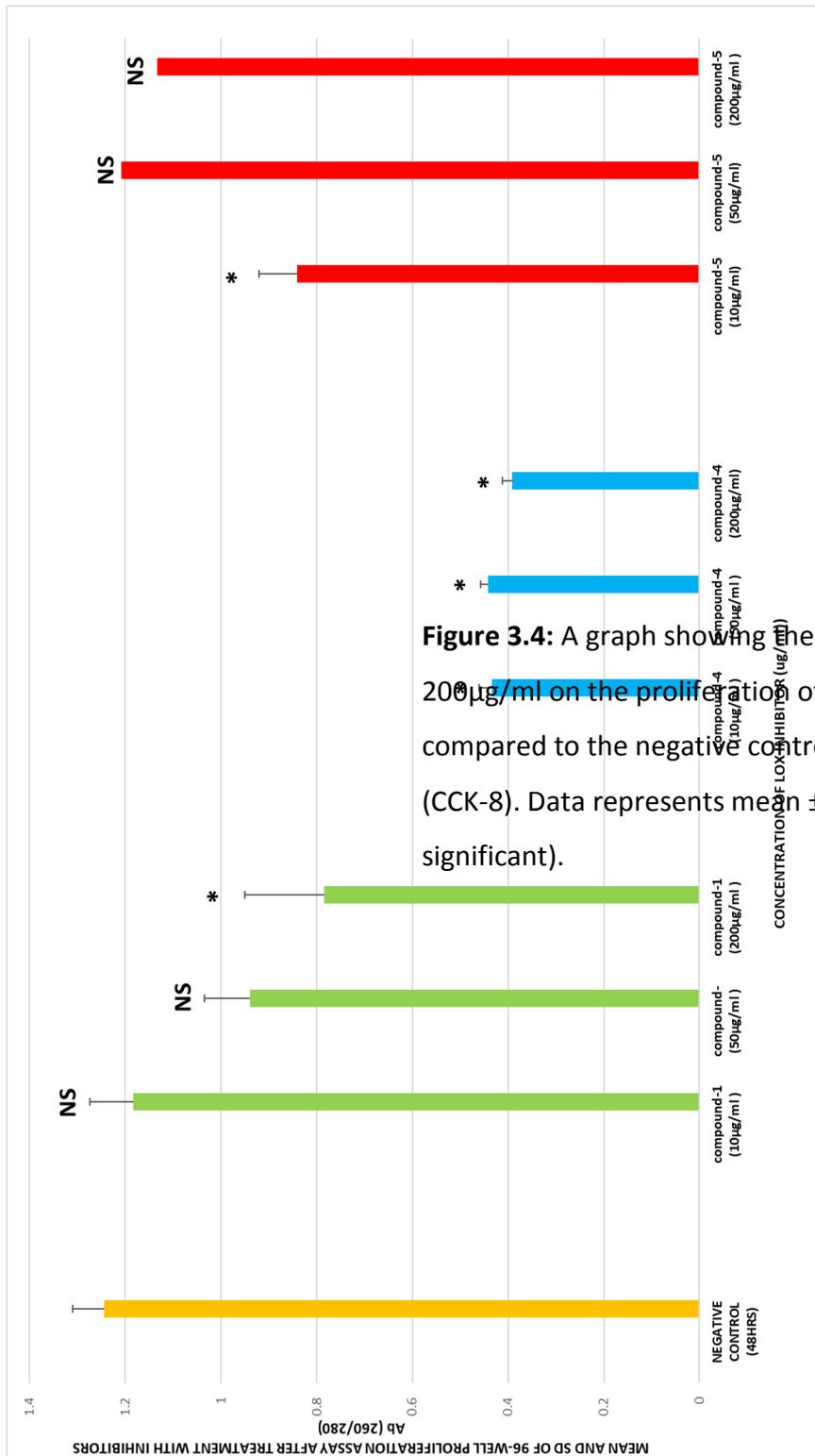
### 3.2 The effects of compound-1, 4 and 5 on the proliferation of Caki-2 cells

Figure 3.4 to 3.6 shows the proliferation results of Caki-2 cells following the treatment with compound-1, 4 and 5 at a concentration of 10,50 and 200 µg/ml for 24, 48 and 72 hours respectively.

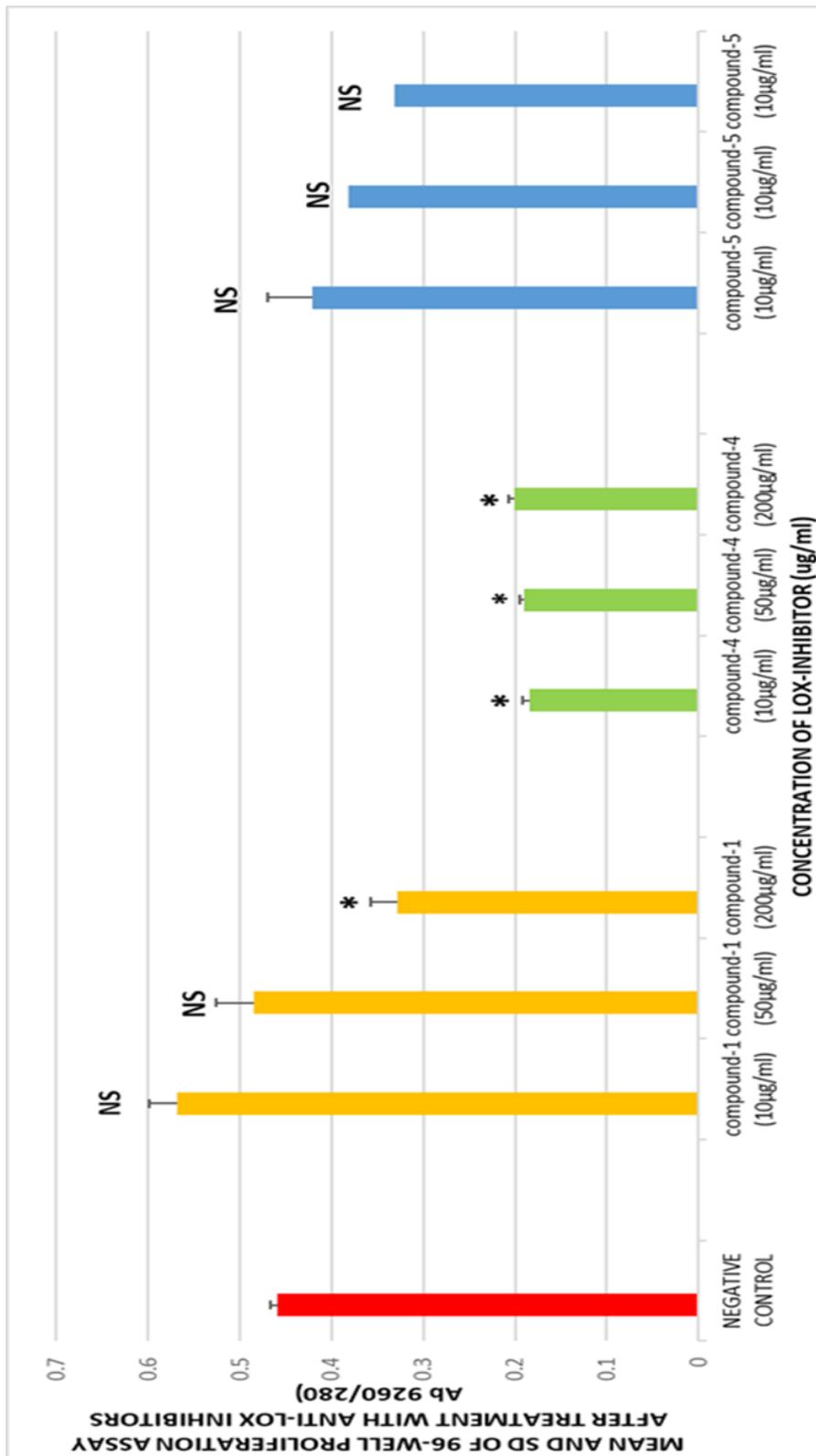
After 24 hours treatment, compound-4 significantly inhibited Caki-2 cell proliferation in comparison to the negative control ( $p < 0.05$ ). While the effects of compound-1 and 5 on cell proliferation were insignificant ( $p > 0.05$ ) (Figure 3.4).

Following 48 hours treatment, compound-4 at concentrations of 10, 50 and 200 µg/ml and compound-1 at a concentration of 200 µg/ml inhibited Caki-2 cell proliferation significantly ( $P < 0.05$ ). However, the effects of compound-1 at lower concentrations of 10 and 50 µg/ml, and compound-5 on cell proliferation were insignificant (Figure 3.5).

After 72 hours treatment, all three compound-1, 4 and 5 at concentrations of 10 to 200 µg/ml significantly inhibited Caki-2 cell proliferation compared to the negative control ( $P < 0.01$ ). Compound-4 had greater impact on inhibiting cell proliferation than compound-1 and 5 (Figure 3.6).



**Figure 3.4:** A graph showing the effect of compound-1, 4 and 5 at a concentration of 10, 50 and 200 µg/ml on the proliferation of the Caki-2 cells after 24 hours of incubation compared to the negative control. The cell proliferation was measured using CCK-8 assay (CCK-8). Data represents mean ± SD, n=6 from three separate experiments (p < 0.05 is considered significant).



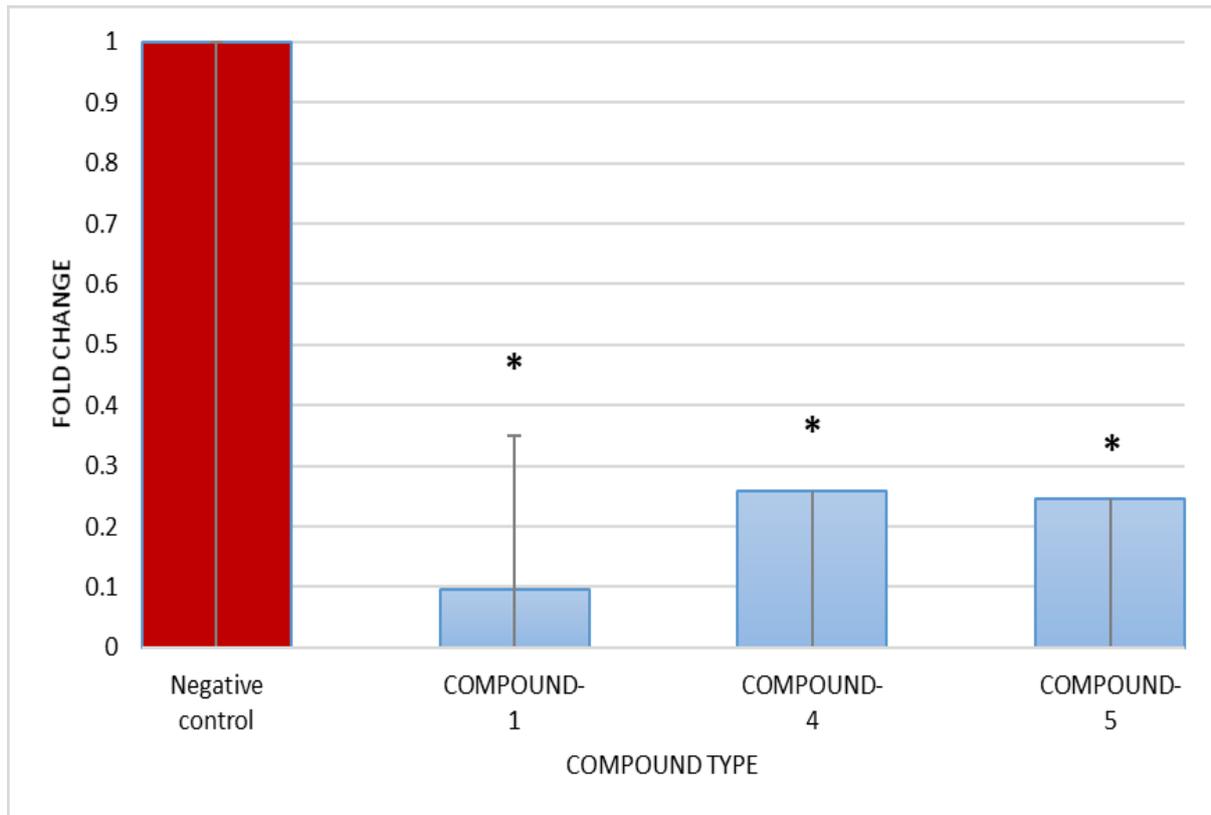
compound-1, 4 and 5 at a concentration of 10, 50 and 200 µg/ml for 24 hours of incubation irrespectively, compared to the control using CCK-8 (CCK-8). Data represents mean ± SD (n=3).



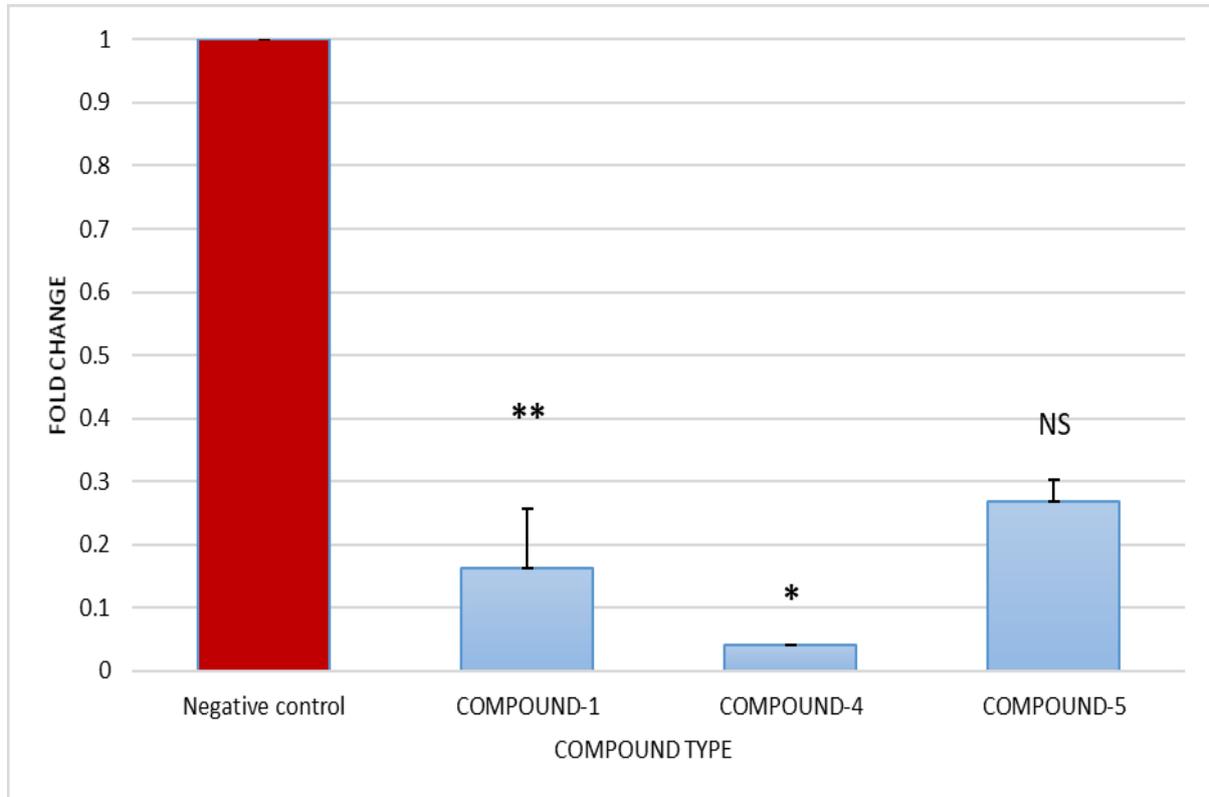
### **3.3 Inhibition of *LOX* and *LOXL2* expression in Caki-2 cells following treatment with compound-1, 4 and 5.**

Figure 3.7 shows the qRT-PCR results for the *LOX* expression in Caki-2 cells following 24 hours of treatment with compound-1, 4 and 5 at a concentration of 200µg/ml. *LOX* mRNA expression in these three compounds treated cells were all significantly ( $P<0.05$ ) lower than in the negative control cells. The results show that the average fold change was between 0.09 and 0.26 for the three compounds treated cells compared to the negative control. It shows compound-1 has the greatest inhibition on the expression of *LOX* than compound-5 and compound-4.

Figure 3.8 shows the change of *LOXL2* mRNA expression in Caki-2 cells after 24 hours of treatment with compound-1, 4 and 5 at a concentration of 200µg/ml using a qRT-PCR test. The average fold change of *LOXL2* was between 0.03-0.27 and across all of the three compounds used. *LOXL2* mRNA expression in the Caki-2 cells treated with compounds 1 and 4 were significantly ( $P<0.05$ ) lower than in the negative control cells but compound-5 did not show significant results.



**Figure 3.7:** The change in *LOX* expression in Caki-2 cells following their treatment with compound-1, 4 and 5 compared to negative control. Expression of the housekeeping genes,  $\beta$ -*Actin* and *GAPDH*, were used for normalization. Data represent mean  $\pm$  SD, n=6 from three separate experiments (\*,  $p < 0.05$ ).

