QATAR UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

# INVESTIGATING THE MOLECULAR MECHANISMS UNDERLYING THE ANTI-CANCER ACTIVITIES OF DIOSMETIN IN COLORECTAL CANCER CELLS: AN IN VITRO STUDY

BY

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# ABSTRACT

AHMAD, SABAH, NISAR Masters: June: [2022], Environmental Sciences Title: Investigating the Molecular Mechanisms Underlying the Anti-Cancer Activities of Diosmetin in Colorectal Cancer Cells: An In Vitro Study

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Diosmetin, a bioflavonoid isolated from citrus fruits, has been reported to have cytotoxic effects against various types of cancer. Although Diosmetin is found to have cytotoxic effects in Colorectal Cancer (CRC) cells, the molecular mechanism remained poorly understood. In this study, we aimed to investigate the anti-cancer activity of Diosmetin in the human CRC HCT116 cell line. The results revealed that Diosmetin exerts significant cytotoxic effects on HCT116 cells as indicated by decreased cell viability and proliferation, increased DNA damage, loss of mitochondrial membrane potential (MMP), cell cycle arrest, and induction of apoptosis. Moreover, Diosmetin treatment downregulated the expression of mesenchymal markers vimentin, snail, and slug and upregulated the expression of the epithelial marker, E-cadherin. These findings demonstrate that Diosmetin inhibits proliferation, induces apoptosis, and suppress Endothelial Mesenchymal Transition (EMT) in HCT116 cells. Thus, Diosmetin can be used as a complementary therapeutic agent for CRC treatment and prevention.

# DEDICATION

*First praise is to Allah, the Almighty, on whom ultimately we depend for sustenance and guidance. Secondly, this thesis is dedicated to my parents, my mentors, and the people who have not only supported me throughout my career but also throughout my life to keep moving forward despite the odds*

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1. Adenomatous polyposis coli (APC)
2. Complementary and alternative medicine (CAM)
3. Chromosomal instability (CIN)
4. CPG island methylator phenotype (CIMP)
5. Colorectal cancer (CRC)
6. Circulating tumor cells (CTCs)
7. Extracellular matrix (ECM)
8. Epithelial to mesenchymal transition (EMT)
9. Fas ligand (Fas-L)
10. Gastrointestinal (GI)
11. Human development index (HDI)
12. Ionizing radiation (IR)
13. Microsatellite instability (MSI)
14. Natural products (NPs)
15. Polycystic ovary syndrome (PCOS)
16. Reactive oxygen species (ROS)
17. Sonic hedgehog (SHH)
18. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCCD)
19. Transforming growth factor-β (TGF-β)
20. Tumor microenvironment (TME)
21. Tumor necrosis factor (TNF)
22. TNF-related apoptosis-inducing ligand (TRAIL)
23. 5-fluorouracil (5-FU)

# 1. INTRODUCTION

Cancer is one of the major causes of death worldwide, accounting for approximately 10 million deaths in 2020 (WHO, 2022). Cancer is a multistage process that arises from the uncontrolled and abnormal transformation of normal cells to tumor cells. These tumor cells can migrate and invade other organs and tissues, a process known as metastasis. The exact cause of cancer is still known, but several external factors can contribute to cancer development. These external agents include physical, chemical, and biological agents, many of which are classified as carcinogens by the International Agency for Research on Cancer (IARC). The interplay between genetics, epigenetics, and metabolism plays a crucial role in cancer pathophysiology (Thakur & Chen, 2019). Many studies have shown that tumor initiation, growth, and metastasis are caused by genetic mutations (Patel et al., 2021). Cancer that occurs due to acquired mutations are known as sporadic cancers, while cancer that occurs due to germline mutations are known as inherited cancers. Sporadic cancers account for 90-95% of all the cancers, while inherited cancers account for 5-10% of all the cancers (Anand et al., 2008).

Carcinogenesis is a multi-step process that involves the accumulation of genetic mutations, epigenetic modifications, disruption of apoptotic and DNA repair mechanisms, the activation of oncogenes and the inactivation of tumor suppressor genes (de Melo et al., 2018). A single cellular alteration is not enough to promote carcinogenesis, but a multitude of different alterations or modifications that disrupt cellular homeostasis and lead to the development of cancer (de Melo et al., 2018).

Carcinomas are the most common type of cancer. They start in epithelial cells or the tissue lining of different organs such as the liver, kidneys, and gastrointestinal (GI) tract. GI cancers account for 35% of cancer-related deaths worldwide and the most

diagnosed malignancies globally (Arnold et al., 2020; Shao et al., 2018). The most common types of GI cancers are esophageal cancer, stomach cancer, liver cancer, colorectal cancer (CRC), and pancreatic cancer, which account for 26% of the global cancer incidence (Arnold et al., 2020). The incidence rates of CRC are approximately 3-fold higher in high human development index (HDI) countries than medium and low HDI countries. The highest incidence of CRC is reported in developed regions such as Northern and Southern Europe, Australia, and New Zealand (Rawla et al., 2019). In addition, CRC is more prevalent among men in middle eastern countries such as Saudi Arabia, Qatar, Kuwait, Yemen, Oman, and UAE (Rawla et al., 2019).

The current treatment options for cancer are surgery, radiotherapy, chemotherapy and immunotherapy. However, side effects are the major limitations of the short- and long-term use of these therapies. On the other hand, natural compounds have long served as a vital source for cancer chemotherapeutic and chemopreventive agents (Cragg & Pezzuto, 2016). Apart from the conventional chemotherapeutic treatments, the use of natural compounds has been embraced mainly as a complementary treatment option in cancer therapeutics. Natural compounds are known for their minimal cytotoxic and side effects. In addition, some of the natural compounds can modulate different cellular signaling pathways and are involved in the regulation of genes that control cell proliferation, cell cycle, cell differentiation, and apoptosis (Pan et al., 2011). Due to the immense anti-cancer effects possessed by the natural compounds, they have been widely employed as therapeutic agents for treating CRC. Besides, their use as potent anti-cancer agents, natural compounds have also been combined with other drugs to enhance the treatment efficacy in CRC and other cancers.

Combination treatment can target multiple signaling pathways and reduce tumor drug resistance, the main challenges in the treatment of CRC. In addition, combination treatment may produce synergistic, additive or antagonistic effects and is a promising

way to overcome chemoresistance in CRC (Redondo-Blanco et al., 2017).

According to the 2015 Qatar National Cancer Registry (QNCR) data, CRC is the second most common cancer (11.87%) amongst males and the third most common cancer (8.31%) amongst females (QNCR, 2015). Increasing global cancer burden and rise in cancer mortality rates have compelled scientists to explore novel therapeutic strategies and discover therapeutic agents that exhibit minimal toxicity and side effects compared to conventional cancer treatment options. Diosmetin, a methoxyflavonoid, is isolated from citrus fruits and is also found to be an active component of traditional Chinese herb, *Galium verum* that has been found to display a wide range of therapeutic properties (Lakić et al., 2010). Pharmacologically, Diosmetin has been found to exhibit anti-microbial, anti-inflammatory, anti-oxidant, and anti-cancer activities (Patel et al., 2013). Diosmetin has been found to play an important role in the management of different metabolic disorders such as cardiovascular diseases, obesity, diabetes and other disorders such as cognitive impairment, polycystic ovary syndrome (PCOS) and hemorrhoids [check review (Garg et al., 2022)]. More recently, Diosmetin has gained widespread attention in cancer therapeutics and has been found to exert growth inhibitory effects through various signaling pathways that are implicated in cancer. This study aims to describe the anti-cancer activities of Diosmetin and delineate the underlying molecular mechanisms mediating its effects in CRC cells.

# 2. LITERATURE REVIEW

## *2.1. Colorectal Cancer*

According to the latest statistics, CRC is the third leading cause of cancer-related mortality worldwide (Rawla et al., 2019). Conventional treatments for CRC include surgery, radiotherapy, and chemotherapy. Despite the many advances in cancer

treatment, CRC remains a leading cause of cancer-related deaths due to chemoresistance, organs toxicity, and undesirable side effects exhibited by chemotherapeutic drugs (Florescu-Ţenea et al., 2019). Therefore, there is a need for developing novel cancer therapeutics with tumor-targeting approaches and minimal adverse effects. CRC development is a sequential process in which genetic mutations accumulate over time and determine phenotypic tumor progression. In contrast, recent evidence has reported that genetic changes during neoplastic progression are uncoupled from histological progression in CRC (Mamlouk et al., 2020). The molecular pathogenesis of CRC starts with the adenoma-carcinoma sequence in which the tubular adenomas that arise within the colon accumulate genetic mutations or inflammation-induced epigenetic changes over time with the activation of oncogenes and inactivation of tumor suppressor genes leading to the transformation of adenoma to adenocarcinoma (Gonzalez et al., 2017; Schmitt & Greten, 2021).

Epidemiological data suggest that in addition to genetics, environmental exposures also predominate the etiology of CRC (Rattray et al., 2017). These environmental elements include an unhealthy lifestyle and diet, exposure to environmental pollutants, infections, and radiation (Baena Ruiz & Salinas Hernández, 2014). Furthermore, multiple factors determine the prognosis of CRC, including tumor location, tumor, node, metastasis (TNM) stage, and histologic subtypes. Additionally, other molecular biomarkers can be used to predict prognosis and therapy response in CRC (Sepulveda et al., 2017).

Three oncogenic molecular pathways include microsatellite instability (MSI), chromosomal instability (CIN), and a CPG island methylator phenotype (CIMP) that is characterized by hypermethylation of promoter CPG island sites (Harada & Morlote, 2020), have been identified for CRC. In addition, genetic and epigenetic changes such as mutations in the *BRAF*, *PIK3CA*, and *KRAS* genes, loss of SMAD4, a protein that

acts as a tumor suppressor, a CIMP, and global DNA hypomethylation are associated with the development of CRC (Huang et al., 2019; Nazemalhosseini Mojarad et al., 2013; Ogino et al., 2011; Wasserman et al., 2019) (Figure 1).

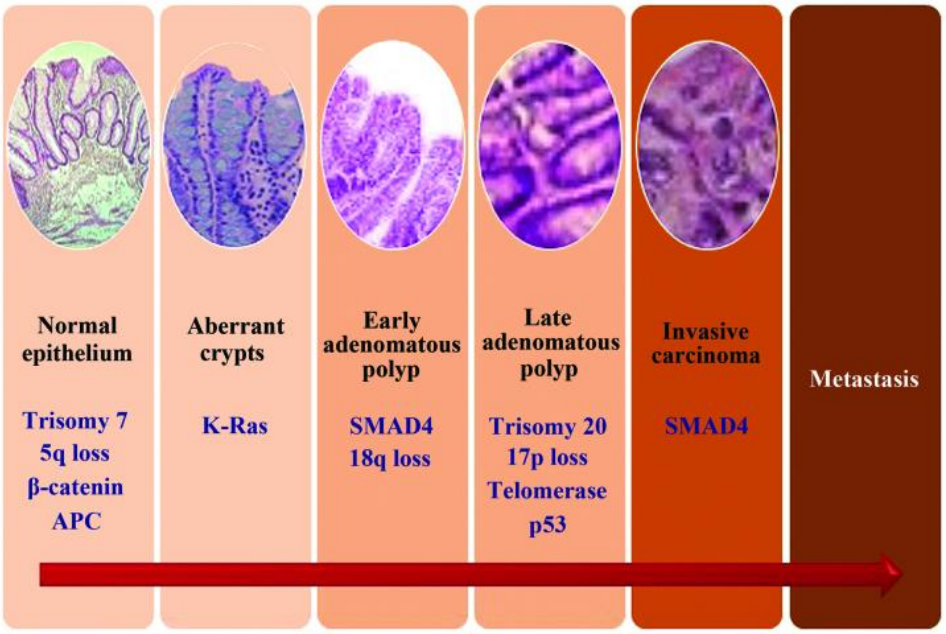


*Figure 1: Steps of CRC progression. Source:(Nikolouzakis et al., 2018)*

Moreover, mutations in the components of signaling pathways such as epidermal growth factor receptor/mitogen-activated protein kinase (EGFR/MAPK), Notch, Sonic hedgehog (SHH), Wnt, PI3K/Akt, and transforming growth factor-β (TGF-β) have been associated with the development and progression of CRC (Koveitypour et al., 2019) (Figure 2).

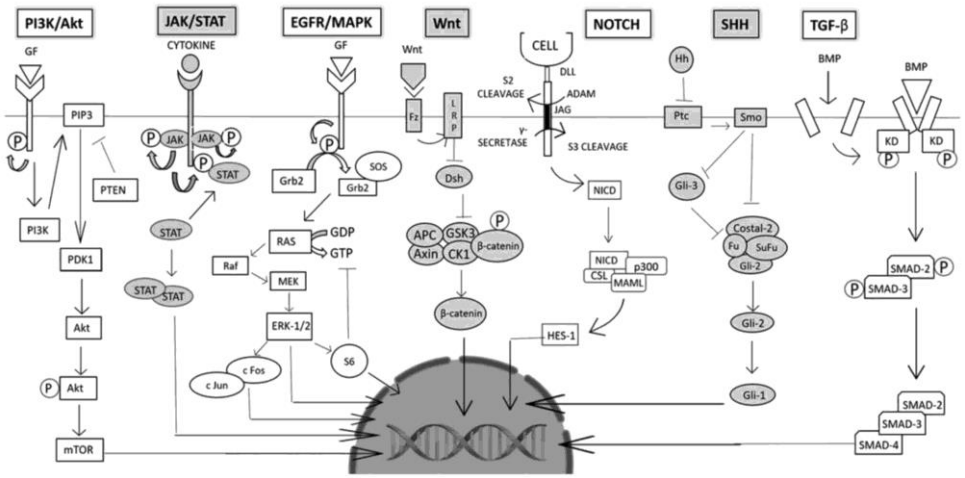


*Figure 2: Signaling pathways involved in CRC. Source: (Ahmad et al., 2021)*

Apart from the genetic instability, CRC is also characterized by high inter-patient and intra-tumor heterogeneity. Intra-tumor heterogeneity refers to the heterogeneity between different parts of the tumor and can be subdivided into spatial and temporal heterogeneity. Spatial heterogeneity is an important feature of the tumor microenvironment (TME) and refers to the differences observed within a single tumor. While temporal heterogeneity results due to the accumulation of genetic alterations within single tumors over time, contributing to the dynamic nature of CRC (Sagaert et al., 2018).

Thus, due to the complex and highly heterogeneous nature of CRC, it is important to identify natural drugs or agents that have high anti-cancer efficacy and minimal cellular toxicity.

### *2.2. Conventional Medicine*

The conventional treatments for CRC are surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy. Radiotherapy uses ionizing radiation beams aimed at the tumor site to control proliferation of cancer cells. Chemotherapy, which includes antimetabolites, plant alkaloids, antibiotics, alkylating agents, hormones, and biological response modifiers, is the most common anti-cancer treatment used for CRC (Mishra et al., 2013). The chemotherapeutic drugs used to treat CRC are oxaliplatin, 5-FU, irinotecan, and capecitabine. These chemotherapeutic drugs lead to cancer cell death by either inducing DNA damage, promoting different cell signaling pathways such as cell cycle arrest, or inhibiting global translation and DNA repair mechanisms (Huang et al., 2019). The use of natural products in combination with conventional drugs is also an effective strategy for improving treatment response in cancer patients. Another type of conventional treatment, targeted therapy, mainly involves incorporating anti-cancer agents/drugs to increase the drug accumulation in cancer tissues. Nanoparticles such as polymeric micelles are ideal delivering agents used for targeted therapy in CRC (Mishra

et al., 2013). These polymeric micelles have a hydrophobic core and a hydrophilic shell that facilitates the delivery of poorly water-soluble anti-cancer drugs. Despite these advances, CRC exerts a high burden on society due to the heterogenic nature of the disease and failure to identify appropriate patient subpopulations for targeted therapies. Moreover, CRC tumors evolve and require long-term treatments (Van Cutsem et al., 2013). Based on the heterogeneous nature of CRC, it is important to identify more natural products or plant-derived anti-cancer agents that are more efficient and less cytotoxic to non-cancerous cells.

### *2.3. Complementary and Alternative Medicine*

In the past few decades, the use of complementary and alternative medicine (CAM) in oncology has significantly increased. CAM encompasses different therapeutic approach: biologically based therapy that involves herbal medicines, acupuncture, dietary supplements, and homeopathy. One of the major areas of research interest in biologically-based therapy is identifying natural products that may reduce disease risk and enhance a patient's quality of life and the chance for long-term survival. One such group of natural compounds is dietary polyphenols prevalent in fruits, grains, and vegetables and have long been used as nutraceuticals due to their various health benefits. Polyphenols are classified into flavonoids and non-flavonoids. Flavonoids are polyphenolic compounds found in plants with potent anti-oxidants and are known to possess anti-cancer properties. In addition, flavonoids are known to modulate reactive oxygen species (ROS)-scavenging activities and are involved in cell cycle arrest, apoptosis and autophagy, and other immunomodulatory and inflammatory processes (Kopustinskiene et al., 2020). As the presence of different environmental pollutants can affect the immune system, certain flavonoids are found to preserve the function of immune and inflammatory cells. A study showed that combination treatment with

flavonoids such as chrysin and quercetin prevented hepatotoxicity induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCCD) in rat's liver (Ciftci et al., 2011). Moreover, another study showed the chemopreventive efficacy of hesperidin, a citrus flavonoid, in benzo(a)pyrene-induced lung cancer in mice (Kamaraj et al., 2009). These studies suggest that apart from the anti-inflammatory and antioxidative properties exhibited by flavonoids, they are also important deterrents of environmental pollutants.

### *2.4. Natural Products*

Nature has long been a source of medicinal products, particularly plants, and will continue to be a major source for identifying novel drugs. Traditional therapies or remedies with products derived from terrestrial plants still dominate throughout many parts of the world, most notably in cancer therapeutics.

Natural products (NPs) contain a rich reservoir of bioactive compounds such as alkaloids, flavonoids, flavones, isoflavones, anthocyanins, lignans, saponins, catechins, and isocatechins, and their derivatives have long been used in traditional therapy for various diseases. More than 60% of anti-cancer drugs in clinical trials are derived from natural sources such as plants, marine organisms, and microorganisms (Seelinger et al., 2012). NPs have been identified as potential antineoplastic agents due to their versatility, structural diversity, molecular characteristics, and the presence of natural anti-oxidants. The anti-oxidants present in the NPs possess reducing, free radical scavenging and quenching properties (Avato et al., 2017; Saidurrahman et al., 2017). Plant-derived and nutraceutical natural compounds as anti-cancer agents have tremendously increased in the last few decades.

Radiotherapy and chemotherapy are the major treatments for solid tumors in the current era. However, they can cause side effects due to toxicity and reduce the quality of life. While in some cases, the effectiveness of biological or natural drugs such as herceptin are limited to specific types of cancer and cannot be used to treat other types

of cancers (Fridlender et al., 2015). Moreover, in many cases, the cancer patients develop drug resistance and are switched to combination treatments that involve the combination of two or more chemotherapeutic drugs. Thus, there is a real need for novel anti-cancer drugs that have reduced side effects and plants serve this purpose and are a promising source for identifying such novel drugs.

The chemopreventive efficacy of natural compounds and their constituents such as flavonoids, anthocyanins, terpenoids, and carotenoids has been reported in CRC [reviewed in (Rejhová et al., 2018)]. Some of the most studied natural compounds in CRC are curcumin, resveratrol, apigenin, quercetin and genistein (Rejhová et al., 2018). Moreover, combination therapy by combining conventional drugs used for CRC treatment such as oxaliplatin, 5-fluorouracil (5-FU), irinotecan, and capecitabine with a natural or bioactive compound has also been shown to be effective against CRC (Kunnumakkara et al., 2009; Riahi-Chebbi et al., 2019; Ruiz de Porras et al., 2016; Su et al., 2018).

### *2.5. Diosmetin*

Diosmetin is a methoxyflavonoid naturally found in citrus fruits and is found in the legume of *Acacia farnesiana* and in the leaves of *Olea europaea* (Patel et al., 2013) plants. It exhibits anti-cancer, anti-inflammatory, anti-oxidant, and antimicrobial activities (Patel et al., 2013) (Figure 3). The anti-cancer effect of Diosmetin has been reported in several cancers such as CRC (S. Koosha et al., 2019; Sanaz Koosha et al., 2019), breast cancer (Androutsopoulos et al., 2009), skin cancer (Choi et al., 2019), prostate cancer (Oak et al., 2018b), non-small cell lung cancer (Chen et al., 2019) and hepatocellular carcinoma (Liu et al., 2016).

Several previous studies have reported the anti-cancer activity of Diosmetin in various cancers. For example, a previous study has shown that Diosmetin inhibits tumor growth, increases the thymus weight, and reduces the levels of TNF-α, TGF-β and IL-

10 in U14-tumor-bearing mice (Zhao et al., 2011). Another study showed that Diosmetin inhibited cell proliferation and migration and induced apoptosis via the caspase pathway in B16F10 melanoma cells (Choi et al., 2019). In another study, Diosmetin inhibited cell proliferation, cell cycle progression and induced apoptosis through p53 activation in HepG2 hepatocellular carcinoma cells (B. Liu et al., 2017). Diosmetin has also been found to suppress proliferation and induce cell cycle arrest and apoptosis via inhibition of the STAT3/c-Myc signaling pathway in Saos-2 and U2OS osteosarcoma cells (Ning et al., 2021). In addition, Diosmetin has been shown to enhance the chemotherapeutic efficacy of paclitaxel by increasing ROS accumulation through the disruption of the PI3k/Akt/GSK3-β pathway (Chen et al., 2019). Another study found Diosmetin to induce apoptosis and caused cell cycle arrest at the G1 and S phase in LNCaP and PC-3 prostate cancer cells. Moreover, Diosmetin was also found to downregulate cyclin D1, Cdk2, Cdk4, c-Myc, and Bcl-2 and upregulate cleaved PARP, caspase-3, Bax, p27 and FOXO3a (Oak et al., 2018a).