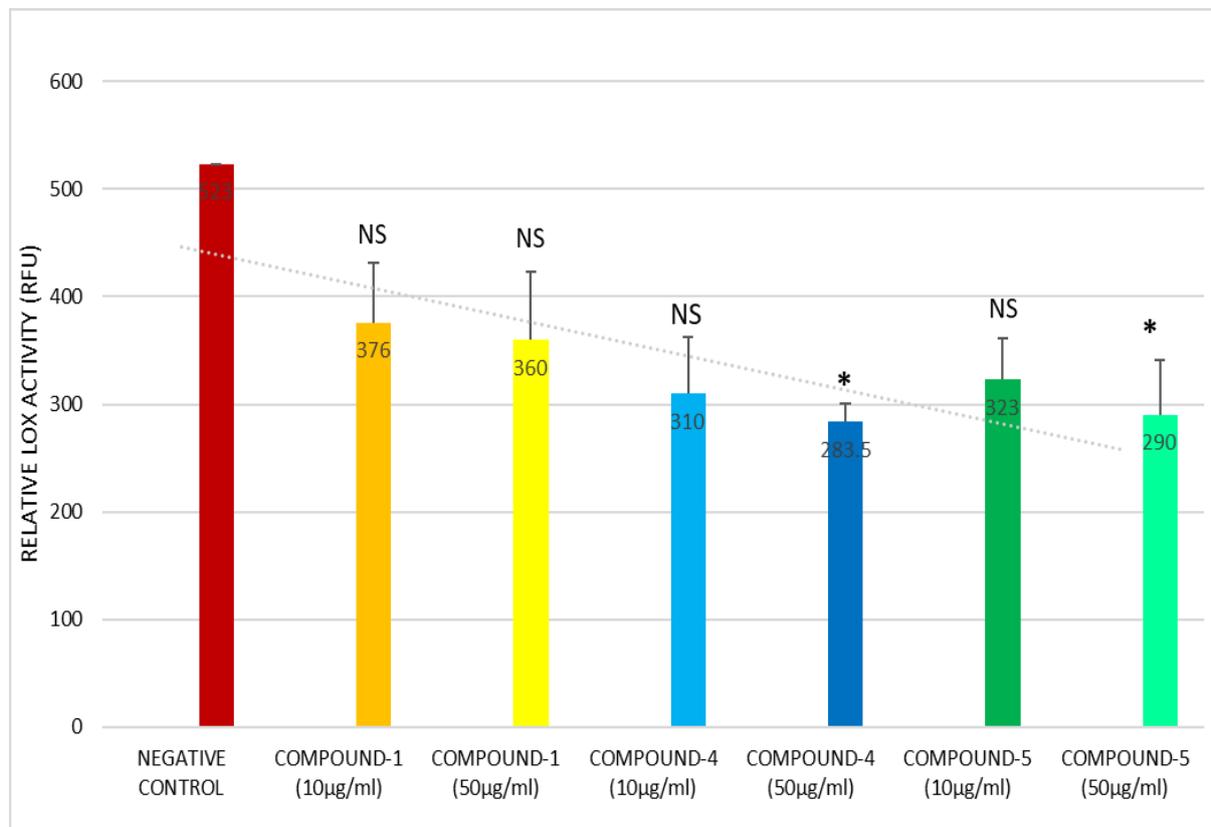


**Figures 3.8:** The fold change of *LOXL2* expression in Caki-2 cells following the treatment with compound-1, 4 and 5 were compared to negative control. Expression of housekeeping genes,  $\beta$ -*Actin* and *GAPDH*, were used for normalization. Data represent mean  $\pm$  SD, n=6 from three separate experiments. (\*,  $p < 0.05$ ).

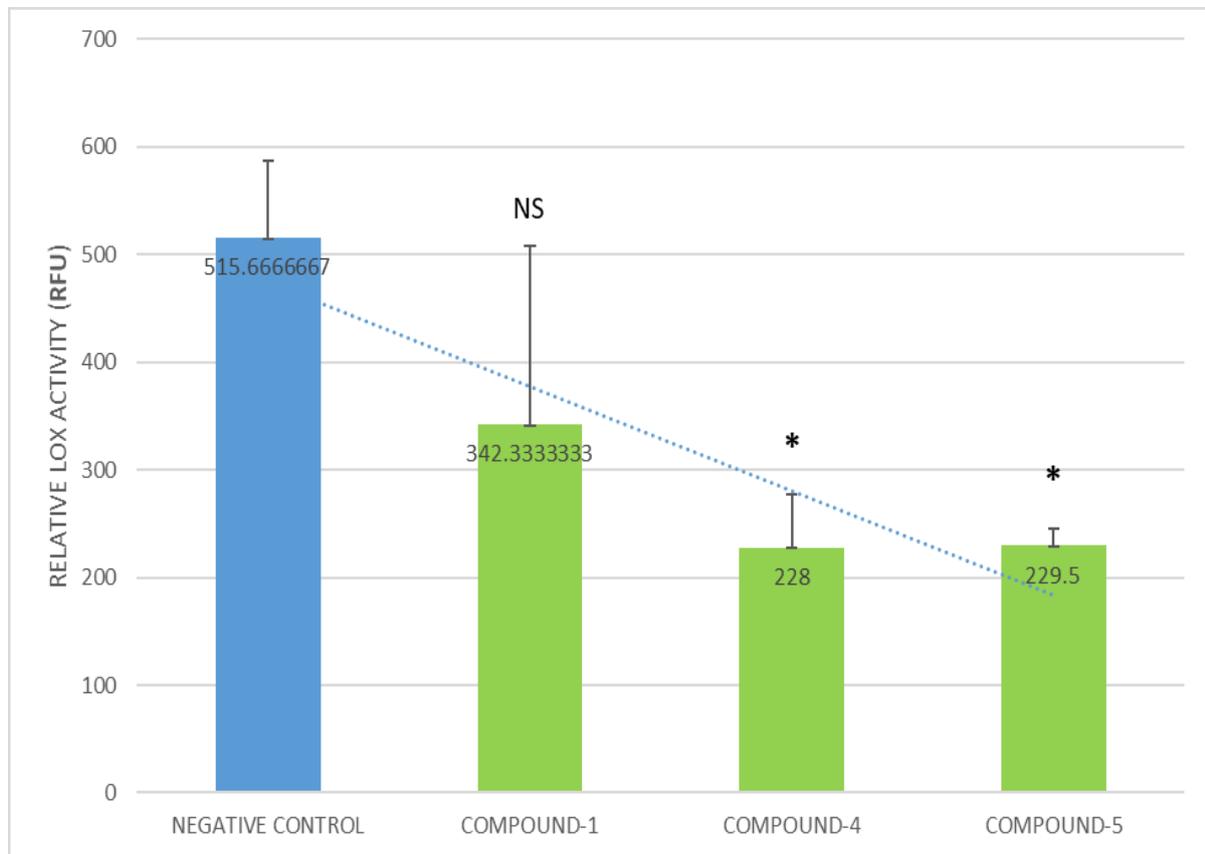
### **3.4 The reduction of LOX activity in Caki-2 cells following treatment with compound-1, 4 and 5**

Figure 3.9 shows the effect of compound-1, 4 and 5 at a concentration of 10 and 50µg/ml on the activity of *LOX* in Caki-2 cells following 24 hours of treatment. *LOX* activities in the Caki-2 cells treated with compound-4 and 5 at 50µg/ml were significantly ( $p<0.05$ ) lower than the negative control cells. Compound-1, at 10/50µg/ml, compound-4 at 10µg/ml and compound-5 at 10µg/ml also reduced the *LOX* activity compared to negative control, but the reduction was not significant.

Figure 3.10 shows the effect of compound-1, 4 and 5 at a concentration of 200µg/ml on the activity of *LOX* in Caki-2 cells following 24 hours of treatment. *LOX* activities in the Caki-2 cells treated with compound-4 and 5 were significantly ( $P<0.05$ ) lower than in the negative control cells. Compound-5 also reduced *LOX* activity compared to negative control, but the reduction was not significant.



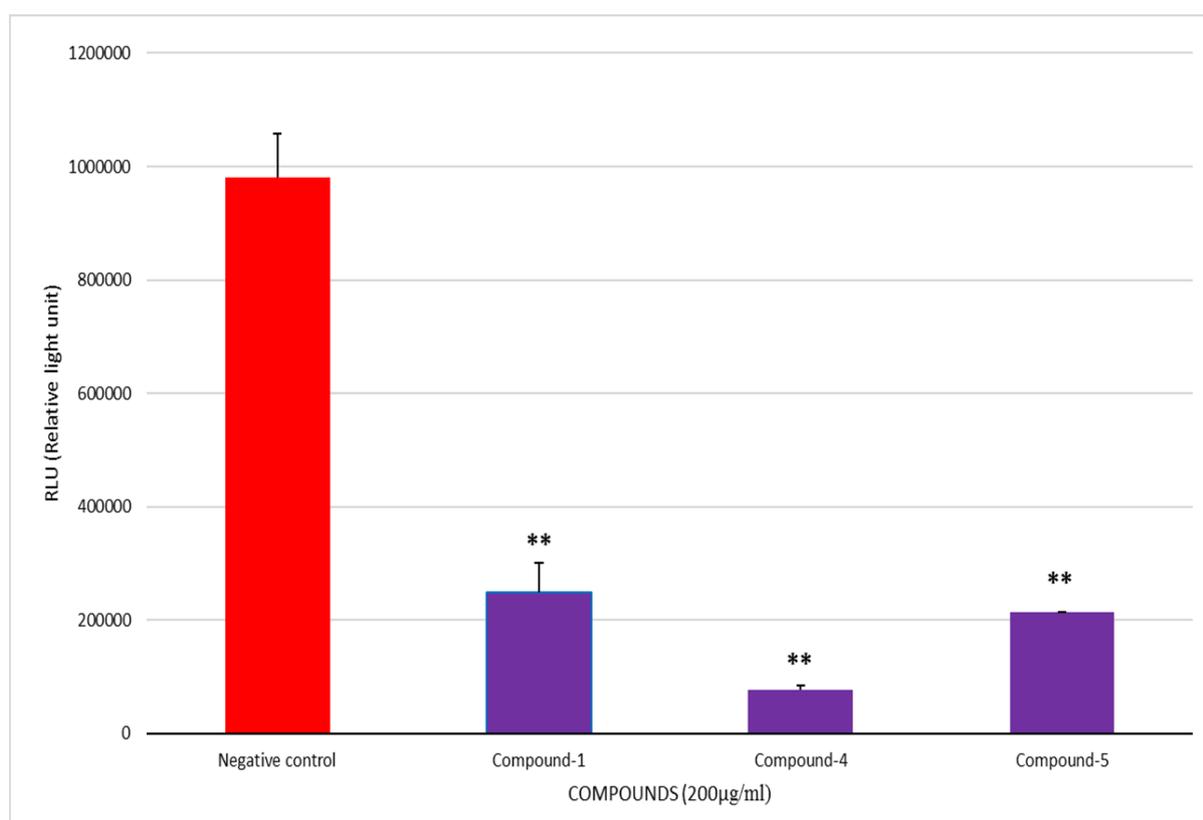
**Figure 3.9:** The lysyl oxidase (*LOX*) activities in Caki-2 cells treated with 10 and 50µg/ml compound-1, 4 and 5 or without compound (negative control) for 24 hours were measured using a fluorescence microplate reader (Ex/Em=540/590nm). Data represents mean  $\pm$  SD, n=6 from three separate experiments. The *LOX* activity in compound treated samples was compared with negative control by student t-test; (\*p<0.05, and NS, not significant).



**Figure 3.10:** The lysyl oxidase (*LOX*) activities in Caki-2 cells treated with 200µg/ml compound-1, 4 and 5 or without compound (negative control) for 24 hours were measured using a fluorescence microplate reader (Ex/Em=540/590nm). Data represents mean  $\pm$  SD, n=6 from three separate experiments. The *LOX* activity in compound treated samples was compared with negative control by student t-test; (\*p<0.05, and NS, not significant).

### 3.5 The effects of compound-1,4 and 5 on the production of NAD(P)H in the Caki-2 cells

Figure 3.11 shows the NAD(P)H production in the Caki-2 cells following 24 hours of treatment with compound-1, 4 and 5 at a concentration of 200µg/ml. NADH and NAD(P)H production in the Caki-2 cells treated with the three compounds were all significantly ( $P<0.01$ ) lower than those in the negative control cells. The inhibitory effect of compound-4 was stronger than compound-1 and 5.



**Figure 3.11:** The production of NADH and NADPH in the Caki-2 cells after treatment with compound-1, 4 and 5 at a concentration of 200µg/ml respectively, compared to the negative control. The data represents mean  $\pm$  SD and  $n=4$ , Student's t-test, (\*\* $P<0.01$  and NS, not significant).

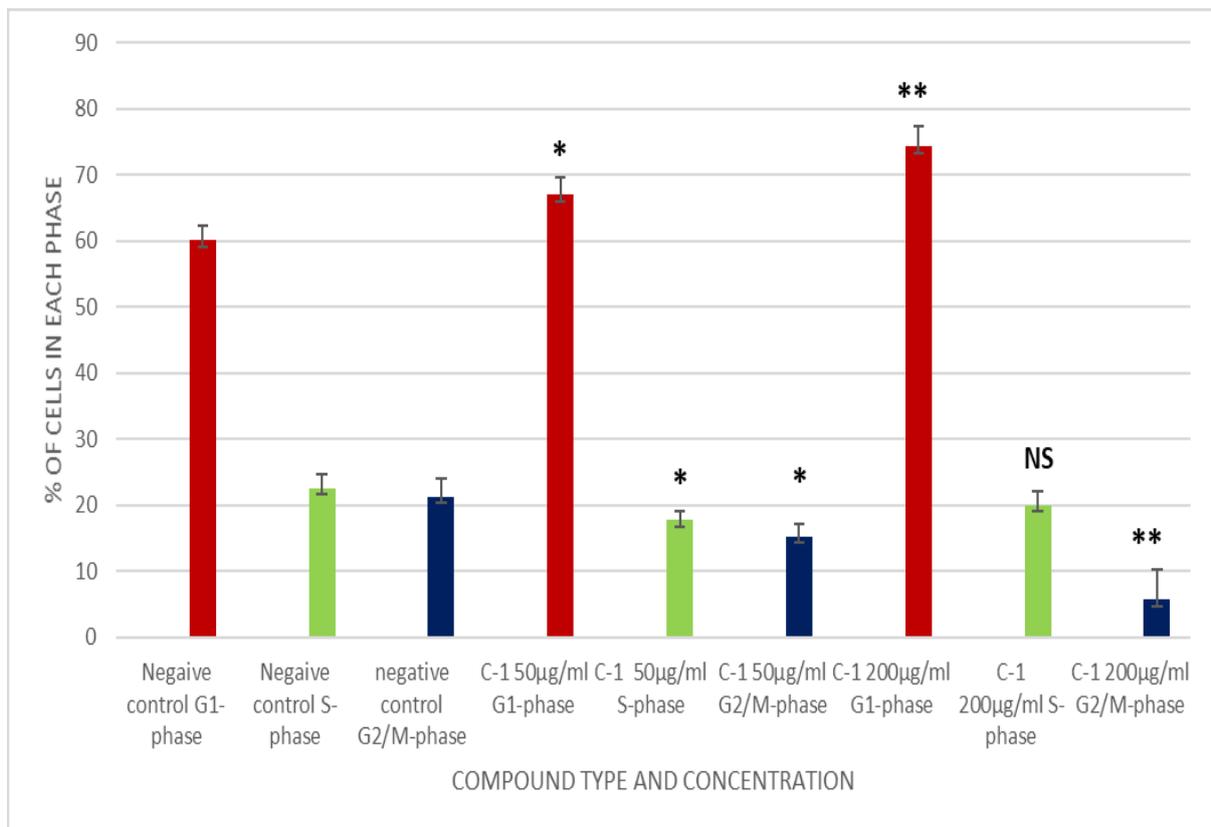
### 3.6 The effects of compound-1, 4 and 5 on Caki-2 cell cycle profile

Figure 3.12 to Figure 3.14 show the phases of cell cycle distribution of Caki-2 cells following 24 hours treatment with compound-1, 4 and 5 at concentrations of 50 and 200µg/ml respectively.