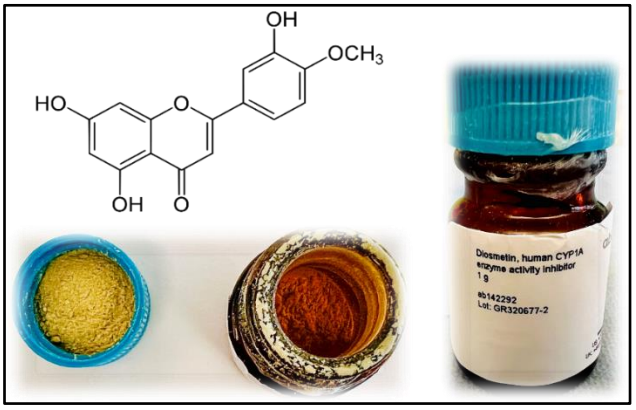


*Figure 3: Chemical structure and physical appearance of citrus flavonoid, Diosmetin.*

### *2.6. Epithelial to Mesenchymal Transition*

Natural compounds are also modulators of epithelial to mesenchymal transition (EMT). There are two types of EMT mechanisms: physiological or developmental EMT that takes place during embryonic development and pathological EMT implicated in

many inflammatory and immunological diseases, most importantly in tumor progression. Physiological EMT is self-regulated and occurs in distinct biological settings such as during embryogenesis, tissue regeneration, wound healing, organ development, and organ fibrosis (Kalluri & Weinberg, 2009). In contrast, pathological EMT is a major hallmark of cancer associated with increased cell proliferation, angiogenesis, invasiveness, and metastasis (Kalluri & Weinberg, 2009). In normal tissues, EMT is a process in which epithelial cells gain a mesenchymal phenotype that enables cell differentiation and increases cellular motility. While abnormal cells are employed during pathological EMT, these cells lose their sensitivity to normal growth regulatory signals and gain a mesenchymal phenotype that enables them to become more motile, invasive, and resistant to apoptosis (Kim et al., 2014) (Figure 4). During EMT, tight junctions, apical-basal polarity and reorganization of the cytoskeletal structure are disrupted, which enables, and reorganization of the cytoskeletal structure, enabling tumor cells to gain more motility to invade other organs or invade other organs tissues.

EMT signatures are associated with poor prognosis in various types of cancers such as breast cancer (Imani et al., 2016), lung cancer (Aruga et al., 2018), esophageal cancer (Liu et al., 2014), and colon cancer (Zhang et al., 2020). Furthermore, the EMT mechanism is also associated with drug resistance and fuels radio/chemoresistance, thus contributing to tumor progression (Dudas et al., 2020).

The tumor microenvironment (TME) plays a critical role in tumor progression and development. The progression of CRC is associated with an inflammatory TME that consists of immune cells, cytokines, stromal cells, growth factors, and extracellular matrix (ECM) (Jang et al., 2021). However, it remains unclear how inflammatory TME induces EMT during CRC progression. EMT regulation in CRC is orchestrated by a multifactorial network that requires the coordination of different factors such as EMT

effectors, EMT core regulators and EMT inducers (Vu & Datta, 2017). Accumulating evidence shows the involvement of signaling pathways such as SMAD/STAT3, NF-kB, Ras-MAPK/Snail/Slug and microRNAs in the development of CRCs via EMT (Zhu et al., 2013). Many recent studies have suggested the importance of circulating tumor cells (CTCs) during metastasis. The CTCs of CRC can serve as early detection biomarkers as they display a mesenchymal phenotype having an increased expression of vimentin and are found to have mutations in *KRAS* and *TP53* key genes that are different from the corresponding tumor tissue (Vu & Datta, 2017). Moreover, the vimentin-positive CTCs are found to display an increased expression of EMT regulators such as ZEB, Snail and Slug (Lim et al., 2014).

Many natural compounds such as resveratrol (Ji et al., 2015), curcumin (Wang et al., 2020), celastrol (Lin et al., 2016), paeonol (Lin et al., 2014), withaferin A (Suman et al., 2016), luteolin (Y. Liu et al., 2017) and magnolol (Chei et al., 2019) have been reported to be effective in inhibiting or reversing EMT in CRC. ,However, no study has explored the EMT mechanism in Diosmetin-treated CRC cells. Thus, the current study results will be a beneficial addition to the current scientific literature.

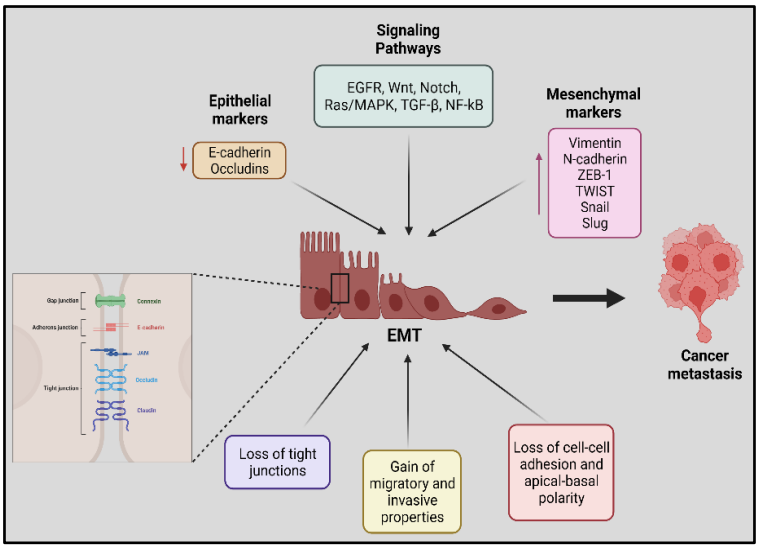


*Figure 4: The process of EMT in cancer. During EMT, the cancer cells lose epithelial markers such as E-cadherin and occludins and gain mesenchymal markers such as*

*vimentin, Snail, Slug, TWIST, ZEB-1, and N-cadherin, resulting in loss of cell identity, which leads to cancer metastasis. (Figure was created using the BioRender Software).*

### *2.7. Apoptosis and Autophagy*

Apoptosis is an orderly orchestrated programmed cell death mechanism in various physiological and pathological conditions. The major hallmarks of apoptosis are chromatin condensation, nuclear fragmentation, cell shrinkage and reduction in cellular volume (Wong, 2011). There are three fundamental types of biochemical changes observed in apoptosis, including activation of caspases, membrane alterations, and degradation of DNA and protein (Abraha & Ketema, 2016). Apoptosis can be triggered by diverse intracellular signals such as increased $Ca^{2+}$ concentration, oxidative damage, hypoxia, and extracellular signals such as pathogens, toxins, growth factors, and hormones (Su et al., 2013). The two core apoptotic pathways involved in inducing apoptosis are the extrinsic and intrinsic pathways. The extrinsic pathway is activated when the extracellular ligands such as tumor necrosis factor (TNF), Fas ligand (Fas-L), and TNF-related apoptosis-inducing ligand (TRAIL) attach to the extracellular domain of the death receptors (Jan & Chaudhry, 2019). While, the intrinsic pathway is activated in response to lethal stimuli such as oxidative stress, hypoxia, genetic impairment, and increased calcium concentrations or the loss of growth factor signals and is controlled by interactions between the proapoptotic (Bax, Bak) and antiapoptotic (Bcl-2, Bcl-xL) Bcl-2 protein family members (Burz et al., 2009) (Figure 5).

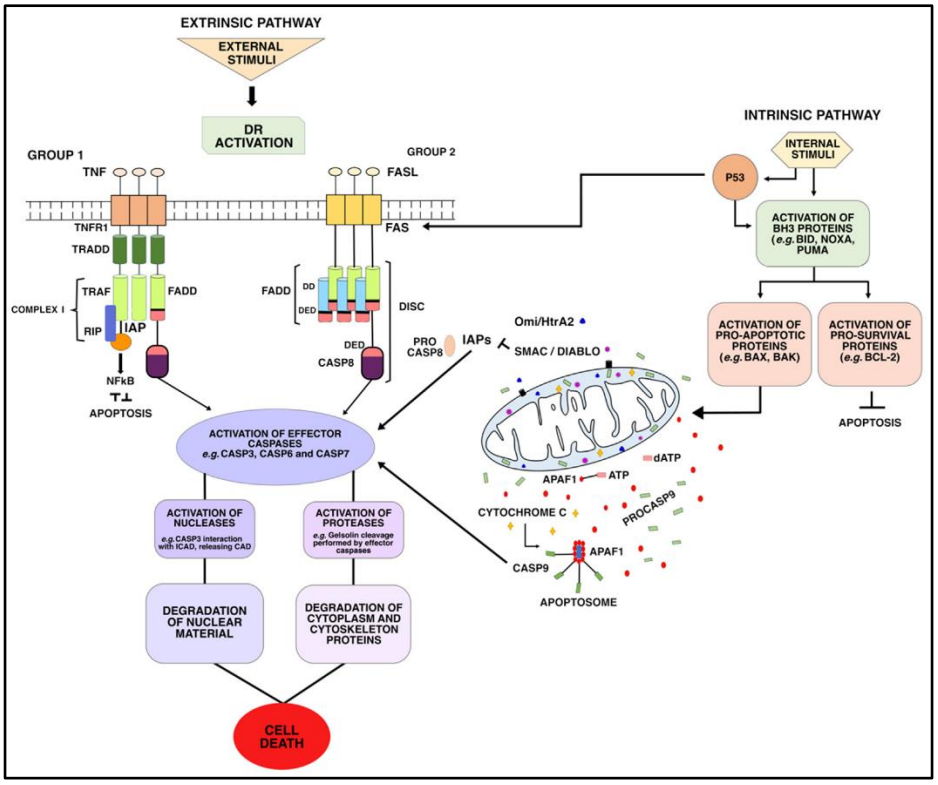


*Figure 5: Extrinsic and intrinsic pathways of apoptosis. Source: (Cavalcante et al., 2019)*

Additionally, the activation of the intrinsic apoptotic pathway is also associated with increased mitochondrial porousness and the release of cytochrome-c in the cytosol (Abraha & Ketema, 2016). In addition, loss of plasma membrane asymmetry is one of the key mechanisms of apoptosis. In apoptotic cells, the phospholipid phosphatidylserine (PS) translocates from the inner surface to the outer surface of the plasma membrane, thereby exposing it to the external cellular environment. Annexin V is a $Ca^{2+}$ dependent cellular protein with a high affinity for PS, binds to the PS exposed cells, and is used to detect apoptosis (Elmore, 2007). Annexin V is sometimes conjugated with fluorochromes such as BV421, which serves as a sensitive probe for the detection of apoptotic cells. Another indicator of apoptosis is the loss of mitochondrial membrane potential (MMP). MMP is a key indicator of mitochondrial health as it reflects the energy storage process of electron transport, and oxidative phosphorylation (OXPHOS), which are required for adenosine triphosphate (ATP)

production (Lee et al., 2019). Cationic fluorescent dyes such as JC-1 and TMRE are commonly used for the detection of MMP.

Many natural compounds such as curcumin, resveratrol, apigenin, quercetin, piperine and many more have been found to induce apoptosis by promoting ROS generation in CRC cells [check review (Ahmed et al., 2019)]. The loss of genomic stability can enable CRC cells to acquire different oncogenic mutations that contribute to CRC's evolution. Tumor suppressor genes such as adenomatous polyposis coli (*APC*) and p53 and proto-oncogene such as *Bcl-2* are associated with apoptosis and CRC development (Güllülü et al., 2021; Ramesh & Medema, 2020; Zhang & Shay, 2017). The accumulation of genetic mutations in CRC can affect apoptotic cell death as many of the genes commonly mutated in CRC are found to control apoptosis (Abraha & Ketema, 2016). In addition to apoptosis, autophagy is also an important catabolic pathway essential for maintaining cellular homeostasis. Several studies have reported autophagy as one of the major factors of drug resistance in CRC.

Autophagy is a naturally conserved lysosome-dependent degradation mechanism that expels unwanted or dysfunctional cytoplasmic proteins or organelles and allows orderly recycling of the cellular components (Xie et al., 2020). In comparison, apoptosis is an autonomic regulated or programmed cell death characterized by blebbing, DNA fragmentation, cell shrinkage, and apoptotic bodies (Xie et al., 2020). Moreover, autophagy is also associated with pyroptosis, a programmed cell death mechanism that is triggered by inflammation, a process that is crucial in the pathogenesis of cancer (Chung et al., 2020). Furthermore, autophagy is found to act as a double-edged sword in CRC as it can either induce cancer cell death or promote tumor survival (Zhang & Liu, 2021). LC3 is an important marker to detect autophagy. The LC3 conjugation system is required for the elongation and maturation of autophagosomes and is widely used to monitor the formation of autophagosomes during the autophagic process (Lee

& Lee, 2016). There are three LC3 isoforms (LC3A, LC3B, LC3C) that undergo post-translational modifications during autophagy (He et al., 2003). The cytosolic LC3-I form converts to LC3-II, which reflects autophagic activity (Tanida et al., 2008). Thus, any dysfunction in the autophagic and apoptotic processes can lead to the development of tumors. The relationship between autophagy and apoptosis is complex as they can have a synergistic, promoting, or antagonistic effect in CRC. Several natural compounds have been reported as autophagy inducers in CRC (Chatterjee et al., 2011; Kim et al., 2016; Ko et al., 2011; Kumar et al., 2016). Qian et al. proposed that an interplay between apoptosis and autophagy plays an important role in regulating CRC cell death. The author proposed that autophagy and apoptosis can be induced concomitantly and can independently regulate cell death. Moreover, autophagy can antagonize the apoptotic process by inhibiting DNA damage and preventing the accumulation of endoplasmic reticulum (ER) stress products (Qian et al., 2017). Several studies have reported the concurrent induction of apoptosis and autophagy in CRC cells in response to chemotherapy or gene interference, and autophagy may act parallel to apoptosis in contributing to CRC cell death [check review (Qian et al., 2017)].

Therefore, exploring the autophagy and apoptotic mechanisms in Diosmetin-treated CRC cells might improve the efficacy of anti-cancer drugs in the clinical treatment of CRC.

### *2.8. Combination therapy*

Current conventional surgical and chemotherapy practices for CRC treatment impair the patient’s quality of life as chemotherapeutic medicines interfere with the cell cycle. So, there is a pressing need for less cytotoxic or non-toxic natural compounds combined with chemotherapeutic drugs that can better respond to therapy and improve patients' quality of life (Rejhová et al., 2018). Combination therapy can prove beneficial for decreasing the concentration of the conventional drug being used, improving the

bioavailability of the drug, reducing the chances of relapse, and most importantly, sensitizing chemotherapy/radiotherapy resistant cancers (Abotaleb et al., 2018). The radiosensitizing effect of Diosmetin has been explored in lung and endometrial cancer (Hu et al., 2020; Xu et al., 2017). The study showed that Diosmetin restrained the ionizing radiation (IR)-induced DNA damage repair in lung cancer cells by inhibiting the Akt signaling pathway (Xu et al., 2017). At the same time, another study showed that Diosmetin enhanced the sensitivity of endometrial cancer cells to radiotherapy by suppressing the homologous repair pathways (Hu et al., 2020). Although the chemosensitizing potential of Diosmetin in CRC is poorly investigated, a recent study showed that the combination of Diosmetin and 5-FU synergistically induced more apoptotic effect and disrupted mitosis in CRC cells compared to 5-FU alone, suggesting that this synergistic combination can be used as a potential treatment for CRC (Kamran et al., 2022). So far, no study has explored the effect of combining Diosmetin with a conventional chemotherapy drug oxaliplatin in CRC.

Thus, this study aims to **assess the effects of Diosmetin on CRC cells, investigate the molecular mechanism by which Diosmetin induces CRC cell death, and evaluate the chemosensitizing potential of Diosmetin combined with conventional chemotherapeutic drug oxaliplatin.**

# 3. OBJECTIVES

The specific aims of this study are to:

1. Assess the effect of Diosmetin on the proliferation, viability, apoptosis, and autophagy of human CRC cells (HCT116)
2. Investigate the role of EMT in Diosmetin-mediated CRC cell death
3. Evaluate the chemosensitizing potential of Diosmetin when combined with conventional chemotherapeutic drug oxaliplatin

# 4. MATERIALS AND METHODS

## *4.1. Reagents and antibodies*

Diosmetin was purchased from Abcam (Cambridge, UK) and was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. Oxaliplatin was purchased from Thermo Scientific (Carlsbad, CA, USA). ATP-Glo Cell Titer for cell viability and DeadEnd™ Fluorometric TUNEL System will be purchased from Promega (Madison, WI, USA). The EMT antibodies (E-cadherin, vimentin, snail, and slug) and autophagy-associated antibody LC3B were purchased from Cell Signaling Technology (Beverly, MA, USA). The BV421 annexin V apoptosis staining kit was purchased from BD Biosciences (San Jose, CA, USA). The mitochondrial membrane assay kit was purchased from Cell Signaling Technology (Beverly, MA, USA). Goat-conjugated secondary antibodies (Rhodamine Red and FITC green) for immunofluorescence were purchased from Invitrogen; Thermo Scientific (Carlsbad, CA, USA). DMEM (1X), FBS and PenStrep will be purchased from Thermo Scientific (Carlsbad, CA, USA).

## *4.2. Cell culture*

The human CRC cell line (HCT116) and the normal epithelial colon cell line (CCD841) were obtained from American Type Culture Collection (ATCC). The cell lines were cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum

(FBS) and 5% penicillin-streptomycin (PenStrep). The cells were incubated in a standard humidified incubator at 37$^{o}$C with 5% $CO_2$.

### *4.3. Cell Viability Assay*

ATP-Glo assay was conducted according to a standard protocol. CCD841 and HCT116 cells were seeded in a 96-well plate (10,000 cells/well; three replicates). The cells were treated with a serial range of Diosmetin concentrations or (Diosmetin and Oxaliplatin) combination for 24 h and 48 h. The cell viability was determined by adding 30 µL of the ATP-Glo substrate and incubating at room temperature (RT) for 10 minutes to stabilize the luminescent signal. The luminescence was recorded at 560 nm. IC50 and the percentage of cell viability were estimated and compared to the control cells using the GraphPad Prism software.

### *4.4. Clonogenic Assay*

Clonogenic assay, also known as colony-formation assay, was used to measure the capability of a single CRC cell to form colonies. The Diosmetin-treated cells were plated in 6 well plates (5000 cells/well) for 10-12 days. The cells were then fixed with 4% paraformaldehyde with PBS and stained with 0.01% (w/v) crystal violet. The cells were then washed with distilled water and allowed to dry overnight. Colonies will be imaged and counted using ImageJ software (National Institutes of Health, MD, USA).

### *4.5. Annexin V BV421 Assay*

To explore the apoptotic effect of Diosmetin, the Annexin V BV421 staining, and flow cytometric analysis were performed. Staining with BV421 Annexin V is used in conjunction with a dye such as 7-Amino-Actinomycin (7-AAD) or sytox Red. Sytox Red is a dead cell stain that penetrates compromised plasma membranes and causes the nucleic acids of dead cells to fluoresce bright red when excited with 633-635 nm red laser light. Cells were seeded and pre-treated with Diosmetin for 48 hours. Treated cells

were then harvested and washed with cold PBS. The cells were then resuspended in the binding buffer, and 100 µL of the cells were transferred to a falcon tube. Subsequently, annexin V-FITC and sytox red dye were added to the treated cells and incubated at RT in the dark for 15 minutes. The cells were then centrifuged and resuspended in binding buffer and analyzed. In addition, the percentage and number of cells undergoing apoptosis/necrosis were analyzed by flow cytometry.

### *4.6. Cell Cycle Analysis by Flow Cytometry*

HCT116 cells were seeded and treated in cell culture flasks with varying concentrations of Diosmetin (25, 35, and 45 µM) for 48h. HCT116 cells without treatment were used as a negative control. The cells were then washed, harvested with trypsin, and centrifuged at 1000 rpm for 5 minutes at 25 ◦C. The resulting cell pellet was fixed with cold 70% ethanol and stored at 4 ◦C overnight. Changes in cell cycle distribution were then analyzed using flow cytometry. The cell cycle arrest was detected by calculating the percentages of the cells accumulated in different cell cycle phases.

### *4.7. Measurement of Mitochondrial Membrane Potential*

To determine the mitochondrial membrane potential (MMP) in HCT116 cells treated with Diosmetin, mitochondrial membrane potential assay kit (II) (Cell Signaling) was used. The cells were washed with 1X PBS, harvested with trypsin, and centrifuged at 1000 rpm for 5 minutes and 1 µl of 50 mM carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a specific proton-ionophore that rapidly dissipates the MMP, was added to the positive control (MMP loss) and incubated at 37°C for 15 minutes. after incubation, 100 µl of the 2 µM tetramethylrhodamine ethyl ester perchlorate (TMRE) Labeling solution was added to each sample and incubated at 37°C and for 30 min. TMRE is a cell membrane-permeable dye that accumulates in healthy cells with intact mitochondria due to their high MMP. After incubation, the samples were centrifuged for 5 minutes at 1000 rpm, and the supernatant was removed. The cells were then washed with 1X PBS,

centrifuged for 5 minutes at 1000 rpm, and then resuspended in 1X PBS. The samples were then analyzed on a flow cytometer. The meanvalues were plotted in the graph for loss of MMP representation.

### *4.8. TUNEL Assay*

DeadEnd™ Fluorometric TUNEL System (Promega) was utilized to evaluate cell apoptosis. The DeadEnd fluorometric TUNEL system catalytically incorporates fluorescein-12-dUTP at 3´-OH DNA ends using terminal deoxynucleotidyl transferase (TdT), which forms a polymeric tail and can be visualized using fluorescence microscopy. HCT116 cells (100,000 cells/well) were seeded on chamber slides and treated with Diosmetin. The slides were then fixed with 4% paraformaldehyde with PBS for 25 minutes at 4$^{o}$C. The slides were then permeabilized with 0.2% Triton-X 100 and later treated with equilibration buffer for 10 minutes. After equilibration, the cells were then incubated with recombinant terminal deoxynucleotidyl transferase (rTDT) buffer for 1 hour in a humidified chamber (37$^{o}$C). The reactions were then terminated by adding 2X saline sodium citrate (SSC) to the cells for 15 minutes at RT. The cells were then washed with PBS and mounted with Vectashield DAPI (Vector Lab Cat.# H-1200) for staining the nuclei. Coverslip was added to the slide and allowed to dry overnight. The slides were then observed under EVOS microscope.

### *4.9. Western Blot*

HCT116 cells treated with and without Diosmetin were extracted using a 200 µL mixture of RIPA lysis buffer, serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF), and phosphatase inhibitor sodium orthovanadate. The cells were placed in a cold room (4$^{o}$C) on a rotor for 60 minutes, and then centrifuged at 12000 rpm for 15 minutes at 4$^{o}$C. The resulting supernatant was transferred to new Eppendorf tubes, and the protein concentration was quantified using the Bradford assay. The gels (%) will be prepared based on the molecular weight of proteins to be investigated. After the run, the gel was

transferred to the PVDF membrane (pore size: 0.45 um; BioRad) using Transblot machine for 45 minutes. The membranes were then blocked in 5% blocking buffer (5 g of non-fat milk powder in 100 mL of TBST) for 1 hour at RT. After blocking, the membranes were probed with primary antibodies (mouse or rabbit), prepared in 3% bovine serum albumin (BSA) and incubated overnight at 4$^{o}$C. After incubation, the membranes were washed 2-3 times with TBST, then probed with HRP-conjugated secondary antibody for 1 hour at RT. After secondary antibody incubation, the membranes were rewashed with TBST 2-3 times. The expression of EMT proteins (E-cadherin, vimentin, snail, slug) and autophagy proteins (LC3B) were detected by adding Immobilon chemiluminescent substrate (Millipore, USA) on the membranes and then visualized using Chemidoc (Bio-Rad, USA).