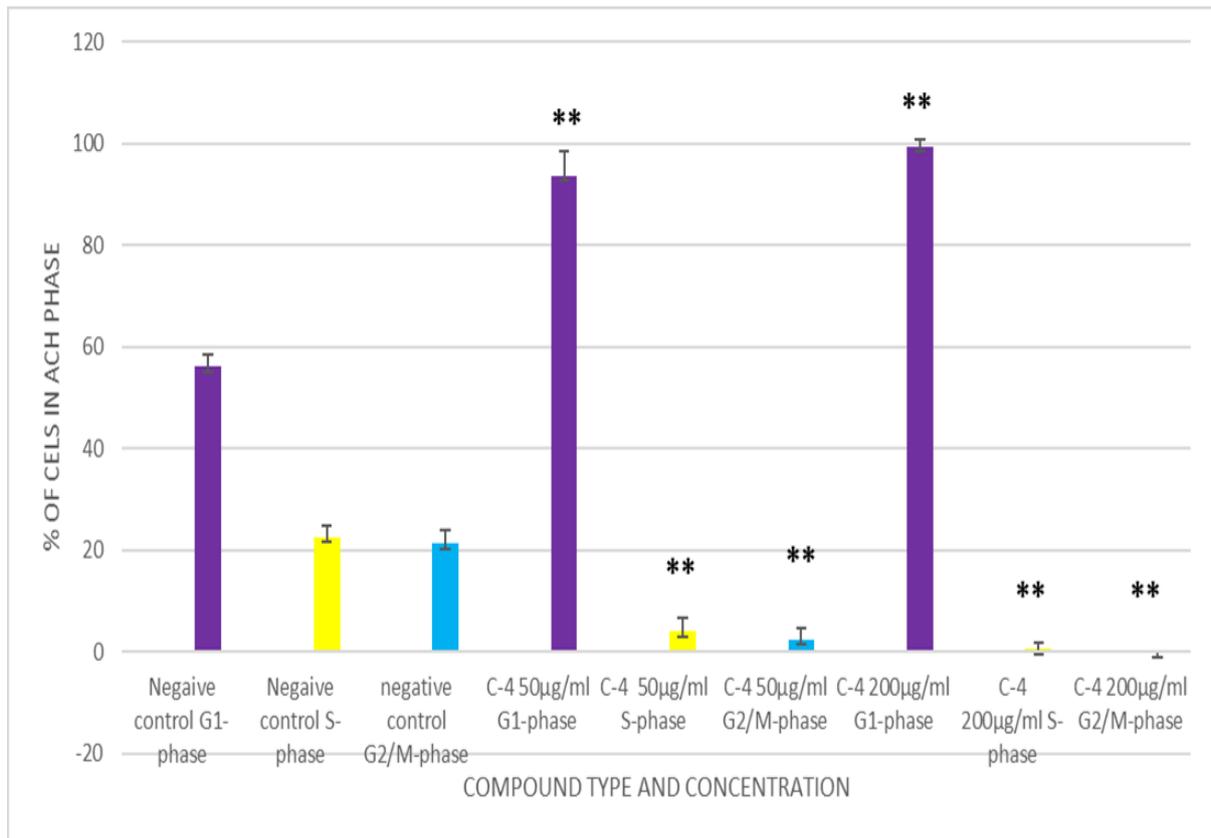
Caki-2 cells treated with 50 or 200µg/ml compound-1 showed that the proportions of G1-phase cells increased, while the proportions of G2/M-phase cells decreased significantly ( $P<0.01$ ) compared to the negative control (Figure 3.9).

Following 24 hours treatment with compound-4 at concentrations of 50 and 200µg/ml, the proportions of G1-phase cells increased significantly, while the percentage of S phase and G2/M-phase cells decreased significantly compared to the negative control ( $P<0.01$ ) (Figure 3.10). High concentration (200µg/ml) of compound-4 had a greater impact on cell cycle distribution than low concentration (50µg/ml) (Figure 3.10).

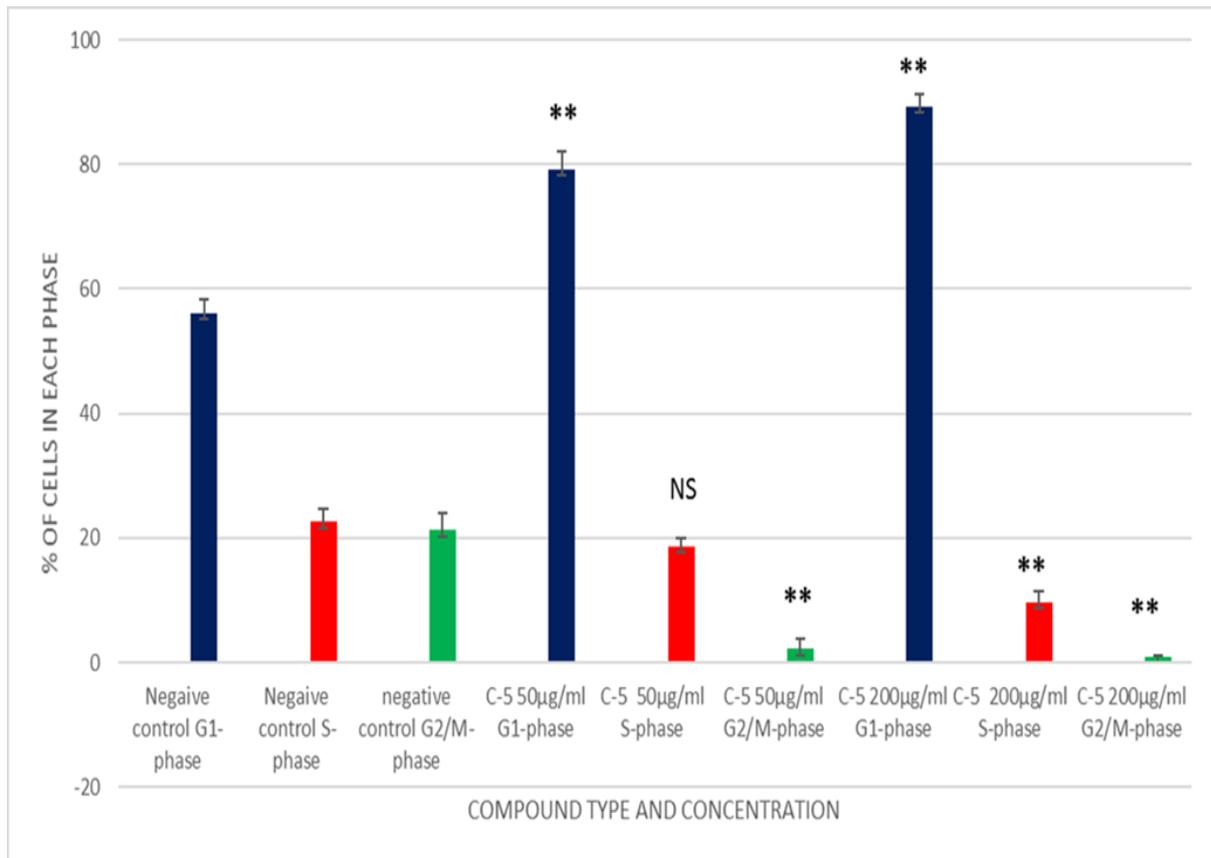
Similar to compound-1, compound-5 treated Caki-2 cells showed that the proportion of G1-phase cells increased significantly, while the proportions of G2/M-phase cells decreased significantly compared to the negative control ( $P<0.01$ ) (Figure 3.11).



**Figure 3.12:** Flow cytometry analysis of cell cycle distribution of Caki-2 cells after 24 hours incubation with 50 or 200 µg/ml of compound-1, compared to the negative control cells. Data represents mean ± SD, n=6 from three separate experiments (\*p<0.05), (\*\*p<0.01), Not significant (NS).



**Figure 3.13:** Flow cytometry analysis of cell cycle distribution of Caki-2 cells after 24 hours incubation with 50 or 200 µg/ml of compound-4, compared to the negative control cells. Data represents mean ± SD, n=6 from three separate experiments (\*p<0.05), (\*\*p<0.01), Not significant (NS).



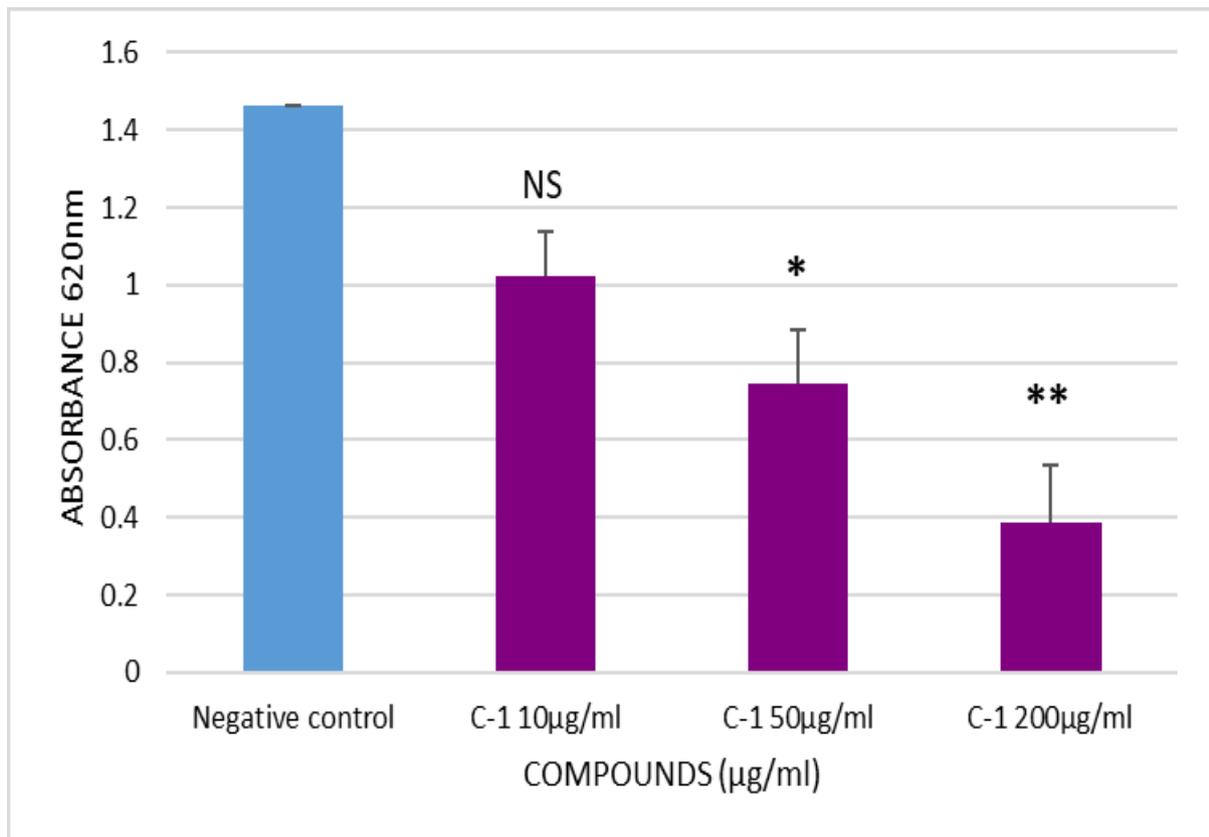
**Figure 3.14:** Flow cytometry analysis of cell cycle distribution of Caki-2 cells after 24 hours incubation with 50 or 200 µg/ml of compound-5 compared to the negative control cells. Data represents mean ± SD, n=6 from three separate experiments (\*p<0.05), (\*\*p<0.01), Not significant (NS).

### 3.7 The effects of compound-1, 4, and 5 on Caki-2 cell adhesion

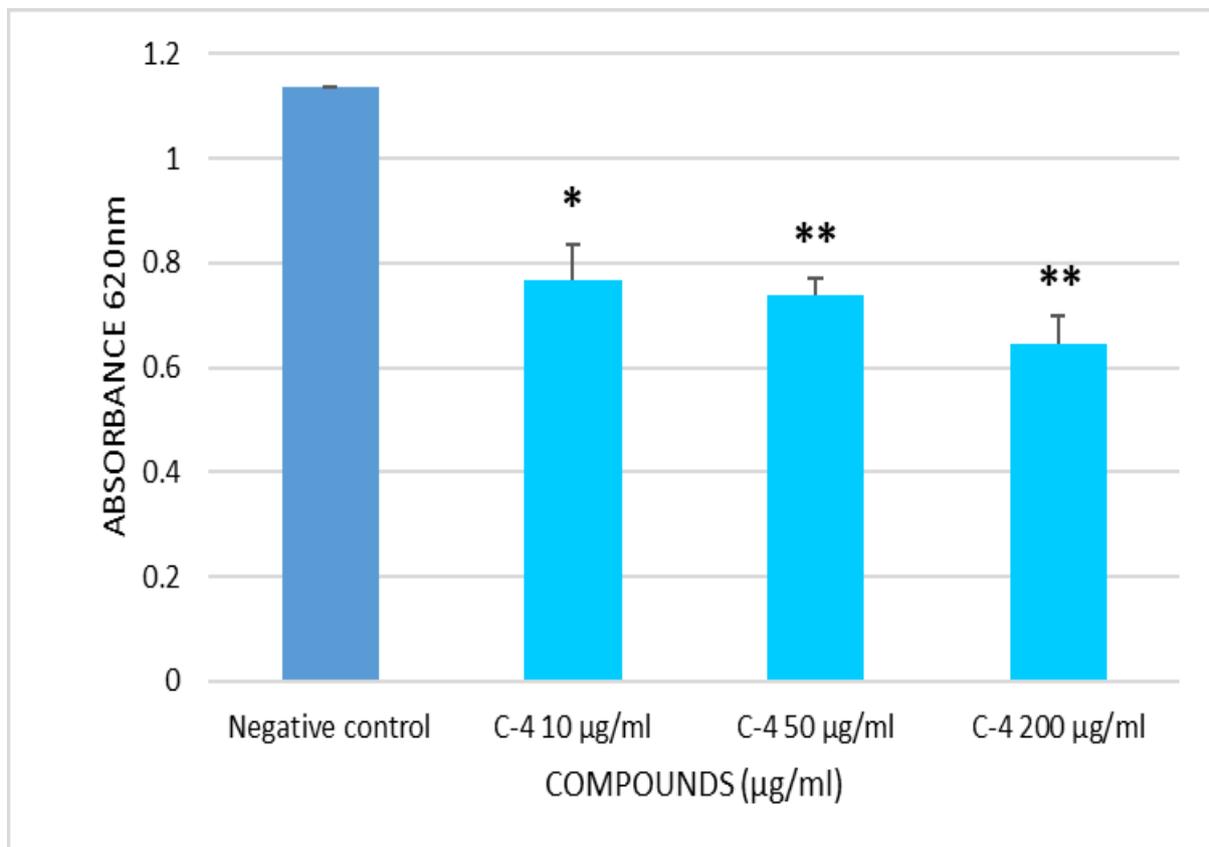
Figure 3.15 to Figure 3.17 show the effects of compound-1, 4 and 5 on Caki-2 cells adhering to the ECM gel *in vitro* following 48 hours of treatment with each compound at concentrations of 10, 50 and 200 $\mu$ g/ml respectively. The Caki-2 cells adhering to the ECM results showed that Compound-1 treatment reduced the adhesion of Caki-2 cells to ECM *in vitro* in a dose-dependent manner. Compound-1 at 10 $\mu$ g/ml did not show significant results when compared to the negative control. While compound-1 at concentrations of 50 and 200 $\mu$ g/ml had significant effects ( $p < 0.05$  or  $p < 0.01$ ) (Figure 3.12).

Similar results were shown on compound-4 treated Caki-2 cells. Cell adhesion to ECM *in vitro* were significantly reduced following 10, 50 and 200  $\mu$ g/ml compound-4 treatment when compared to the negative control ( $p < 0.05$  or  $p < 0.01$ ) (Figure 3.13).

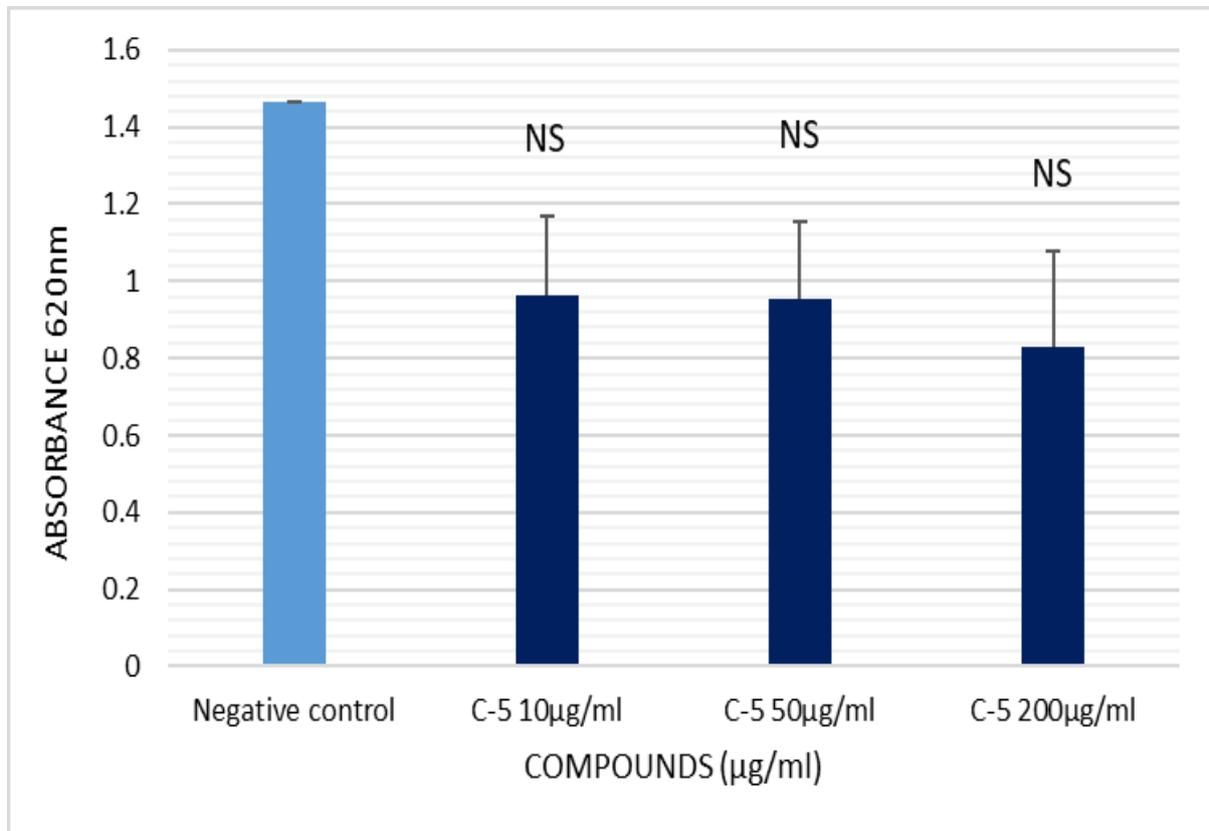
Although there were noticeable decreases in the adhesion of the Caki-2 cells to the ECM following treatment with compound-5 at concentrations of 10, 50 and 200 $\mu$ g/ml when compared to the Caki-2 cells treated with the negative control. However, none of the results were significant ( $p > 0.05$ ) (Figure 3.14).



**Figure 3.15:** The effect of compounds-1 on the Caki-2 cells adhering to ECM gel in vitro. Caki-2 cells had been treated with 10, 50 or 200µg/ml for 48 hours before they were added to ECM gel-coated 96-well plates. The capacity of compound-treated cells adhering to ECM gel were compared to the negative control cells. Data represents mean  $\pm$  SD, n=6 and (\*p<0.05), (\*\*p<0.01), Not significant (NS).



**Figure 3.16:** The effect of compounds-4 on the Caki-2 cells adhering to ECM gel *in vitro*. Caki-2 cells had been treated with 10, 50 or 200µg/ml for 48 hours before they were added to ECM gel-coated 96-well plates. The capacity of compound-treated cells adhering to ECM gel were compared to the negative control cells. Data represents mean  $\pm$  SD, n=6 and (\* $p$ <0.05), (\*\* $p$ <0.01), Not significant (NS).

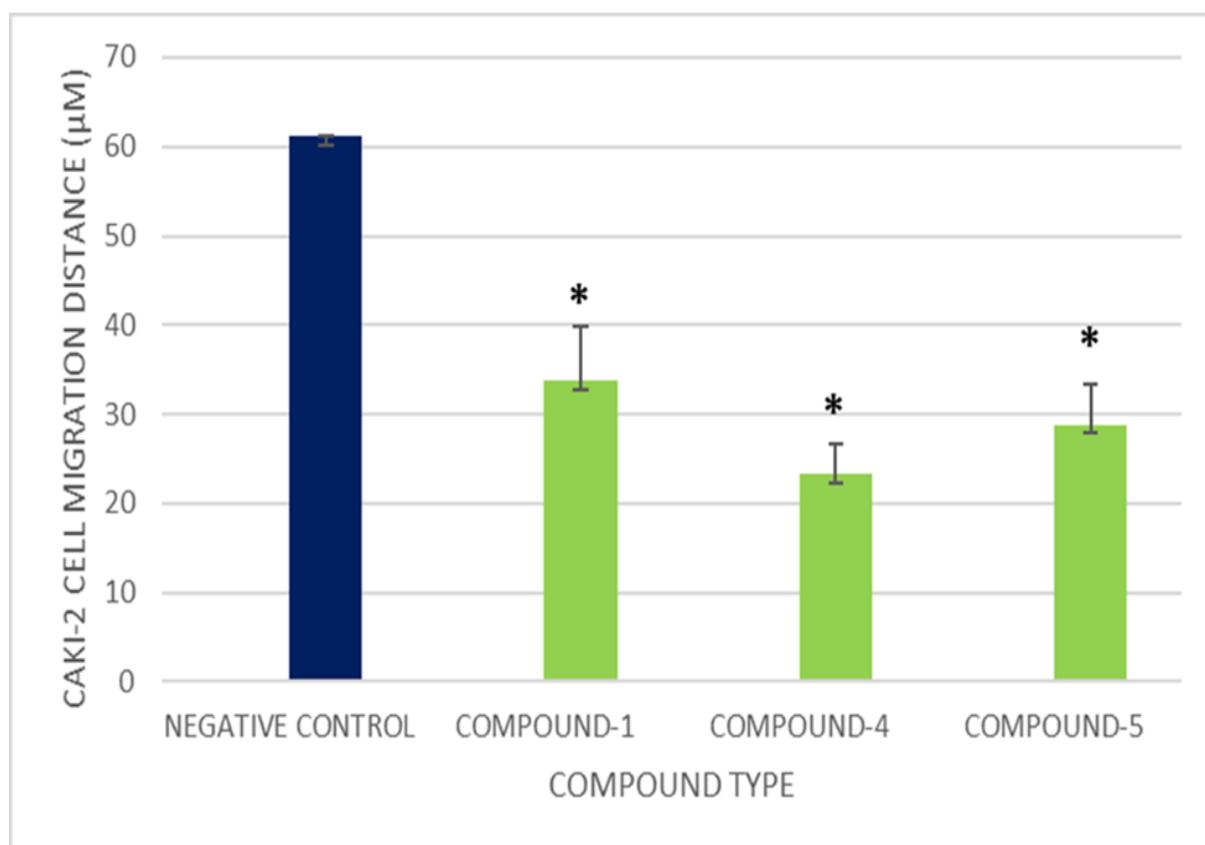


**Figure 3.17:** The effect of compound-5 on the Caki-2 cells adhering to ECM gel in vitro. Caki-2 cells had been treated with 10, 50 or 200 $\mu\text{g/ml}$  compound-5 for 48 hours before they were added to ECM gel-coated 96-well plates. The capacity of compound-treated cells adhering to ECM gel were compared to the negative control cells. Data represents mean  $\pm$  SD,  $n=6$  and ( $*p<0.05$ ), ( $**p<0.01$ ), Not significant (NS).

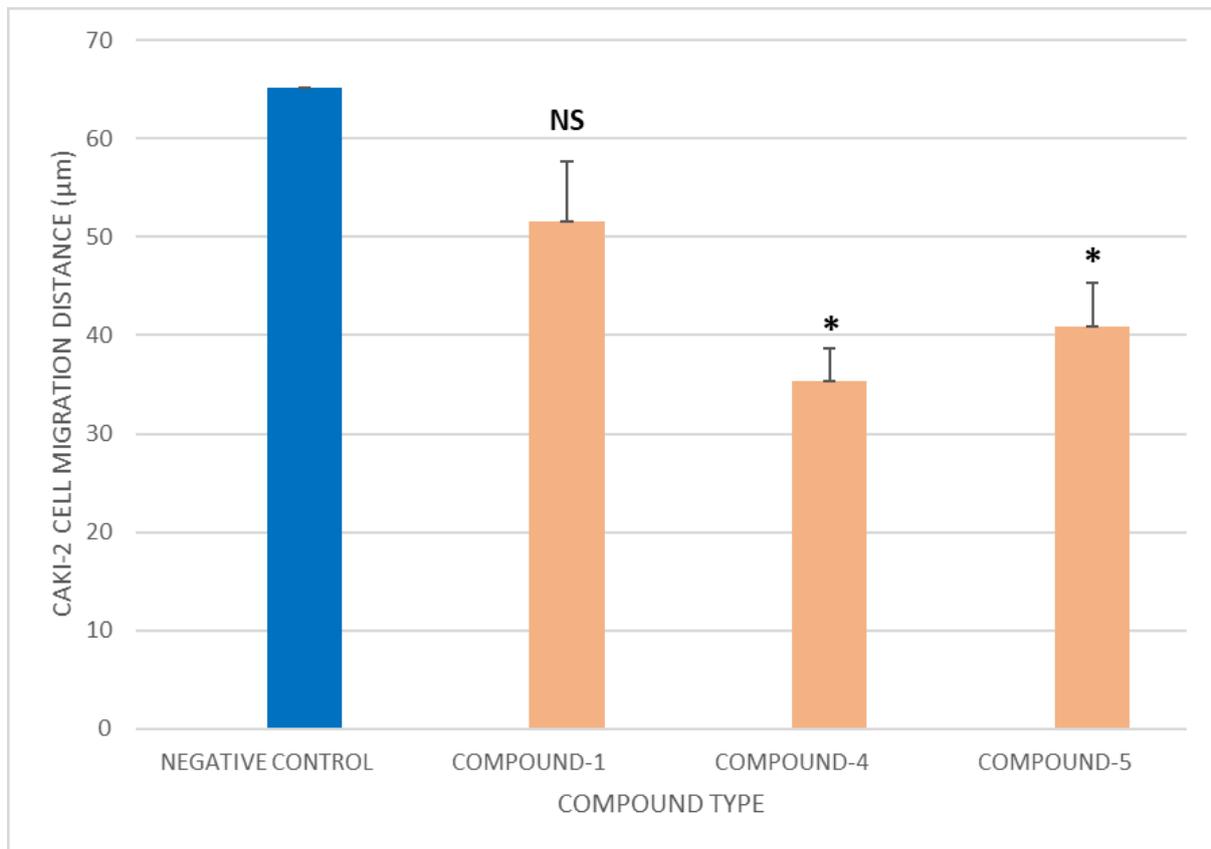
### 3.8 The effects of the three compounds- (1, 4 and 5) on the migration distance of the Caki-2 cells *in vitro*

Figure 3.18 shows the effect of compound-1, 4 and 5 at 200 $\mu$ g/ml on Caki-2 cell migration over a period of 24 hours *in-vitro* compared to the negative control, compound-1, 4 and 5 significantly inhibited Caki-2 cell migration ( $p < 0.05$ ).

Figure 1.19 shows the effect of compound-1, 4 and 5 at 200 $\mu$ g/ml on Caki-2 cell migration over a period of 48 hours *in-vitro* compared to the negative control, compound-4 and compound-5 significantly inhibited Caki-2 cell migration ( $p < 0.05$ ). While the effect of compound-1 was not significant



**Figure 3.18:** The effect of 200 $\mu$ g/ml compound-1, 4 and 5 on Caki-2 cells migration over a period of 24 hours *in-vitro*. The migration distance of compound-treated Caki-2 cells was compared to the negative control cells. Data represent mean  $\pm$  SD,  $n=6$  from three separate experiments (\* $p < 0.05$ ) and Not significant (NS).



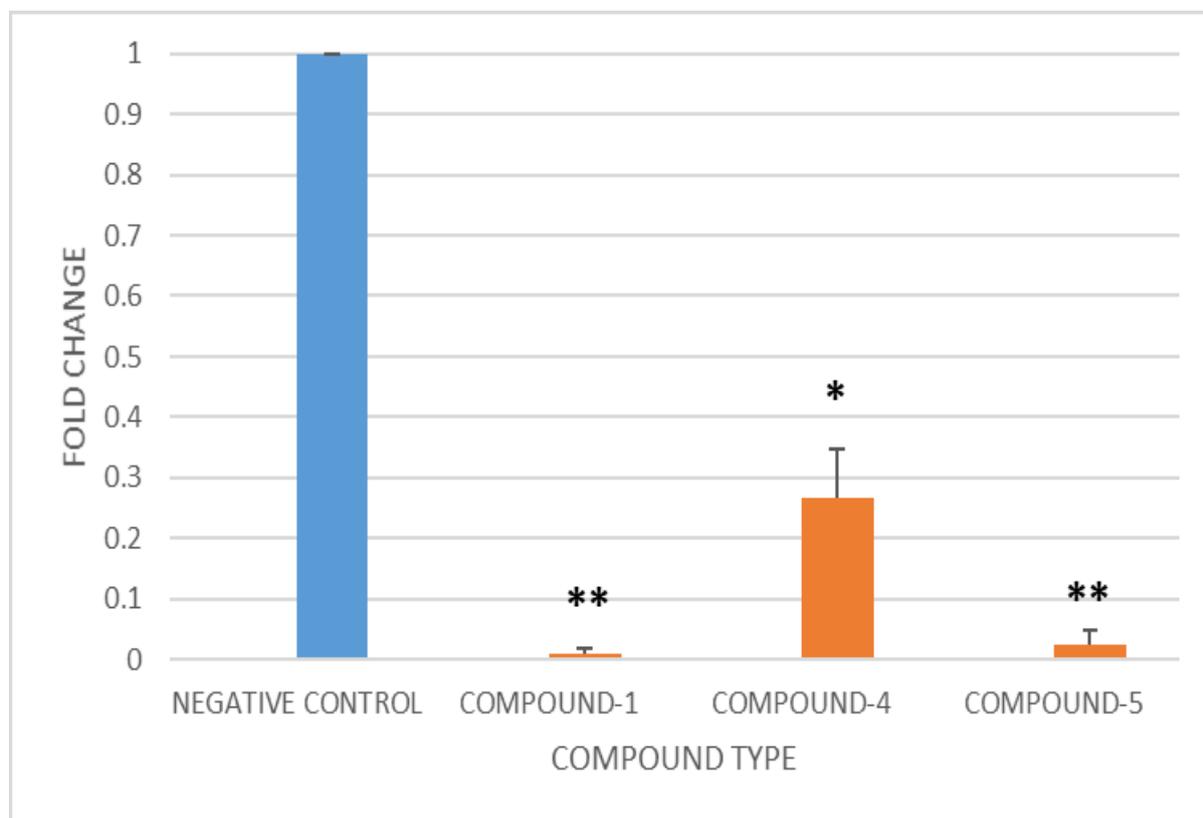
**Figure 3.19:** The effect of 200µg/ml compound-1, 4 and 5 on Caki-2 cells migration over a period of 48 hours *in-vitro*. The migration distance of compound-treated Caki-2 cells was compared to the negative control cells. Data represent mean  $\pm$  SD, n=6 from three separate experiments (\*p<0.05) and Not significant (NS).

### 3.9.1 Alterations in gene expression in Caki-2 cells following the treatment with compound-1, 4 and 5

Following 24 hours of incubation with compound-1,4 and 5 at a concentration of 200µg/ml, the expressions of genes associated with cell proliferation, migration, adhesion and apoptotic signalling pathways were tested using qRT-PCR, and results are shown in Figure 3.20 to Figure 3.27.

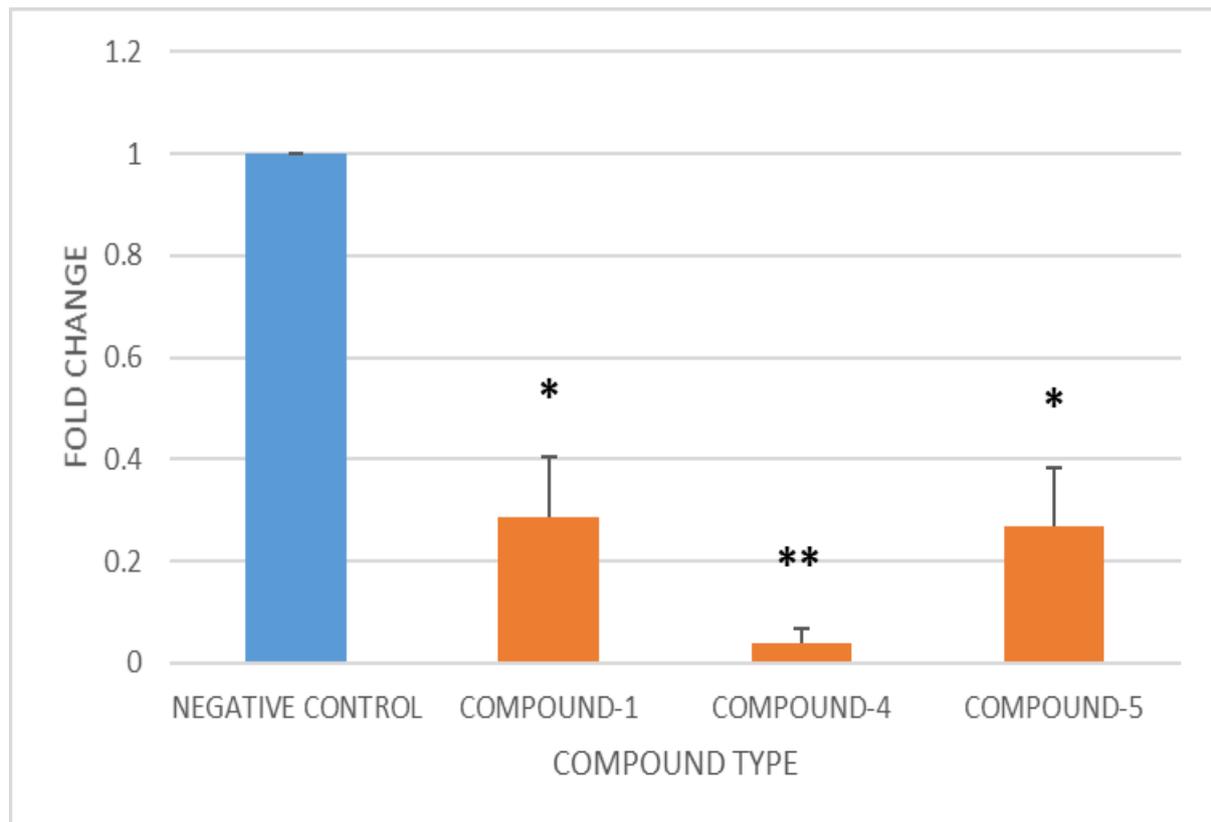
### 3.9.2 Changes in the expression of cell growth-controlling genes *RRM2* and *CDKN1B*

Figure 3.20 shows *RRM2* mRNA expression in the Caki-2 cells treated with compounds 1, 4 and 5 were significantly reduced between 0.01-fold to 0.27-fold compared to its expression in the negative control cells ( $p < 0.05$ ).