### *4.10. Immunofluorescence Assay*

Immunofluorescence assay was utilized to analyze the cellular content, translocation (nuclear or cytoplasmic), and subcellular localization of EMT proteins (E-cadherin, vimentin, cleaved caspase-3, and cleaved PARP) to further confirm its expression. Approximately 100,000 cells/well of HCT116 cells were seeded in chamber slides and were treated with Diosmetin. The cells were then fixed with 4% paraformaldehyde with PBS for 15 minutes at RT. After fixation, the cells were then incubated for 30 minutes in $NH_4Cl$ solution to remove aldehydes. After incubation, the cells were washed with cold PBS twice and then permeabilized with 0.5% triton x100 in PBS for 10 minutes. The cells were washed again with cold PBS (3x) and then incubated in 10% goat serum at RT for 1 hour. After incubation, the primary antibodies (1:50 dilution) prepared in 0.1% BSA solution were added to the cells and incubated overnight at 4$^{o}$C. The next day, after discarding the primary antibody solution, the cells were then washed with wash buffer (0.05% Tween 20 in 1X PBS) (3x) and then incubated with goat-conjugated FITC and Rhodamine secondary antibodies (1:50 dilution) for 1 hour at RT in the dark.

After incubation, the cells were rewashed with wash buffer (3x) and with cold PBS, then mounted with Vectashield DAPI (Vector Lab Cat.# H-1200). Coverslip was added to the slide and allowed to dry overnight. The slides were then observed under EVOS microscope.

### *4.11. Statistical Analysis*

The statistical analysis was performed using GraphPad Prism software (San Diego, CA, USA). Statistical differences between mean ± SEM or SD values were evaluated by one-way ANOVA (non-parametric) and Dunnetts's multiple comparison tests. Differences between groups with $p \leq 0.05$ were considered statistically significant.

# 5. RESULTS

## *5.1. Diosmetin reduces the cell viability and cell proliferation of HCT116*

The first objective of this study was to determine the IC50 and the effect of Diosmetin on the viability of HCT116 cells.Thus, the normal colon cell line CCD841 and CRC cell line HCT116 were treated with an increasing concentration of Diosmetin for 24 h and 48 h. At 24 h, the half-maximal inhibitory concentration (IC50) was not achieved for HCT116 cells. However, at 48h, IC50 values for HCT116 cells were observed between 38.7 µM (Figure 6A). To assess the cell viability, ATP-Glo substrate was used. Results show a significant decrease in the HCT116 cell viability in a concentration-dependent manner when treated with 0-200 µM of Diosmetin at 48 h (Figure 6B).

The optimal Diosmetin treatment period for this study was fixed at 48 h. To determine the effect of Diosmetin on normal colon cells, CCD841 cells were treated with the same concentrations of Diosmetin mentioned above for 48 h. After the treatment period, ATP-Glo substrate was added to the cells to assess cell viability. Results showed that Diosmetin does not have any toxic effects on CCD841 cells as the IC50 value was observed at 361.524 µM (Figure 6C) and there was no significant difference observed in the cell viability when compared to Diosmetin treated HCT116 cells (Figure 6D). The CellTiter-Glo® luminescent cell viability assay determines the number of viable cells by measuring the amount of ATP present, which is directly proportional to the amount of metabolically active cells present in the culture.

To investigate the effect of Diosmetin on cell proliferation and survival, a clonogenic assay was performed. Compared with control, Diosmetin treatment caused a significant inhibition of colony formation in HCT116 cells in a concentration-dependent manner (Figure 6E).

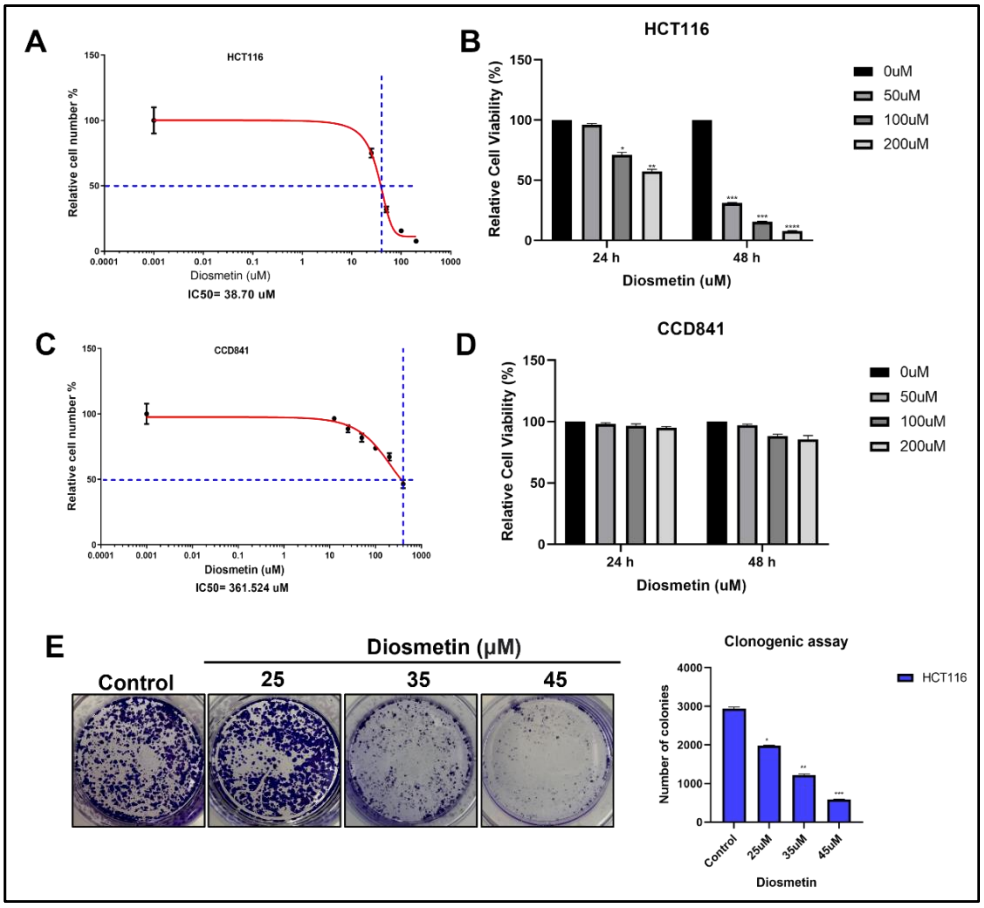


*Figure 6: Effect of Diosmetin on cell viability and proliferation of HCT116 cells. IC50 values were determined for HCT116 and CCD841 cell lines treated with varying concentrations of Diosmetin. The cells were treated 24h after seeding cells, and the viability was assayed 48h after the treatment using CellTiter-Glo* ***(A)*** *The IC50 of HCT116 cells was observed at 38.70 µM at 48h. (B) Diosmetin significantly inhibits the viability of HCT116 cells in a concentration-dependent manner at 48h* ***(C)*** *The IC50 of CCD841 (normal colon cells) was observed at 361.524 µM at 48h* ***(D)*** *Diosmetin does not inhibit the viability of CCD841 (normal colon cells) after treatment with 50, 100, and 200 µM of Diosmetin for 24h and 48 h* ***(E)*** *Diosmetin inhibited the colony formation ability of HCT116 cells. The graphs display the mean ± SD (standard deviation) of three independent experiments with replicates. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. IC50 values were calculated using the GraphPad Prism Software. Cell viability was determined using CellTiter-Glo cell viability assay, and results are presented as % viable cells (relative to control). The colonies were counted using the ImageJ software. The graphs were generated using the GraphPad Prism Software.*

*5.2. Diosmetin showed no synergistic or additive effects when combined with Oxaliplatin*

We conducted the next set of experiments to determine if there is any combined synergistic or additive effect when Diosmetin is combined with chemotherapeutic drug Oxaliplatin. Our results showed that treatment with Diosmetin alone reduced the viability of HCT116 cells to 50% at the concentration of 35.253 µM, and treatment with Oxaliplatin reduced the cell viability to 50% at the concentration of 12.765 µM. Moreover, the combination treatment reduced the viability of HCT116 cells to 50% at the concentration of 26.516 µM (Figure 7). Our results show that Diosmetin shows no additive or synergistic effect when combined with Oxaliplatin. It was observed that Diosmetin did not increase the chemosensitivity of HCT116 cells to Oxaliplatin treatment as the IC50 value obtained upon combination treatment was higher than when treated with Oxaliplatin alone.

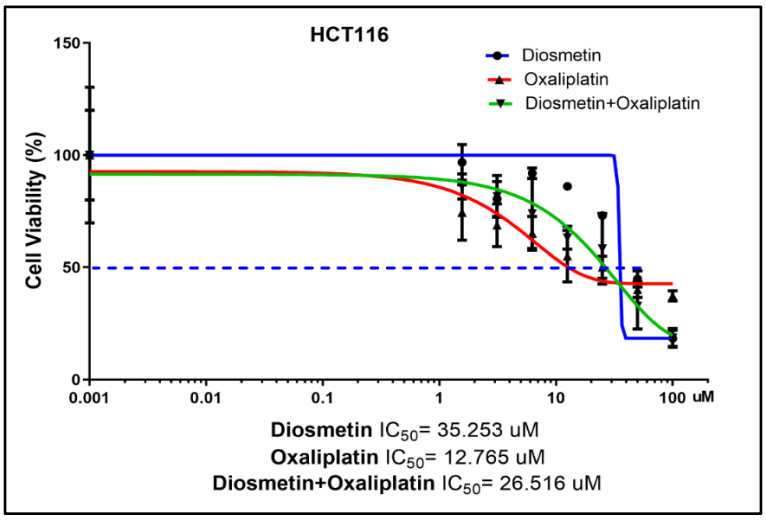


*Figure 7: Effect of Diosmetin when combined with Oxaliplatin in HCT116 cells. The cells were treated with Diosmetin and Oxaliplatin at varying concentrations (0-100 µM) and the IC50 values were determined 48h after the treatment using CellTiter-Glo. The concentrations used in combination treatment were based on the individual IC50 values of Diosmetin and Oxaliplatin. IC50 values were calculated using GraphPad Prism Software.*

### *5.3. Diosmetin induces apoptosis, cell cycle arrest, and causes loss of mitochondrial membrane potential in HCT116 cells*

To determine whether the decreased viability of HCT116 cells occurs as a result of apoptosis, BV421 Annexin V was performed using flow cytometry.

Figure 8 shows four different quadrants (Q1, Q2, Q3, and Q4). Q1 represents necrotic cell populations, Q2 represents late apoptotic cells, Q3 represents early apoptotic cells, and Q4 represents viable cells (Figure 8A). Incubation of HCT116 cells without (control) and with increased concentration of Diosmetin 25, 35, and 45 µM resulted in a concentration-dependent increase in apoptosis (2.33% in control vs. 22.2% with 45 µM Diosmetin).