**Figure 3.20:** The fold change of *RRM2* expression in Caki-2 cells following the treatment with compound-1, 4 and 5 were compared to negative control. Expression of housekeeping genes,  $\beta$ -Actin and *GAPDH*, were used for normalization. Data represent mean  $\pm$  SD,  $n=6$  from three separate experiments. (\*,  $p < 0.05$ , \*\* $p < 0.01$ ).

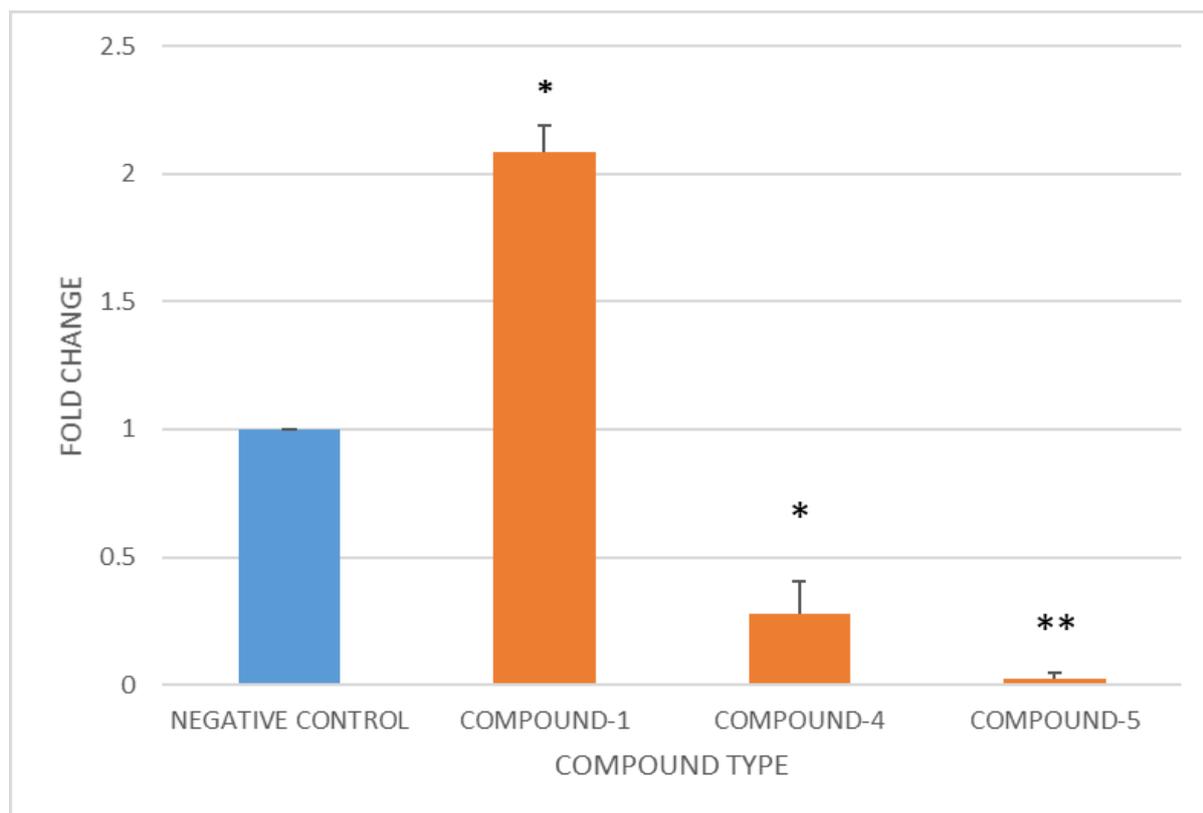
Figure 3.21 shows the downregulation of *CDKN1B* mRNA expression in Caki-2 cells following treatment with compound-1,4 and 5 at a concentration of 200µg/ml. The average fold change of *CDKN1B* expression was between 0.03 and 0.28 in the three compounds treated cells compared to negative control. The effects of compound-4 on *CDKN1B* expression was greater than compound-1 and 5.



**Figure 3.21:** The fold change of *CDKN1B* expression in Caki-2 cells following the treatment with compound-1, 4 and 5 were compared to negative control. Expression of housekeeping genes,  $\beta$ -Actin and *GAPDH*, were used for normalization. Data represent mean  $\pm$  SD, n=6 from three separate experiments. (\*,  $p < 0.05$ , NS,  $p > 0.05$ ).

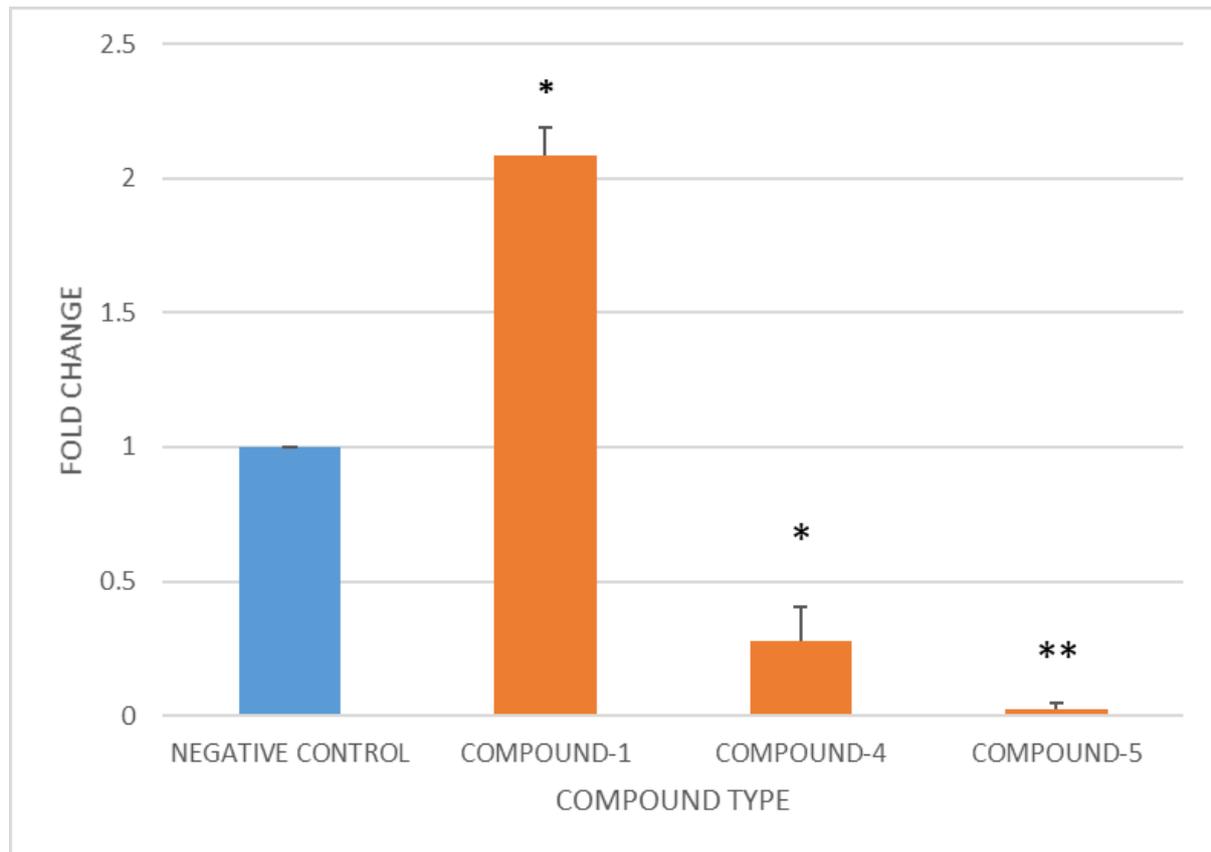
### 3.9.3 Changes in the expression of cell migration and adhesion-associated genes

Figure 3.22 shows that *MMP9* mRNA expressions in Caki-2 cells following 24 hours incubation with 200 $\mu$ g/ml compound-4 and 5 were significantly downregulated compared to its expression in the negative control ( $p < 0.05$ ). While in compound-1 treated cells, *MMP9* expression was upregulated.



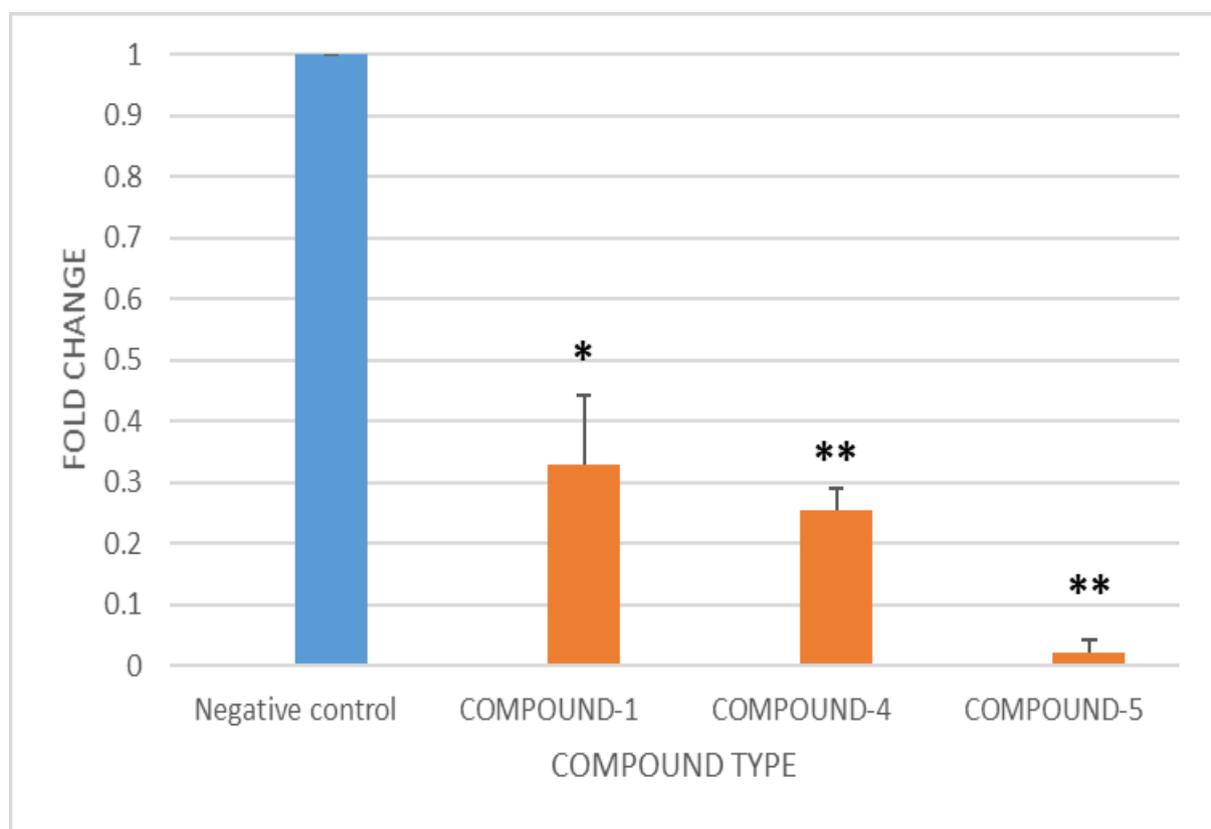
**Figure 3.22:** The fold change of *MMP9* expression in Caki-2 cells following the treatment with compound-1, 4 and 5 were compared to negative control. Expression of housekeeping genes,  $\beta$ -Actin and *GAPDH*, were used for normalization. Data represent mean  $\pm$  SD,  $n=6$  from three separate experiments. (\*,  $p < 0.05$ , NS,  $p > 0.05$ ).

Figure 3.23 shows that the changes of *CXCH12* mRNA expression in Caki-2 cells after 24 hours incubation with 200µg/ml compound-1, 4 and 5 were similar to the changes of *MMP9* expression. *CXCH12* expression was upregulated in compound-1 treated cells, but downregulated in compound-4 and 5 treated cells ( $p < 0.05$ ) compared to its expression in negative control.



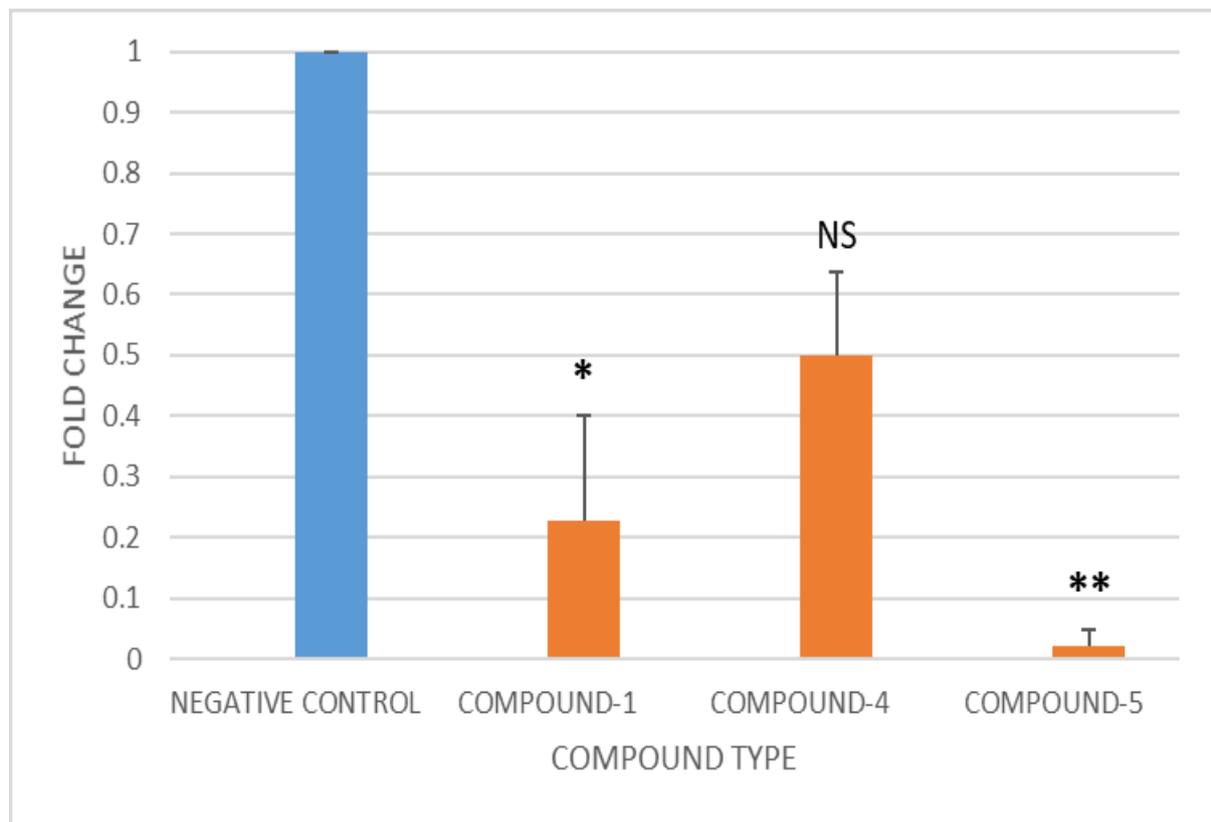
**Figure 3.23:** The fold change of *CXCH12* expression in Caki-2 cells following the treatment with compound-1, 4 and 5 were compared to negative control. Expression of housekeeping genes,  $\beta$ -Actin and *GAPDH*, were used for normalization. Data represent mean  $\pm$  SD, n=6 from three separate experiments. (\*\* $p < 0.01$ , \* $p < 0.05$ , NS,  $p > 0.05$ ).

Figure 3.24 shows the changes of *VMP1* mRNA expression in Caki-2 cells after 24 hours of incubation with compound-1, 4 and 5 at a concentration of 200µg/ml using a qRT-PCR test. The average fold change of *VMP1* expression was between 0.01 and 0.32 across all of the three compounds used. *VMP1* mRNA expression in the Caki-2 cells treated with compound-4 and 5 was significantly ( $P < 0.01$ ) down-regulated compared to the negative control cells, compound-1 also showed significantly ( $p < 0.05$ ) lower results when compared to the negative control. The impact of compound-5 on *VMP1* expression was greater than compound-4 and compound-1.



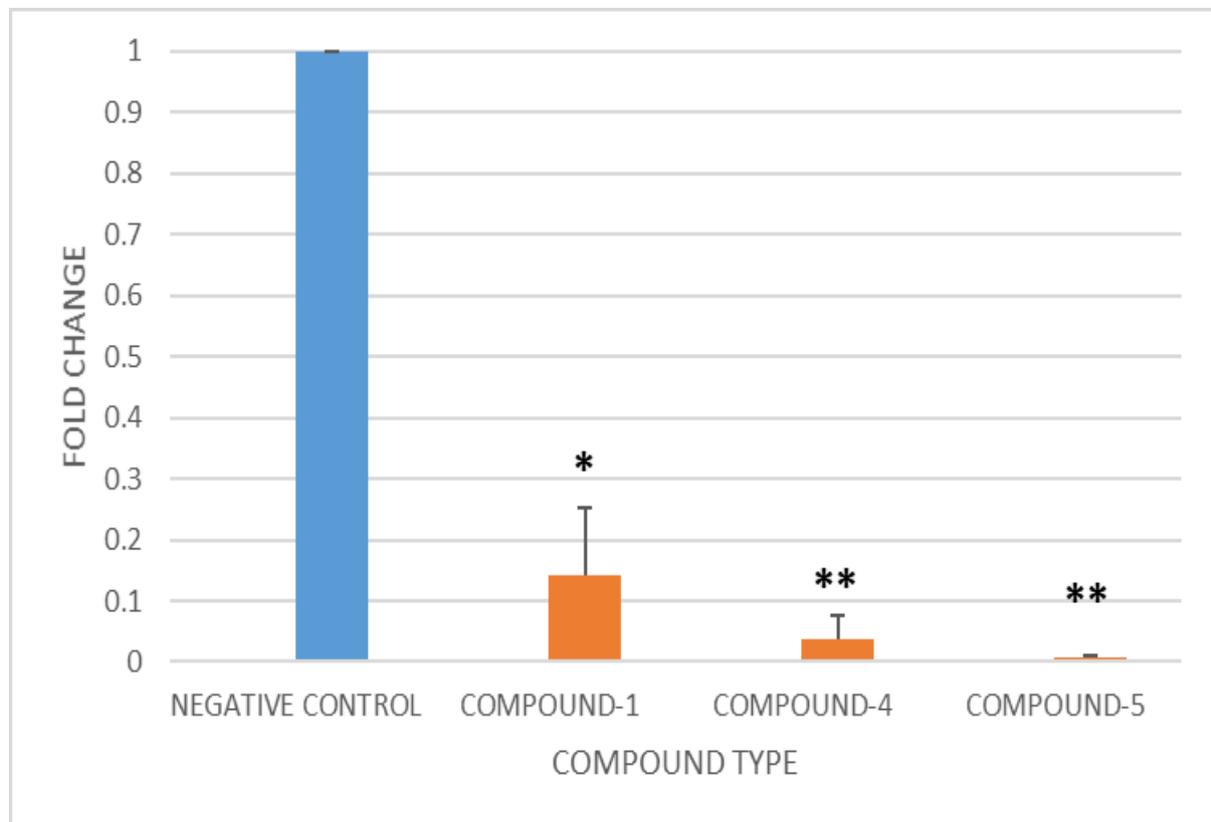
**Figure 3.24:** The fold change *VMP1* expression in Caki-2 cells following the treatment with compound-1, 4 and 5 were compared to negative control. Expression of housekeeping genes,  $\beta$ -Actin and *GAPDH*, were used for normalization. Data represent mean  $\pm$  SD, n=6 from three separate experiments. (\*\* $p < 0.01$ , \*,  $p < 0.05$ , NS).

Figure 3.25 Shows *CD98HC* mRNA expression in Caki-2 cells following 24 hours of incubation with 200µg/ml compound-1, 4 and 5 were downregulated 0.01-fold to 0.5-fold compared to its expression in the negative control. However, the downregulation was only significant in compound-1 and 5 treated cells ( $p < 0.05$ / $p < 0.01$ ).



**Figure 3.25:** The fold change *CD98HC* expression in Caki-2 cells following the treatment with compound-1, 4 and 5 were compared to negative control. Expression of housekeeping genes,  $\beta$ -Actin and *GAPDH*, were used for normalization. Data represent mean  $\pm$  SD,  $n=6$  from three separate experiments. (\*,  $p < 0.05$ , NS,  $p > 0.05$ ).

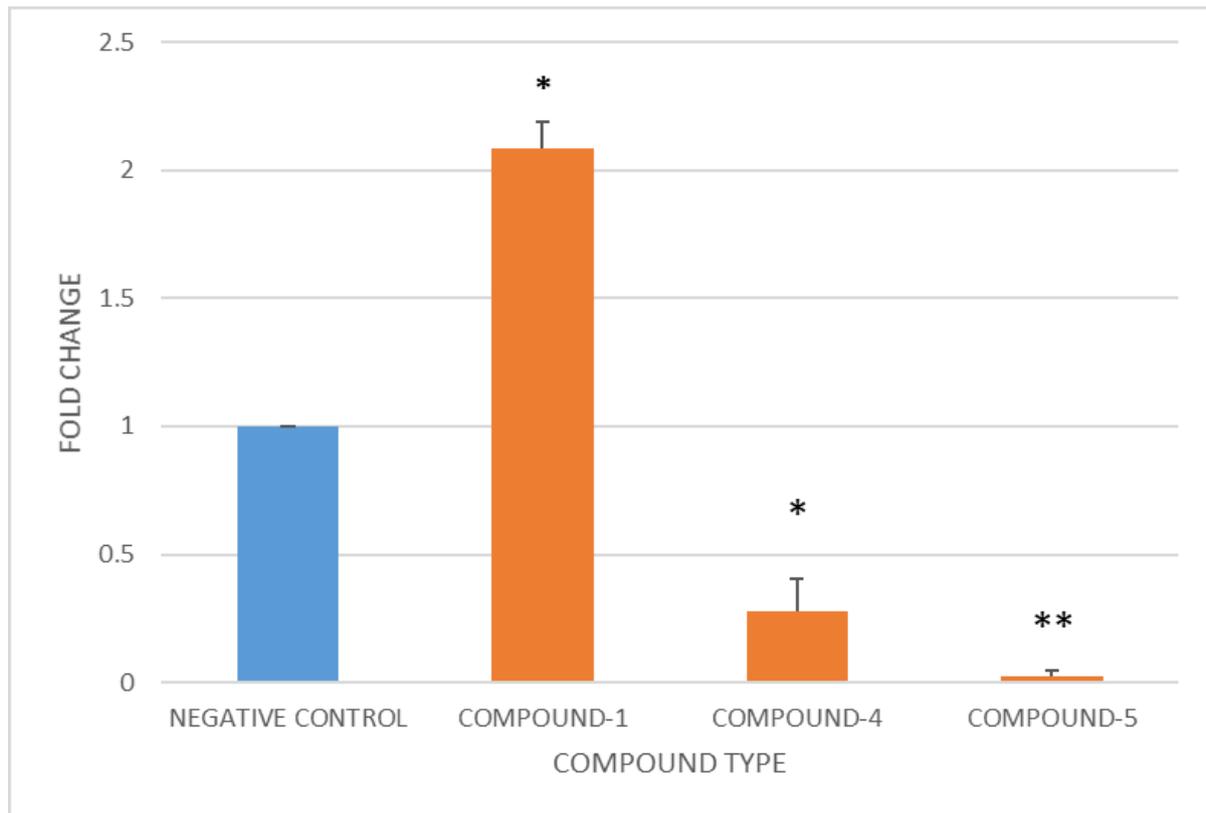
Figure 3.26 shows that *CXCR16* mRNA expression in Caki-2 cells following 24 hours incubation with 200 $\mu$ g/ml compound-1, 4 and 5 were significantly downregulated 0.005-fold to 0.13-fold compared to its expression in the negative control ( $p < 0.01$ ).



**Figure 3.26:** The fold change *CXCR16* expression in Caki-2 cells following the treatment with compound-1, 4 and 5 were compared to negative control. Expression of housekeeping genes,  $\beta$ -Actin and *GAPDH*, were used for normalization. Data represent mean  $\pm$  SD,  $n=6$  from three separate experiments. (\*\*,  $P < 0.01$ , \*,  $p < 0.05$ ).

### 3.9.4 Changes in the expression of cell apoptosis-associated gene *CASPA9*

Figure 3.27 shows that *CASPA9* mRNA expression in Caki-2 cells following 24 hours incubation with compound-1 was significantly upregulated 2.2-fold ( $p < 0.05$ ) compared to its expression in negative control. However, in compound-4 and 5 treated cells, *CASPA9* expression were downregulated.

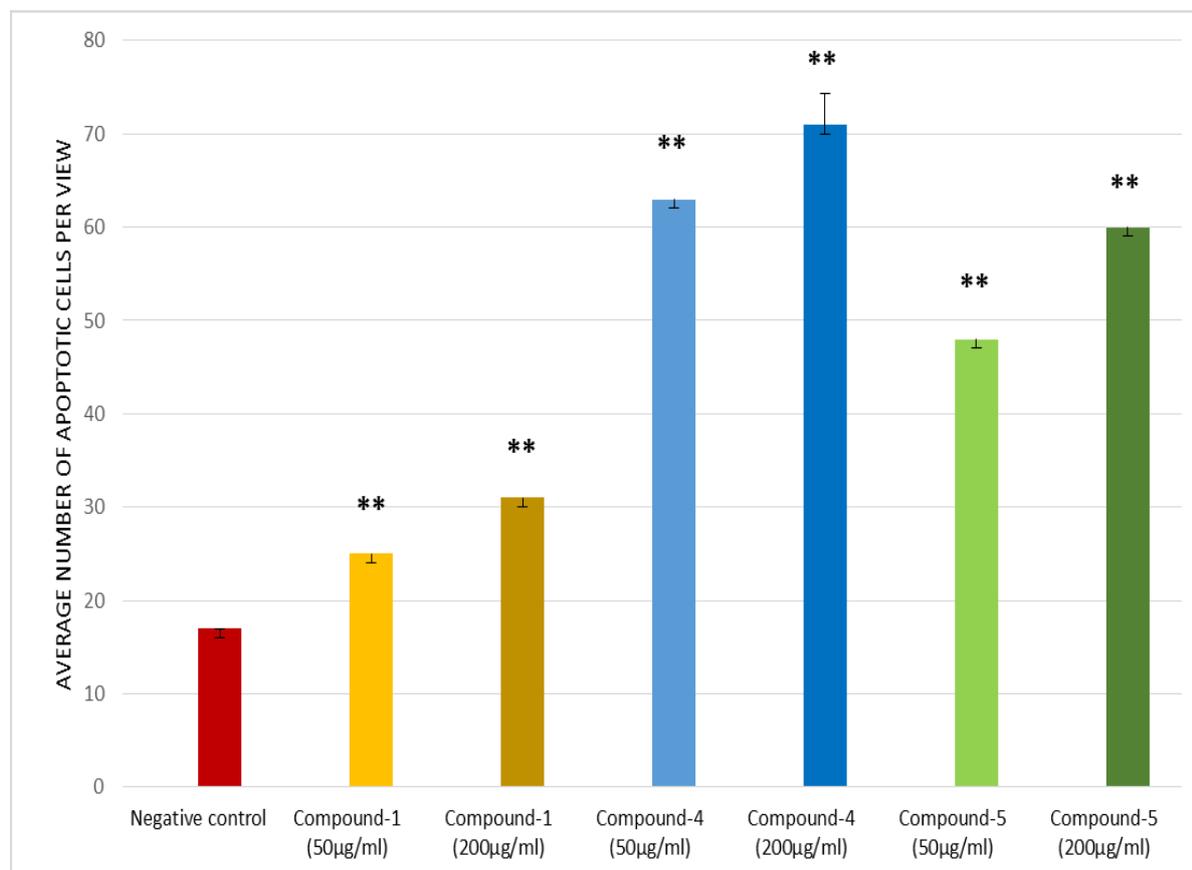


**Figure 3.27:** The fold change *CASPA9* expression in Caki-2 cells following the treatment with compound-1, 4 and 5 were compared to negative control. Expression of housekeeping genes,  $\beta$ -Actin and *GAPDH*, were used for normalization. Data represent mean  $\pm$  SD,  $n=6$  from three separate experiments. (\*,  $p < 0.05$ , NS,  $p > 0.05$ ).

### **3.10 The effect of compound-1, 4 and 5 on the apoptotic activity of the Caki-2 cells**

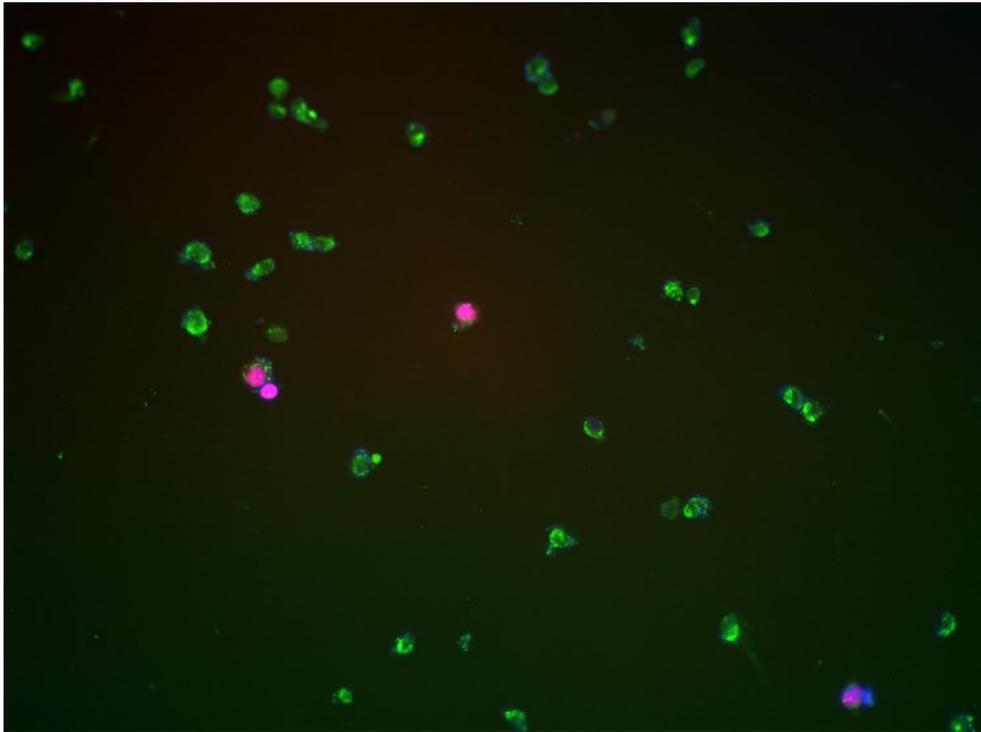
Figure 3.28 shows the apoptotic activity of the Caki-2 cells following 24 hours of treatment with compound-1, 4 and 5 at a concentration of 50 and 200 $\mu$ g/ml. More apoptotic cells were observed when Caki-2 were treated with compound-1, 4 and 5 compared to the negative control cells. The differences were significant ( $p < 0.01$ ). The higher concentration 200 $\mu$ g/ml had a greater effect than the lower concentration 50  $\mu$ g/ml.

Figure 3.29-3.32 shows fluorescent images of the apoptotic activity of the Caki-2 cells following 24 hours of treatment with compound-1, 4 and 5 at a concentration of 50 and 200 $\mu$ g/ml. More apoptotic cells (red) cells were observed in the images of the Caki-2 cells treated with compound-1, 4 and 5 compared to the negative control which showed more viable (green) cells. The Caki-2 cells treated with higher (200 $\mu$ g/ml) concentrations showed more apoptotic cells compared to the lower concentrations (50 $\mu$ g/ml).

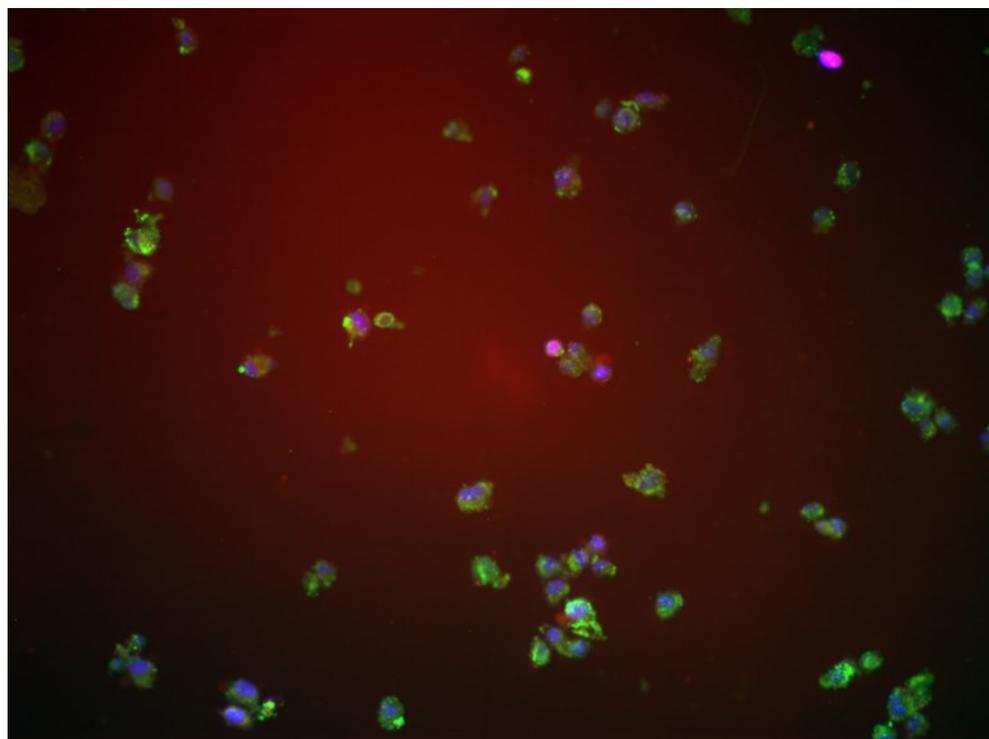


**Figure 3.28:** The effect of compound-1, 4 and 5 at a concentration of 50 and 200 µg/ml on the apoptosis of Caki-2 cells after 24 hours of incubation irrespectively, compared to the negative control. The apoptotic cells were measured using fluorescent microscope. Data represent mean  $\pm$  SD, from six measurements in three separate experiments ( $n = 3$ ). Student's t-test, (\*\* $p < 0.01$ , and NS, not significant).