Cell cycle analysis was performed using flow cytometry to investigate whether Diosmetin induced cell cycle arrest in HCT116. HCT116 cells were treated without (control) and with different concentrations of Diosmetin 25, 35, and 45 µM for 48h. As shown in Figure 8B, Diosmetin induced S phase cell cycle arrest as the % of cells increased in the S phase at 25, 35, and 45 µM concentrations (22.87%, 23.61%, and 28.7%) respectively compared to the control (15.01%) in a concentration-dependent manner. In addition, 3.47% of the cells accumulated in the G2 phase at the highest concentration (45 µM). Thus, our results showed that Diosmetin induced S and G2 phase arrest in HCT116 cells.

To get more insights into the mechanism of apoptosis, we first performed a mitochondrial membrane potential assay. using the flow cytometry technique. HCT116 cells were treated without (control) and with increased concentrations of Diosmetin 25, 35, and 45 µM for 48h followed by flow cytometry analysis. Figure 8C shows that MMP loss reached a value of 55.7% with a concentration of 45 µM of Diosmetin compared to a MMP loss of 5.67% in untreated HCT116 cells.

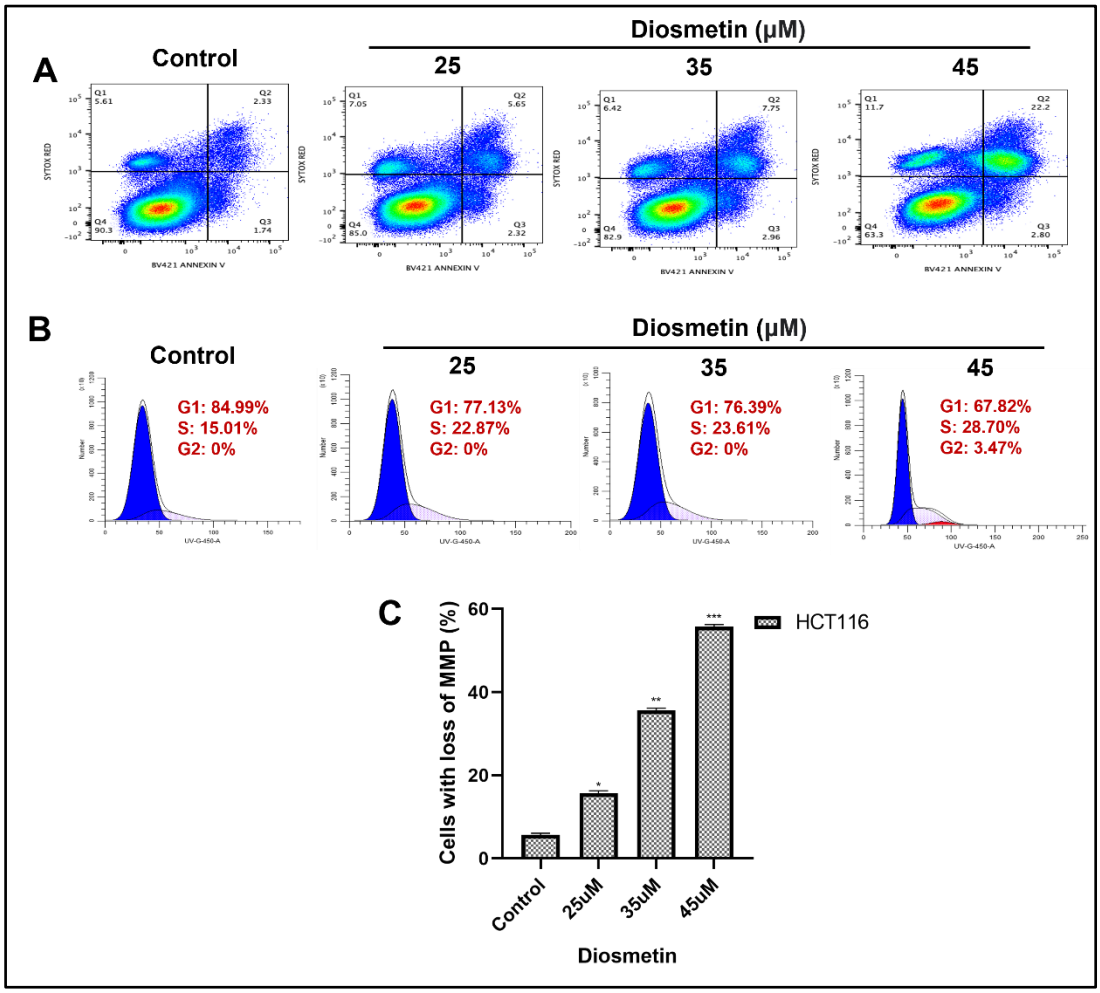


*Figure 8: Diosmetin-induced apoptosis in HCT116 cells **(A)** Cells were treated without (control) and with 25, 35, and 45 μM of Diosmetin for 48 h and stained with BV421 annexin-V and sytox red dye. The cells were then analyzed via flow cytometry **(B)** Cell cycle analysis revealed that Diosmetin arrested cells at the S phase and a small percentage of cells were arrested at the G2 phase at the highest concentration **(C)** Diosmetin treatment causes a loss of MMP in HCT116 cells. HCT116 cells were treated with increasing doses of Diosmetin for 48 h. After TMRE labeling, cells were analyzed by flow cytometry. Data are presented as mean ± SD of results from three independent experiments. *p<0.05, **p<0.01, ***p<0.001.*

In another set of experiments, TUNEL assay was performed to detect DNA fragmentation in HCT116 after treatment with Diosmetin. HCT116 cells were seeded in a 4 chamber-well and treated without (control) and with different concentrations of

Diosmetin (25, 35, and 45 µM) for 48h. Diosmetin induced DNA fragmentation in HCT116 cells in a concentration-dependent manner, as shown in Figure 9.

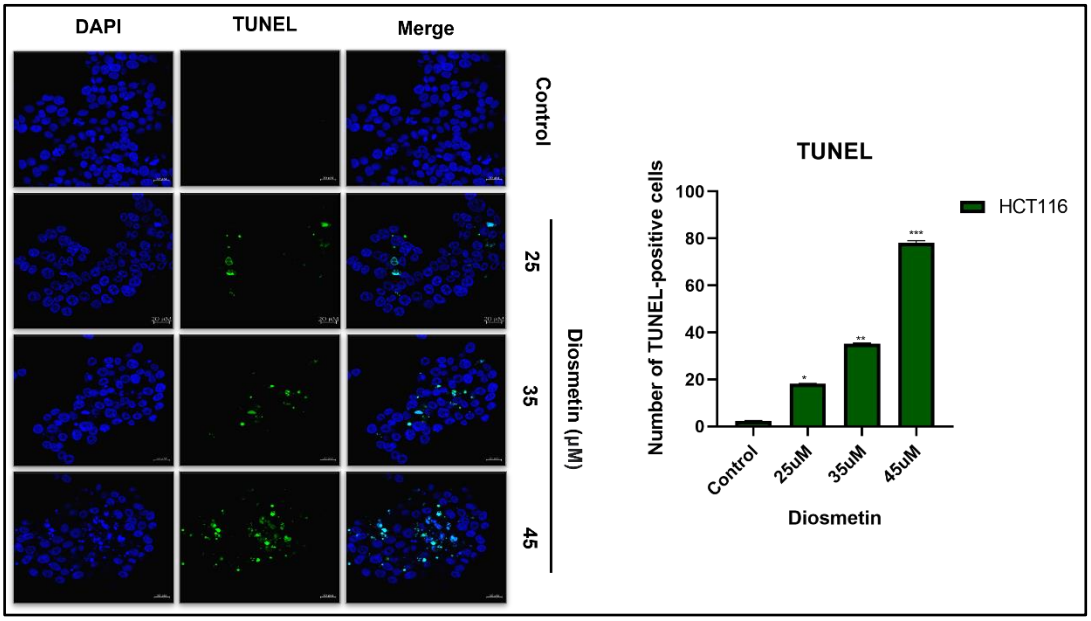


*Figure 9: Diosmetin-induced DNA damage in HCT116 cells. Cells were treated without (control) and with 25, 35, and 45 µM Diosmetin for 48 h. Diosmetin induced DNA fragmentation in HCT116 cells in a concentration-dependent manner. Images were visualized using confocal microscopy. TUNEL-positive cells were counted using the ImageJ software. Data are shown as mean ± SD from three independent experiments $p<0.05$, $**p<0.01$, $***p<0.001$.*

### *5.4. Diosmetin suppresses the expression of EMT markers in HCT116 cells*

The major hallmark of EMT in cancer is the downregulation of epithelial marker, E-cadherin, and upregulation of mesenchymal marker, vimentin. This transition allows the cancer cells to gain migratory and invasive properties to migrate from the primary site (Ramos et al., 2017). To investigate the effect of Diosmetin treatment on EMT in HCT116 cells, we performed western blot to assess the expression of epithelial and mesenchymal markers to determine whether the observed inhibitory effects of Diosmetin on HCT116 involved alterations in the EMT process (Figure 10). Western blot imaging showed an increase in the expression level of the epithelial marker E-

cadherin at the highest concentration (45 µM) of Diosmetin, although a slight decrease in E-cadherin expression was observed at concentrations of 25 µM and 35 µM (Figure 10A). In contrast, the expression of mesenchymal markers, vimentin, and snail decreased when treated with Diosmetin in a concentration-dependent manner (Figure 10B, 10C). In addition, a significant decrease was observed in the mesenchymal marker slug at all the concentrations compared to the control (Figure 10D). Thus, our results show that Diosmetin suppresses the expression of EMT markers in HCT116 cells. However, the EMT mechanism in CRC cells upon treatment with Diosmetin requires more detailed and in-depth investigations.