(A)

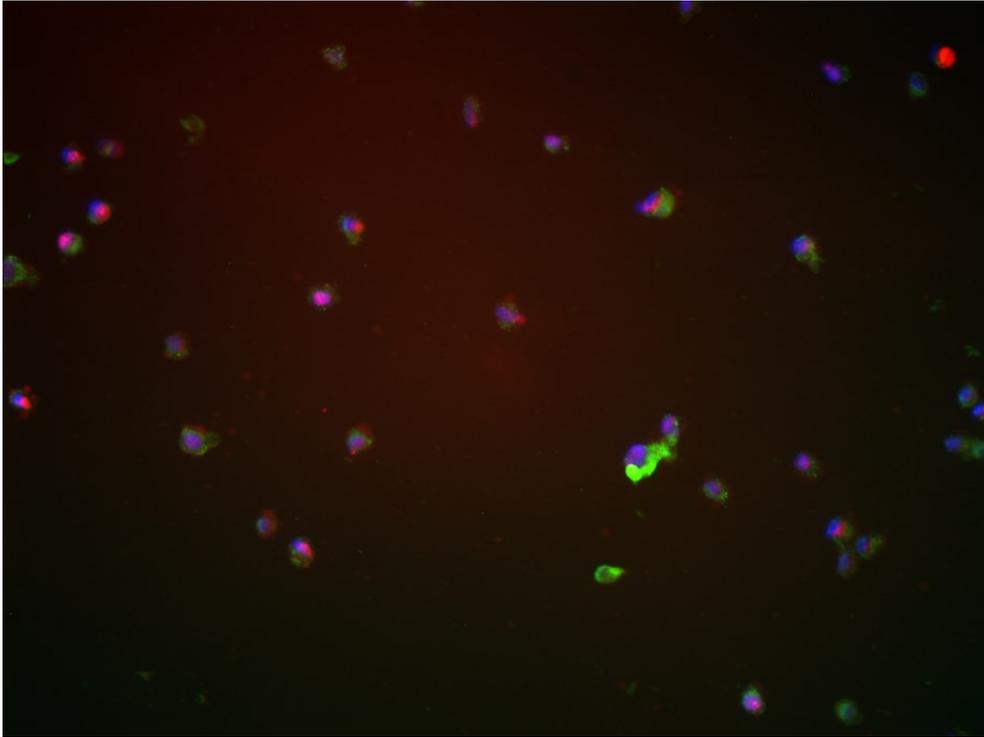


(B)

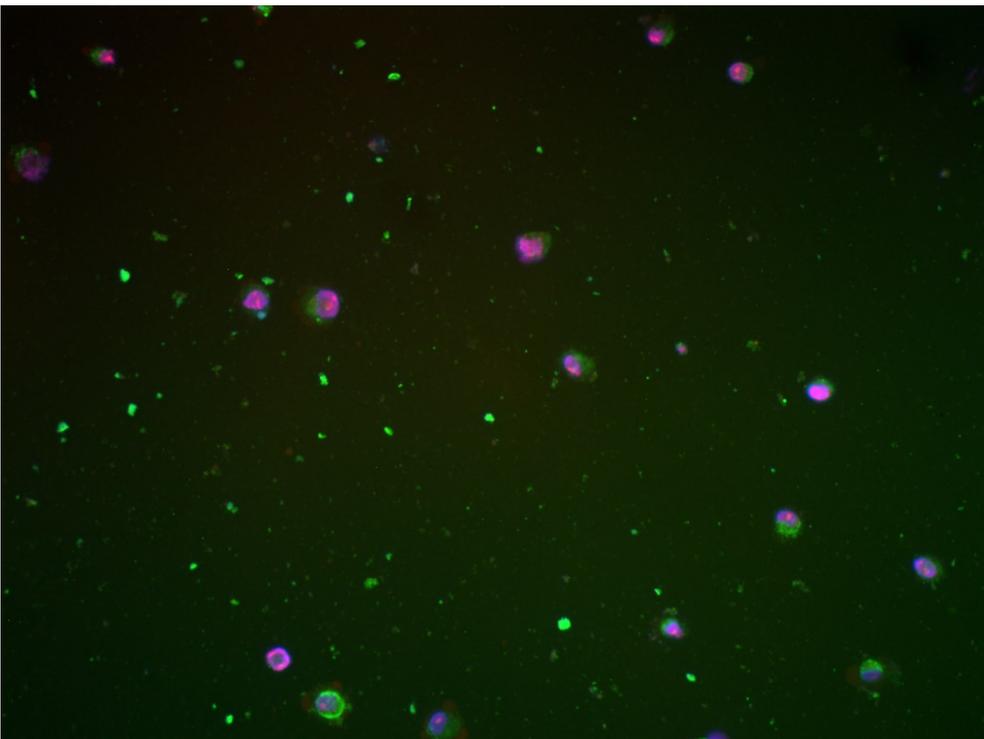


**Figure 3.29:** Fluorescent images showing apoptotic activity of Caki-2 cells using fluorescent microscopy assay following 24 hours treatment with negative control. Then the Cak-2 cells were then fixed and treated with both DAPI (blue) and Anti-Fluorescein antibody (green).

(A)

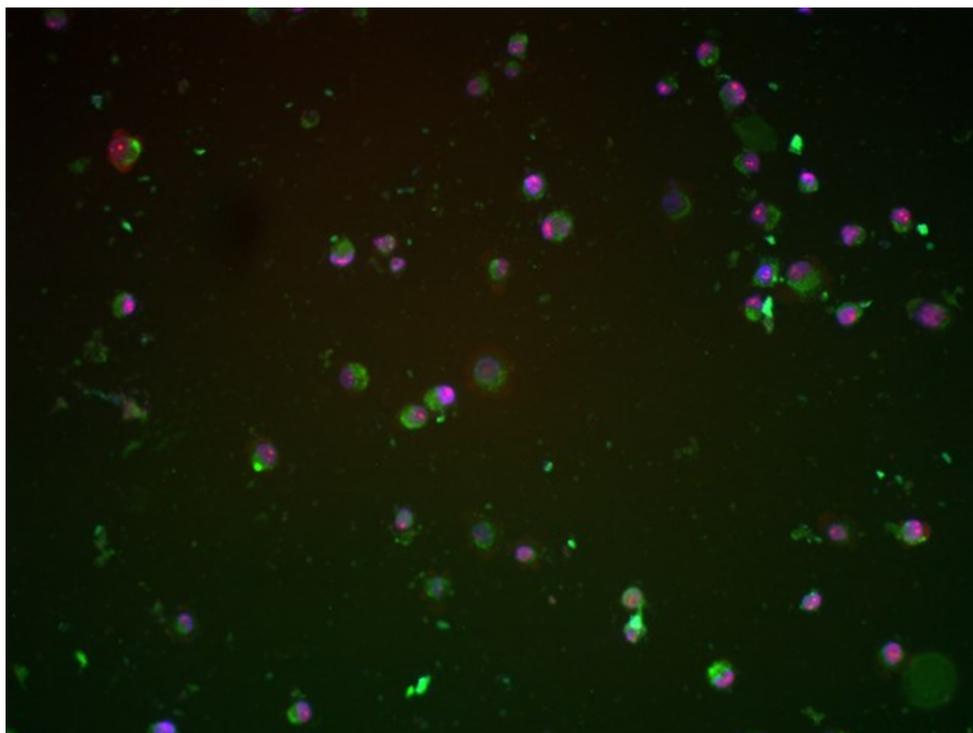


(B)

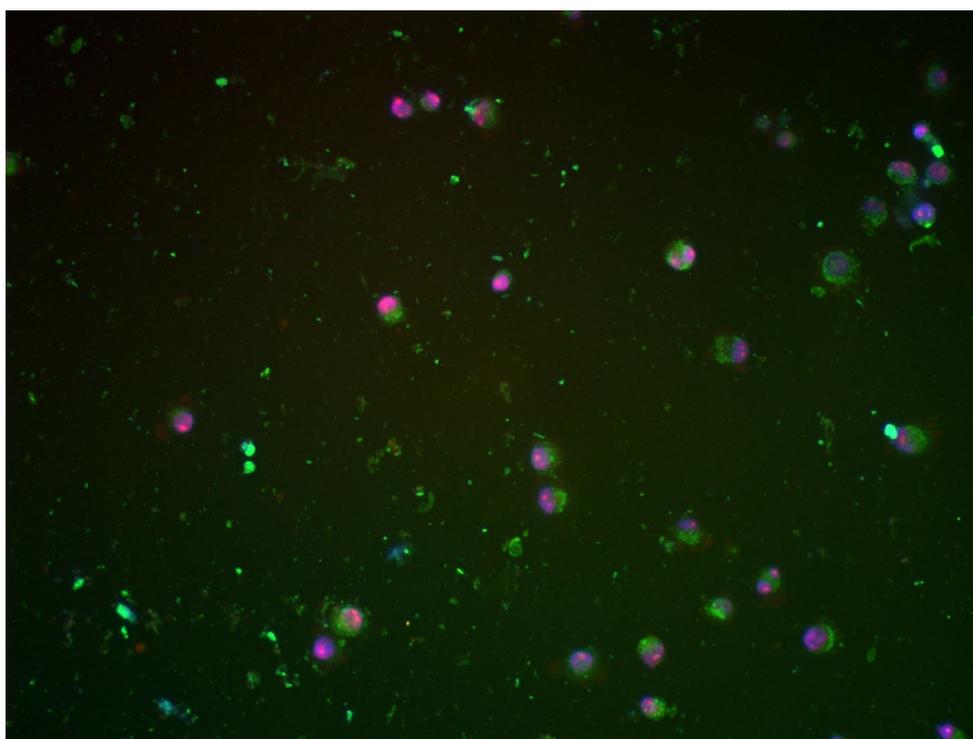


**Figure 3.30:** Fluorescent images showing apoptotic activity of Caki-2 cells using fluorescent microscopy assay following 24 hours treatment with compound-1 at a concentration of (A) 50µg/ml and (B) 200µg/ml. Then the Caki-2 cells were then fixed and treated with both DAPI (blue) and Anti-Fluorescein antibody (green).

(A)

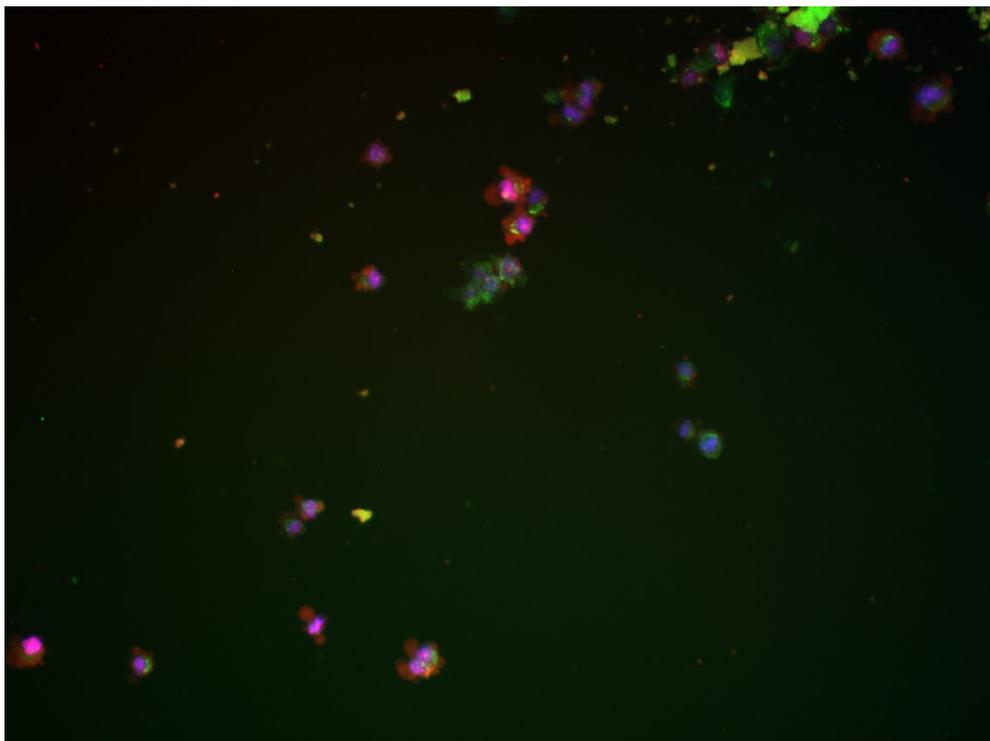
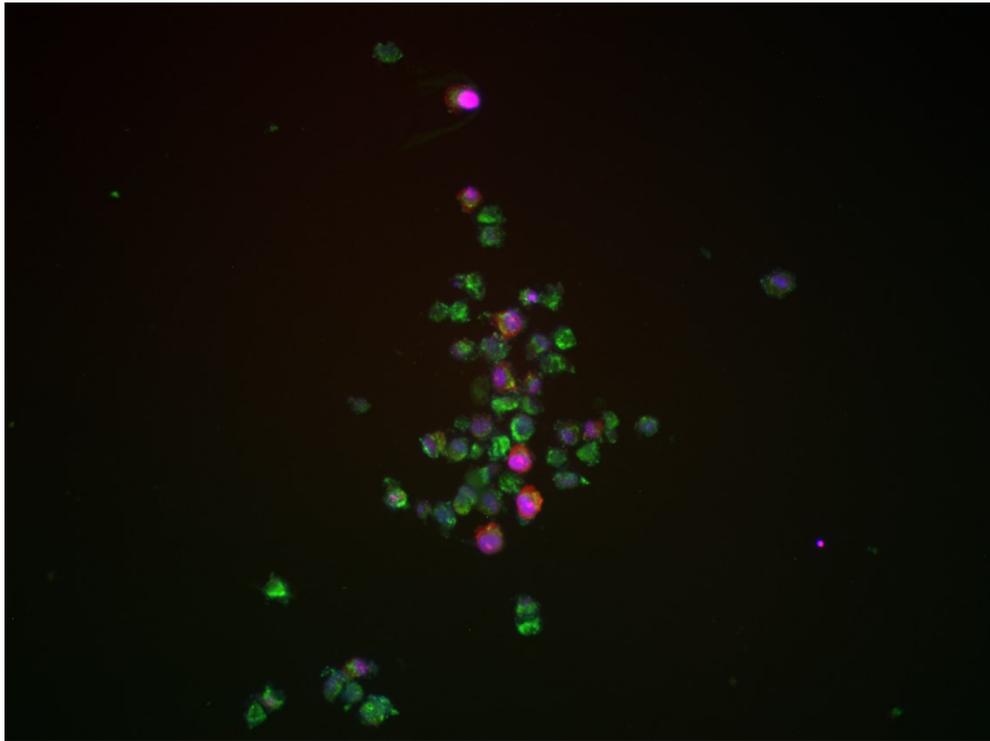


(B)



**Figure 3.31:** Fluorescent images showing apoptotic activity of Caki-2 cells using fluorescent microscopy assay following 24 hours treatment with compound-4 at a concentration of (A) 50µg/ml and (B) 200µg/ml. Then the Caki-2 cells were then fixed and treated with both DAPI (blue) and Anti-Fluorescein antibody (green).

(A)



**Figure 3.32:** Fluorescent images showing apoptotic activity of Caki-2 cells using fluorescent microscopy assay following 24 hours treatment with compound-5 at a concentration of (A) 50µg/ml and (B) 200µg/ml. Then the Caki-2 cells were then fixed and treated with both DAPI (blue) and Anti-Fluorescein antibody (green).

#### 4. Discussion

The novel compounds (compound-1 to 5) not previously tested for anti-cancerous properties were investigated for their potential as therapeutic agents for ccRCC by targeting LOX/LOXL2. ccRCC is the most frequent malignancy of the kidneys. Despite all the current therapies for ccRCC, the tumours eventually become resistant to them (Rizzo and Porta, 2017). Identifying new molecular targets and developing potentially novel drugs against them is essential for treatment of ccRCC patients.

Emerging evidence indicate that tumour microenvironment is pivotal in tumour initiation and progression. Increased ECM deposition and remodelling creates a microenvironment that promotes tumour progression by affecting cell-cell adhesion and cell migration. ECM remodelling also elevates growth factor signalling (Bissell and Hines, 2011; Gilkes *et al*, 2013; Sangaletti *et al*, 2017); Aberrant expression and activity of LOX and LOXL2 have been reported in many cancers, including in ccRCC. LOX and LOXL2 initiate the crosslinking of elastin and collagens, play important roles in remodelling the tumour microenvironment. Inhibitions of LOX and LOXL2 have shown considerable promise and have become exciting novel targets for the treatment and prevention of tumour metastasis (Schietke *et al*, 2012; Barker *et al*, 2012; Wang *et al*, 2016). In this study, three newly synthesised drugs (compound-1, 4 and 5) were evaluated on their inhibition of LOX and LOXL2 expression and on LOX activity.

It was found that compound-4 and 5 significantly inhibited LOX-activity in Caki-2 cells ( $P < 0.05$ ) following 24 hours' treatment. The inhibitory effect on LOX-activity by compound-1 was shown, but not significant (Figure 3.9 and 3.10). The significant downregulations of both LOX and LOXL2 mRNA expressions in Caki-2 cells following the treatment with compound-1, 4 and 5 were demonstrated by qRT-PCR (Figures 3.7 and 3.8). These results indicate three newly synthesised compound-1, 4 and 5 are potential inhibitors of LOX and LOXL2. They can be used to target overexpressed LOX/LOXL2 in cancer cells if they are able to interfere cancer cell properties. Therefore, the effects of the three compounds on cell proliferation, adhesion, migration, and survival of Caki-2 cells in vitro and changes in cell signalling gene expressions were evaluated in the present study.