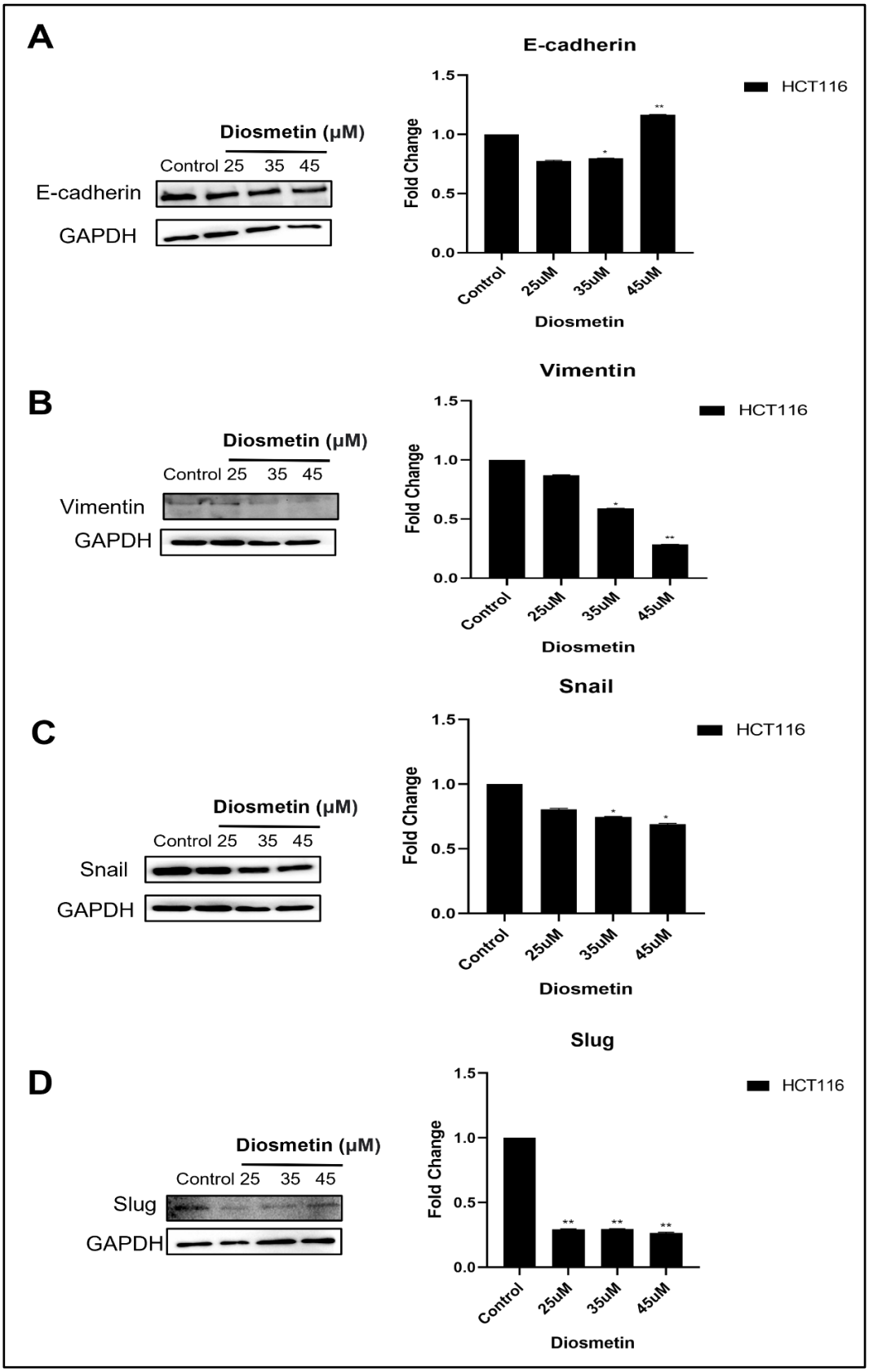


*Figure 10: Effect of Diosmetin treatment on the expression of EMT markers in HCT116 cells. Cells were treated without (control) and with 25, 35, and 45 μM Diosmetin for 48 h.*

*Cell lysates were prepared and immunoblotted with antibodies against* ***(A)*** *E-cadherin* ***(B)*** *Vimentin* ***(C)*** *Snail, and* ***(D)*** *Slug. GAPDH was used as a loading control. Data are shown as mean ± SD from three independent experiments. *p<0.05, **p<0.01. Western blot bands quantification was performed using GraphPad Prism Software.*

To further validate the above western blot results regarding the EMT mechanism, immunofluorescence was performed to detect the expression of EMT markers, E-cadherin, and vimentin. E-cadherin is a junctional protein localized in the membrane and cytoplasm. Fluorescence microscopy images showed that staining of E-cadherin in the membrane increased with Diosmetin treatment. In contrast, vimentin is localized in the nucleus, and staining decreased following treatment with Diosmetin (Figure 11).

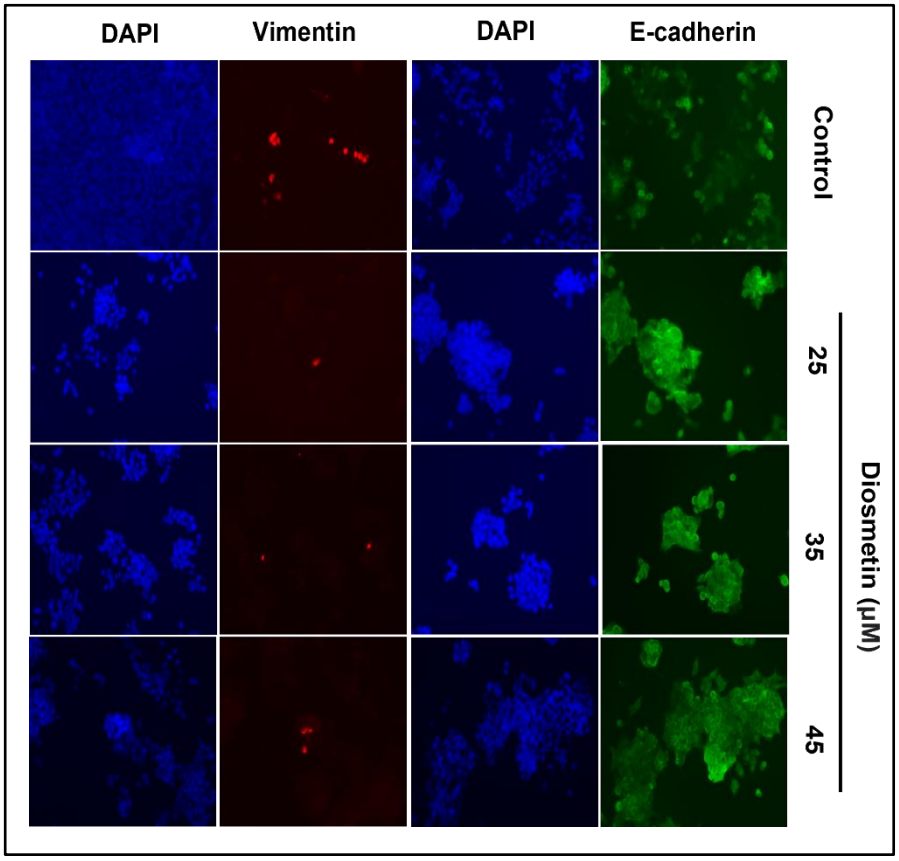


*Figure 11: Immunofluorescence assay of E-cadherin, and vimentin expression in HCT116 cells. Cells were treated without (control) and with 25, 35, and 45 µM Diosmetin for 48 h. Cell nuclei were counterstained with DAPI (blue). Immunostaining was performed using antibody against vimentin (red) and antibody against E-cadherin (green). Images were visualized using EVOS microscope (magnification, 20x).*

### *5.5. Diosmetin activates apoptosis and autophagy in HCT116 cells*

In the next set of experiments, we wanted to investigate the expression of apoptotic markers in HCT116 cells after treatment without (control) and with increasing concentrations of Diosmetin (25, 35, 45 μM) for 48h. Caspase-3, a member of the cysteine protease family, is found to play a fundamental role in the execution phase of apoptosis. During apoptotic cell death, caspase-3 is primarily responsible for PARP cleavage, which causes the suppression of DNA repair mechanisms (Mashimo et al., 2021). The intrinsic apoptotic pathway is regulated by mitochondrial proteins, one of them being cytochrome-c that is released upon stimulation by various external and internal stimuli. Cytochrome-c release causes the activation of downstream executor caspases 3, 6, 7 that are involved in the breakdown of PARP, thus leading to mitochondrial-mediated apoptosis (Shanmugam & Sethi, 2022).

Our results showed that treatment of HCT116 cells with Diosmetin increased the expression of cleaved caspase-3 (Figure 12A) and cleaved PARP (Figure 12B) in a concentration-dependent manner, indicating that Diosmetin induces apoptosis in HCT116 cells through the activation of the intrinsic apoptotic pathway.

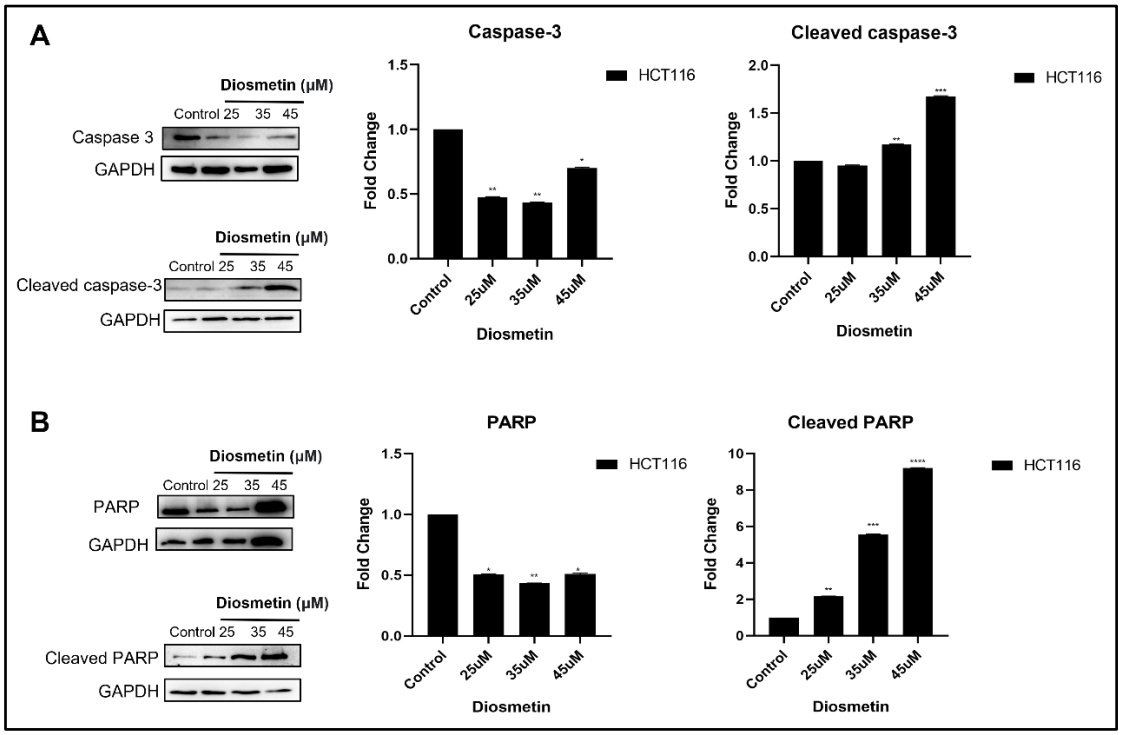


*Figure 12: Effect of Diosmetin on the expression of apoptotic markers caspase-3 and PARP in HCT116 cells. Cells were treated without (control) and with 25, 35, and 45 µM Diosmetin for 48 h. After cell lysis, the proteins were immunoblotted against* ***(A)*** *caspase-3, cleaved caspase 3,* ***(B)*** *PARP, and cleaved PARP. GAPDH was used as a loading control. Data are shown as mean ± SD from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Western blot bands quantification was performed using GraphPad Prism Software.*

For further confirmation of the above findings, immunofluorescence was performed to detect the expression of apoptotic markers, cleaved caspase-3, and cleaved PARP. As shown in Figure 13, the expression of cleaved PARP increased in the nucleus of Diosmetin-treated cells, while it was undetectable in the control cells. Similarly, cleaved caspase-3 was distributed in small clumps in the cytoplasm of Diosmetin-treated cells, and both proteins levels showed an increased expression in a concentration-dependent manner.