The results show that compound-1, 4 and 5 decreased the viability of Caki-2 cells significantly following the treatment for 24 and 48 hours *in vitro* (Figures 3.5 and 3.6). The cell proliferation was also inhibited significantly following the 72 hours' treatment with compound-1, 4 and 5 respectively in comparison with negative control (Figure 3.6). The results indicate that compound-4 has greater impact on Caki-2 cell viability and proliferation than compound-1 and 5. Downregulation of LOX activity and LOX/LOXL2 expression may directly linked to the decreased proliferation and viability of Caki-2 cells.

These findings were supported by similar previous studies carried out to investigate the roles of LOX and LOXL enzymes in proliferation of prostate cancer (Nilsson *et al*, 2016) and colorectal cancer (Tang *et al*, 2017). Inhibition of LOX enzymes, using beta-aminopropionitrile (BAPN) reduced rat prostate tumour AT-1 cell growth *in vitro* (Nilsson *et al*, 2016). Tang *et al* (2017) demonstrated that knocked-down of LOX expression in colorectal cancer cell line (SW480) cells displayed a significant reduction in cell proliferation compared to the negative control cells. Their study also showed that the role of LOX in colorectal cancer cell proliferation was completely dependent on the activation of SRC. Interestingly, downregulation of ribonucleotide reductase subunit 2 (RRM2) gene expression was found in compound-1, 4 and 5 treated cells in this study (Figure 3.20). RRM2 expression is essential for the proliferation of cancer cell (Yoshida *et al*, 2016). The stimulation of RRM2 gene expression in proliferating cells involves SRC and ERK1/2 kinases (Fredriksson and Nedergaard, 2002). It suggests that the anti-proliferation effects of compound-1, 4 and 5 on Caki-2 cells may through inhibition of LOX/LOXL2 mediated via RRM2 and SRC involved cell signalling pathways. Further studies are required to confirm it. Moreover, the cell cycle analyses reveal that the treatment with three compounds caused increased percentage of Caki-2 cells in the G1 phase, while percentage of cells in the S phase and G2/M phase decreased compared to the negative control (Figures 3.12, 3.13, and 3.14). Cell apoptotic analyses show that treatment with compound-1, 4 and 5 increased apoptotic cell numbers in Caki-2 cells (Figure 3.28). The apoptotic data were consistent with the cell viability data; and both showed the inhibitory effects of compound-4 was greater than compound-1 and 5 on cell survival. These results indicate that compound-1, 4 and 5 inhibit the proliferation of Caki-2 cells might by arresting cells in the G1 phase and inducing cell apoptosis. Unexpectedly, gene expression analysis showed that CDKN1B was downregulated

in compound-1, 4 and 5 treated cells compared to negative control (Figure 3.21). CDKN1B encodes p27Kip1 protein, which belongs to the cyclin dependent kinase family. Increased p27Kip1 is usually associated with the cell cycle arrest in the G1 phase (Sharma and Pledger, 2016).

Recent studies have identified CDK-independent functions of p27Kip1. Depending on the cell context, the activities of CDKN1B encoded protein might turn pro-oncogenic and even stimulate cancer progression (Bencivenga *et al*, 2017). Thus, the roles of CDKN1B gene in the anti-cancer activities of compound-1, 4 and 5 need further investigation.

The mRNA expression of cell apoptosis-associated gene CASPA9 in Caki-2 cells was significantly upregulated following compound-1 treatment, however, its expression were downregulated in compound-4 and 5 treated cells when compared to negative control (Figure 3.27). CASPA9 gene encodes an initiator caspase protein, caspase-9. The process of apoptosis is mediated by the sequential activation of caspases. Two pathways lead to the activation of caspases: the intrinsic pathway (mitochondrial apoptosis) and the extrinsic pathway associated with membrane receptors and their ligands (Matsuura *et al*, 2016). This result indicates compound-1 may induce Caki-2 cell apoptosis by activating intrinsic apoptosis pathway, which includes the stimulation of caspase-9 expression. While whether compound-4 and 5 mediated apoptosis through extrinsic apoptotic pathway need further study.

Chang *et al*, (2017) demonstrated that LOXL2 inhibition did not directly induce apoptosis in the MDA-BM-321 breast cancer cells when PXS-S2A inhibitor was used, whereas, a dual inhibition of LOX and LOXL2 when PXS-S1A inhibitor was used, a significant increase in apoptosis was shown when compared to the negative control. Therefore, a combination of compound-4 and compound-1 or 5 can be used on Caki-2 cells in the future to see whether they can synergistically increase the anti-apoptotic effects.

Cancer cells exhibit higher glucose consumption and NAD(P)H production. The amount of intracellular NAD(P)H is higher in tumour cells than in normal cells (Matsuura *et al*, 2016). This study shows the reduction of NAD(P)H in compound-4 treated Caki-2 cells was greater than in compound-1 and 5 treated cells (Figure 3.11). Cancer-associated alterations in metabolism have been viewed as an indirect response to cell proliferation and survival

signals (Heiden 2011). The reduction of NAD(P)H generation caused by the compounds treatment further supports the proliferation and viability results obtained in this study.

Metastasis is one of the hallmarks of cancer cells. It is the major cause of cancer-associated death. The capability of cancer cell to invade and metastasize depends on the ability of cell migration, cell-cell and cell-ECM adhesion (Hanahan and Weinberg, 2011). LOX and LOXL2 promote tumour progression and metastasis (Wang *et al*, 2016). Overexpression of LOXL2 in N87 and MKN45 gastric cancer cell lines led to a significant enhancement of cell adhesion to fibronectin and collagen I matrix in vitro (Peng *et al*, 2009). In this study, compound-1 and 4 significantly reduced the adhesion of Caki-2 cells to ECM in vitro ( $p < 0.05$ ). The reduction was in a dose-dependent manner, higher dose caused a greater effect (Figures 3.15 and 3.16). While the decrease in cell adhesion to the ECM caused by compound-5 was not significant ( $p > 0.05$ ) (Figure 3.17). Wound-healing cell migration assays showed that compound-4 and compound-5 significantly inhibited Caki-2 cell migration ( $p < 0.05$ ) following 24 hours treatment compared to the negative control (Figures 3.18, 3.19). Compound-1 treatment also slowed down the cell migration, but was not significant compared to the negative control (Figure 3.19). These results are in agreement with previous finding by Hase *et al* (2014). They found that knockdown LOXL2 expression by siRNA significantly inhibited migration of human ccRCC cell line 786-O cells in vitro. Similar result was shown in colorectal cancer cells that LOXL2 knockdown led to a significant reduction in the migratory potential of SW480 cells (Park *et al*, 2017). Data from current study and previous studies suggest that compound-1, 4 and 5 have potential to control ccRCC metastasis by inhibiting LOX and LOXL2. Compound-4 may have a greater potential than compound-1 and 5. As the three compounds affected the properties of Caki-2 cell migration and adhesion differently, it is probable that different mechanisms might be involved in their regulations.

In this study, the changes in expression of cell migration and adhesion-related genes in Caki-2 cells were identified following the treatment with compounds. Compound-1, 4 and 5 treatments significantly downregulated CXCL16 and CD98HC mRNA expression in Caki-2 cells (Figures 3.25 and 3.26). CXCL16 is a transmembrane chemokine, not only found on immune cells, but also on cancer cells. High expression of CXCL16 promotes metastasis of lung cancer (Liang *et al*, 2018). CXCL16-CXCR6 interactions in prostate cancer cells promote  $\alpha\beta 3$  integrin clustering, modify cellular adhesion as well as enhance migration (Singh *et al*,

2016). Overexpression of CD98HC occurs in a variety of cancers. CD98HC regulates adhesion-induced intracellular signal transduction via integrin. A CD98hc/integrin interaction was required for ccRCC cell migration (Poettler *et al*, 2013). Hase *et al*, (2014) have shown that LOXL2 promotes tumour progression by regulating integrin  $\alpha 5$  levels in RCC. These data suggest that compound-1, 4 and 5 suppressed Caki-2 cell adhesion and migration by targeting LOX/LOXL2 might link to down-regulation of CXCL16 and CD98HC and their interaction with integrins.

MMP9 is involved in the breakdown of the extracellular matrix in a normal physiological process including embryonic development, reproduction and tissue remodelling (Human Genome Resources at NCBI - NCBI, 2018). Expressions of Snail, MMP9 and CXCL12 promote RCC metastasis. Snail stimulates MMP9 upregulation of in RCC (Mikami *et al*, 2011; Pan *et al*, 2006). Park *et al* (2017) found that LOXL2 participates in the regulation of the expression of Snail. In this study, the expressions of MMP9 and CXCL12 were downregulated in compound-4 and 5 treated cells (Figures 3.22 and 3.23). This explains the cell migration results that down-regulation of MMP9 and CXCL12 led to the suppressed cell migration. In compound-1 treated cells, both genes were upregulated, which may link to insignificant effects of compound-1 on Caki-2 cell migration.

VMP1 is a novel cell-cell adhesion protein. VMP1 mRNA level was significantly reduced in kidney cancer metastases as compared to primary tumours (Sauer mann *et al*, 2008). However, in this study, VMP1 expressions in the Caki-2 cells were decreased following compound-1, 4 and 5 treatment (Figure 3.24). Whether these compounds can efficiently suppress ccRCC cell metastasis *in vivo*, and the mechanisms underline their control of cell migration and adhesion need further studies.

## 5. Conclusion

In relation to this, there was significant difference in proliferation rates between the treated and untreated cells. Therefore, the proliferation and viability of the Caki-2 cells.

Additionally, a noticeable and significant decrease was observed in the lox-activity, cell cycle, migration, adhesion and gene expression after the three inhibitors were introduced. these findings highlight the potential of the three compounds in order for them to be used after further research as pro-apoptotic drugs and anti-ccRCC therapy.

In conclusion, this study found that three newly synthesized compound-1, 4 and 5 decreased the expressions of LOX and LOXL2 and reduced LOX activity of Caki-2 cells in vitro. They are potentially novel inhibitors for LOX and LOXL2. The three compounds showed anti-cancer properties by inhibiting proliferation, migration, cell-ECM adhesion, and stimulating apoptosis of Caki-2 cells. Compound-4 has greater effects than compound-1 and 5. Probably, different cellular signalling pathways and mechanisms are underline their effects. These findings highlight the potential of the three compounds in their usage in anti-ccRCC therapy. This project represents a preliminary investigation of a new potential therapy for ccRCC. The three compounds exhibited promising anti-ccRCC properties worth further studies in the further.

## 6. The recommended future work

This study enabled us to identify the three potential LOX inhibitors and their effects on the proliferation, migration, adhesion and survival of Caki-2 cells *in vitro*. Further studies related to the current project may be carried out in the following areas in the future:

1. To evaluate the inhibition of both extra- and intra-cellular LOX and LOXL2 protein expression by western blotting.
2. To understand the molecular mechanisms underlying the anti-ccRCC effects of compound-1, 4 and 5, protein microarray can be utilized to investigate the signalling pathways induced by the inhibition of LOX/LOXL2 in a broad spectrum.
3. To see whether there is synergistic increase on the anti-apoptotic effects, a combination of compound-4 and compound-1 or 5 can be used on Caki-2 cells.
4. To reveal whether compound-1, 4 and 5 can efficiently suppress ccRCC cell growth and metastasis *in vivo*, animal models with ccRCC tumour can be applied with the administration of these three compounds.

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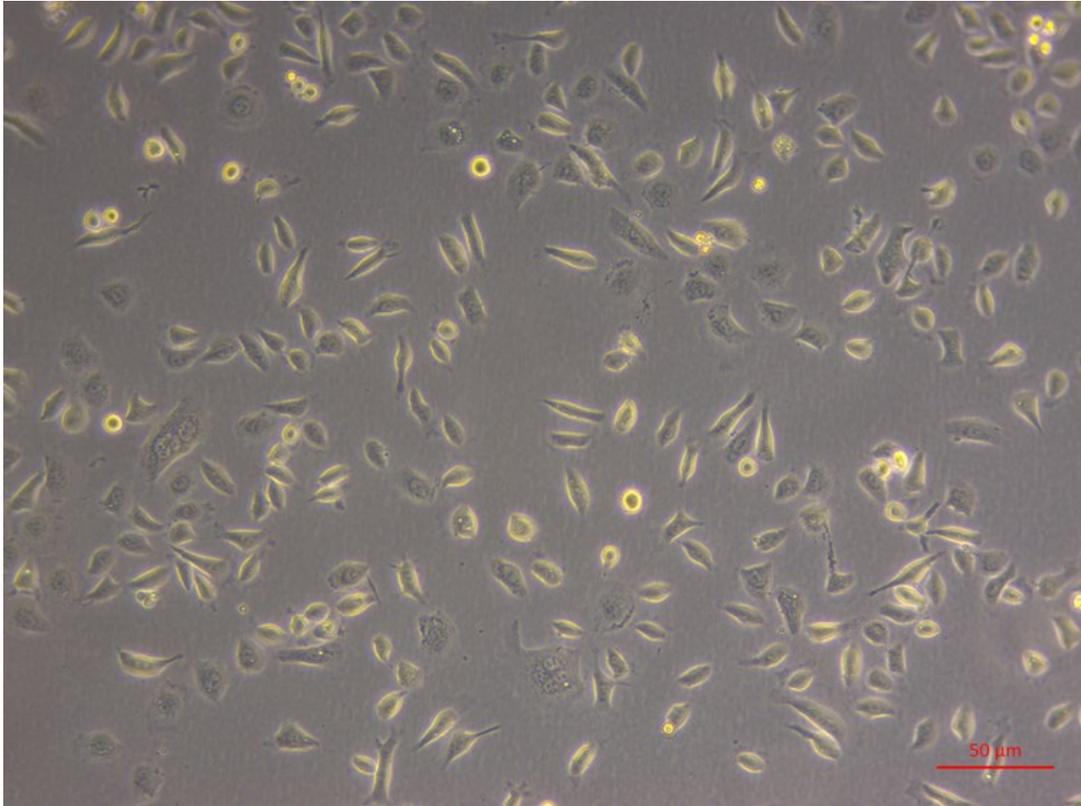
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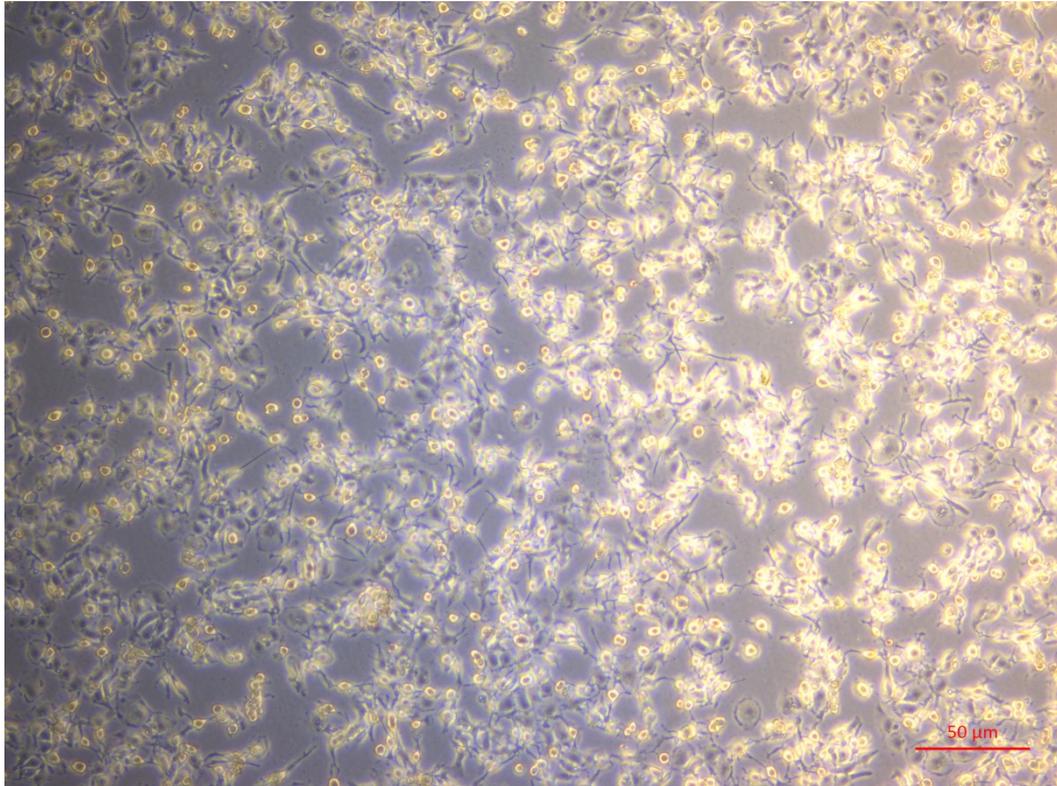
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## APPENDICES

**Appendix 1:** Microscopic images showing Caki-2 cell morphology at 20% confluence



**Figure A:** Microscopic image showing morphology of the Caki-2 cells at 20% confluence and x100 magnification.

**Appendix 2:** Microscopic images showing Caki-2 cell morphology at 90% confluence

**Figure B:** Microscopic image showing morphology of the Caki-2 cells at 90% confluence and x100 magnification.