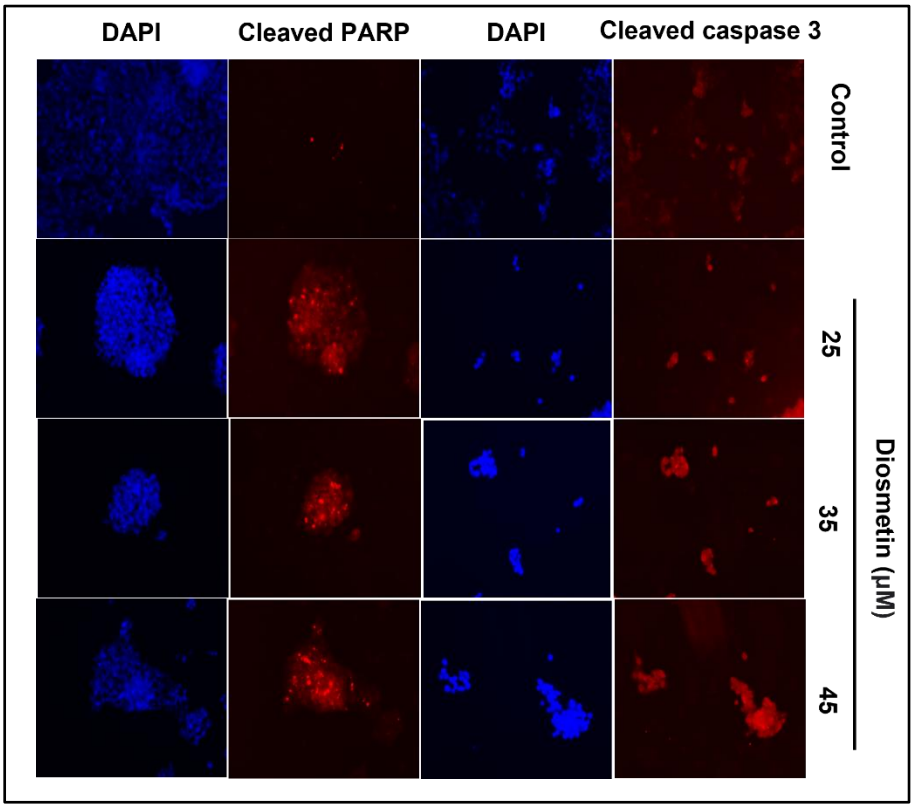


*Figure 13: Immunofluorescence assay of cleaved caspase-3 and cleaved PARP expression in HCT116 cells. Cells were treated without (control) and with 25, 35, and 45 µM Diosmetin for 48 h. Cell nuclei were counterstained with DAPI (blue). Immunostaining was performed using antibody against cleaved PARP (red) and cleaved caspase-3 (red). Images were visualized using EVOS microscope (magnification, 20x).*

To further investigate whether the antiproliferative effect of Diosmetin on HCT116 cells involved other cell death mechanisms apart from apoptosis, we assessed the expression of autophagy marker LC3. After treatment of HCT116 cells with increasing concentrations of Diosmetin for 48h, cell lysates were separated on SDS-PAGE and immunoblotted with antibodies against LC3B. The results show that LC3 was upregulated in HCT116 cells after Diosmetin treatment in a concentration-dependent manner (Figure 14).

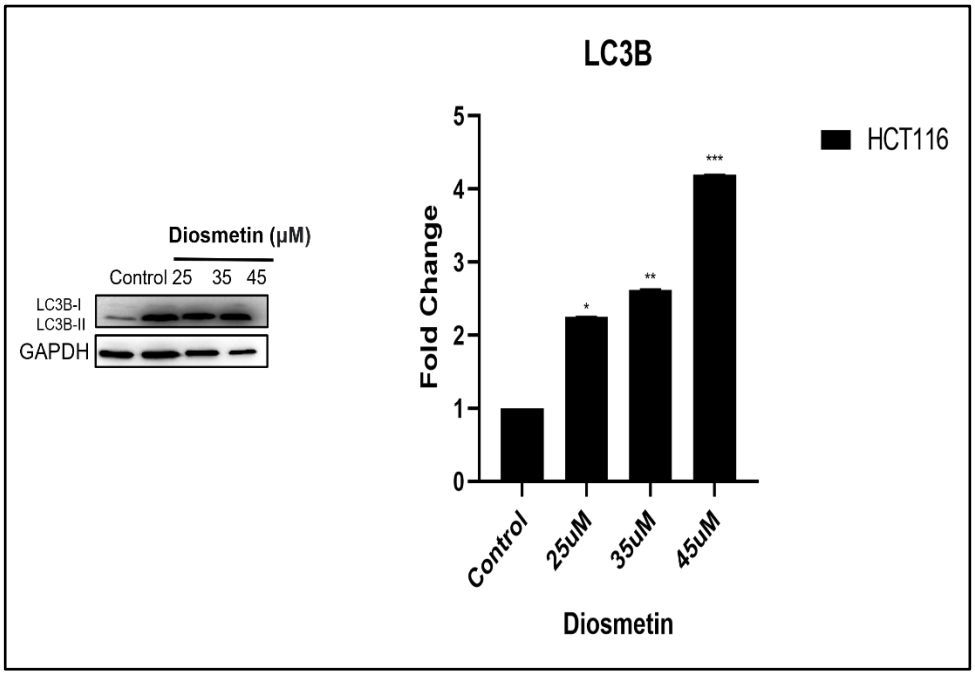


*Figure 14: Diosmetin induces autophagy in HCT116 cells. Cells were treated without (control) and with 25, 35, and 45 µM Diosmetin for 48 h. After cell lysis, the proteins were immunoblotted against LC3B. GAPDH was used as a loading control. Data are shown as mean ± SD from three independent experiments. *p<0.05, **p<0.01, ***p<0.001. Western blot bands quantification was performed using GraphPad Prism Software.*

# 6. DISCUSSION

The heterogeneous nature and the constant evolution of CRC have contributed to drug resistance and the failure of conventional chemotherapeutic treatments, which poses a challenge and calls for the discovery of novel anti-cancer therapeutic agents. NPs have been widely studied for centuries and have long been used as anti-cancer agents. Many plant-derived NPs with less cellular toxicity and fewer side effects have been used as potential alternatives for CRC treatment.

Diosmetin, a citrus bioflavonoid, has been found to exhibit antioxidant, anti-inflammatory and anti-apoptotic properties. In this study, we sought to evaluate the anti-cancer potential of Diosmetin on CRC cells. Data obtained from this study showed that Diosmetin inhibited the proliferation and reduced the cell viability of HCT116 cells in a concentration-dependent manner. Moreover, Diosmetin induced DNA damage, causing cell cycle arrest and apoptotic cell death in HCT116 cells via the intrinsic apoptotic pathway. Previous studies have also shown Diosmetin to induce apoptosis through the upregulation of cleaved caspase-3 and cleaved PARP in melanoma and ovarian cancer cells (Choi et al., 2019; Zhao et al., 2021).

Combination therapy involving two therapeutic agents for cancer treatment has gained widespread attention in recent years. The combination of anti-cancer agents or drugs acting synergistically or additively can enhance the efficacy compared to the mono-therapy approach (Bayat Mokhtari et al., 2017). In our study, the combination of Diosmetin with conventionally used chemotherapeutic drug Oxaliplatin reduced the IC50 values for HCT116 cells compared to when treated with Diosmetin alone. However, the combination of Diosmetin did not increase the chemosensitivity of the cells to Oxaliplatin treatment as the IC50 value obtained upon combination treatment was higher than when treated with Oxaliplatin alone, suggesting no additive or

synergistic effect of Diosmetin when combined with Oxaliplatin.

Accumulating evidence suggests that alteration of the cell cycle can prevent or induce an apoptotic mechanism, and many of the genes involved in cell cycle progression are found to regulate apoptosis (Pucci et al., 2000). The cell cycle checkpoints are critical for maintaining DNA integrity and regulating cells through the cell cycle, and loss of these checkpoints are associated with cancer progression. In the present study, treatment of HCT116 cells with Diosmetin resulted in S and G2 phase arrest in a concentration-dependent manner. Previous studies have also shown Diosmetin to induce G2/M phase cell cycle arrest in osteosarcoma and liver cancer cells (Ma & Zhang, 2020; Ning et al., 2021).

Caspase signaling cascades are important in the process of apoptosis. The activation of caspases initiates cell death by cleaving other proteins such as nuclear protein PARP (Elmore, 2007). PARP has been found to regulate many cellular functions, including multiple DNA repair pathways. Cleavage of PARP prevents DNA repair and blocks energy-depletion-induced necrosis, thus promoting apoptosis (D'Amours et al., 2001). Our study showed that treatment with Diosmetin resulted in the activation of caspase-3 and subsequent cleavage of PARP, suggesting the activation of the intrinsic apoptotic pathway in HCT116 cells. These findings are aligned with other studies, as Diosmetin was found to induce apoptosis in other cancers such as osteosarcoma (Ning et al., 2021), non-small cell lung cancer (NSCLC) (Chen et al., 2019), liver cancer (Ma & Zhang, 2020) and renal carcinoma (Qiu et al., 2020). Another early indicator of the initiation of cellular apoptosis is the loss of MMP in cells. Cells with depolarized or inactive mitochondria cannot accumulate TMRE and exhibit decreased MMP. Herein, we assessed the MMP in HCT116 cells upon treatment with Diosmetin and found that Diosmetin increased the MMP loss in HCT116 cells. Our

results are consistent with previous studies that showed Diosmetin to induce MMP loss in CRC and breast cancer cells (Sanaz Koosha et al., 2019; Wang et al., 2019). Moreover, Diosmetin was found to reduce hydrogen peroxide-induced MMP loss in normal liver cells L02, thus showing its cytoprotective effects (Wang et al., 2018).

Next, we sought to determine the involvement of another potential Diosmetin-mediated cell death mechanism, such as autophagy. Autophagy is a non-apoptotic form of programmed cell death. It is a natural conserved degradation mechanism in a cell that promotes proteolytic degradation of dysfunctional organelles or other cytosolic components at the lysosome (Glick et al., 2010). LC3 is an important protein in the autophagy pathway and is one of the most widely used autophagosomal marker with a molecular mass of 17 kDa. The pro-LC3 form is cleaved by a cysteine protease Atg4 to generate a cytosolic form LC3-I which Atg7 then activated and transferred to Atg3, generating the LC3- phosphatidylethanolamine conjugate (LC3-II) processed form (Deng et al., 2019). Thus, LC3-II is an important autophagosomal marker that is used to detect autophagic activity in cells (Tanida et al., 2008). In our study, we observed the expression of LC3 to be upregulated in HCT116 cells after Diosmetin treatment, thus indicating autophagy induction.

Next, we wanted to determine the effect of Diosmetin on EMT markers in HCT116 cells. EMT is a process that is characterized by loss of cell polarity and cell-cell adhesion resulting in the loss of epithelial markers and gain of mesenchymal markers, which allow cancer cells to gain motility and invasiveness, a key process in cancer metastasis and progression (Ribatti et al., 2020). Our data showed that treatment with Diosmetin resulted in an upregulation of the epithelial marker E-cadherin and downregulation of the mesenchymal markers vimentin, snail, and slug, thus showing that Diosmetin has a role in reversing EMT. The epithelial marker E-cadherin is an

important cellular protein that helps maintain epithelial structural integrity, and loss of this epithelial marker is associated with cancer metastasis (Na et al., 2020). A previous study showed that treatment with Diosmetin increased the expression of E-cadherin in U251 glioma cells (Yan et al., 2020). Transcription factors such as snail and slug are involved in the orchestration of the EMT process and are responsible for the sustenance of the mesenchymal phenotype (Ribatti et al., 2020; Stemmler et al., 2019). Therefore, our study demonstrated that Diosmetin might inhibit metastasis of CRC cells by reversing EMT. However, more detailed studies are required to explore the effect of Diosmetin on EMT in CRC and in different cancers.

In conclusion, our study provides evidence that Diosmetin could inhibit CRC proliferation by inducing apoptosis and autophagy, S and G2 cell cycle arrest, and reversal of EMT. Thus, Diosmetin may be used as a potential target for cancer therapy. More specifically, the role of Diosmetin in EMT needs more investigation. Finally, more *in vitro* and *in vivo* studies are required to explore the therapeutic efficacy of Diosmetin in CRC and other types of cancer.

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