ABSTRACT

Title of Thesis: ANTICANCER ACTIVITY OF NATURAL PRODUCTS IN HUMULUS LUPULUS(HOPS) IN HUMAN COLORECTAL CANCER CELLS.

Gillian Tamia, Master of Science, 2023

Thesis Directed Dy: Doctor Seong-Ho Lee
Department of Nutrition and Food Science

Cancer is a major public health problem and the second-leading cause of death in the world. Colorectal cancer (CRC) is the third most diagnosed cancer in the U.S. CRC is highly associated with daily diet and eating patterns. A plant-based diet rich in phytochemicals has been known to be protective against the initiation and progression of CRC occurrence. The hop plant, a key ingredient in beer, contains a diverse form of bioactive compounds that possess biological benefits in tumorigenesis. Xanthohumol (XN), the most abundant prenylated flavonoid, has been used over the years to treat a broad range of chronic diseases such as diabetes, obesity, and cancer. Several derivatives of XN, including isoxanthohumol (IXN), 8-prenylnaringenin (8-PN), and tetrahydroxanthohumol (TXN), possess similar and greater biological benefits compared to XN. While XN's anti-cancer properties are well known, the effects of these derivatives have not been evaluated in human CRC models. Our study aimed to test the cancer-suppressive activities of these derivatives and elucidate anti-cancer mechanisms using human adenocarcinoma CRC cells. The results indicate that four hop compounds (XN, IXN, 8-PN, and TXN) significantly suppressed the proliferation of different types of human CRC cell lines.

We selected TXN and XN for further studies due to their more significant and promising anti-proliferative activity compared with other forms. Flow cytometry analysis indicated that TXN and XN led to significant induction of S-phase and G2/M-phase arrest. An apoptotic assay showed a huge induction of early and late apoptosis in cells treated with TXN and XN at doses of 12 μM and 18 μM. Western blot data indicate that TXN and XN induce the cleavage of PARP and increase the expression of CHOP, IRE1α, and ATF4, indicating activation of caspase-dependent apoptosis and ER stress. In addition, a dose-dependent increase in intracellular ROS was observed in cells treated with 12 and 18 μM of TXN and XN, affecting mitochondrial dysfunction. Taken together, our current study proposes an anti-cancer mechanism by TXN and XN through their action on the induction of ROS release and mitochondrial dysfunction, ER stress, and apoptosis in human CRC cells.

# ANTICANCER ACTIVITY OF NATURAL PRODUCTS IN HUMULUS LUPULUS(HOPS) IN HUMAN COLORECTAL CANCER CELLS.

by

GILLIAN TAMIA

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2023

ADVISORY COMMITTEE:

Associate Professor: Dr. Seong-Ho Lee, Chair
Associate Professor: Dr. Byung-Eun Kim
Distinguished University Professor: Dr. Liangli Yu

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# CHAPTER 1: General Overview of Cancer

## 1.1 Hallmarks of Cancer

Cancer, which has infested multicellular living organisms for decades, is the second leading cause of death in the United States [1] [2] [3]. Cancer is a major public health problem with about 11 million diagnosed cases and an estimated increase of 16 million by 2020 [1] [4]. The longitudinal observation of tumor growth in humans remains impractical, which makes cancer an even harder topic to grasp [5]. Data collected at a single point in time provide an understanding of tumor evolution [5]. Human cells are usually recruited and transformed into tumors [6]. When cell division occurs, any error during DNA replication leads to new mutations and genetic alterations, which can be transferred to the genome of the daughter cell. Evolutionary selection plays a significant role in shaping cancer genomes by determining the distribution of mutations within tumors, in addition to mutations [5]. In this process, one group of cells in a tumor is evolutionarily favored over others. These favored cells grow and evolve a new phenotypic trait that provides them with an adaptive advantage in a tumor microenvironment. So, any mutation in this population becomes more common in the entire tumor population [5].

The six hallmarks of cancer explain the different pathways cancer circumvents to enable tumor growth and metastatic dissemination [7] [3]. Firstly, sustaining chronic and chaotic proliferation is the most fundamental trait of cancer cells. Cancer cells dysregulate the release of growth-promoting signals that control the cell cycle [7] [8]. In order to maintain these signals, growth factors bind to cell-surface receptors, primarily tyrosine kinase domains [8]. Emission of signaling proceeds via branched intracellular signaling pathways, which regulate the progression of the cell cycle and cell growth as well as other biological activities like cell survival and energy metabolism [7]. Sustenance of cell growth signaling in cancer cells is regulated in two ways: cancer

cells either produce their growth factor ligands or signal normal cells with supporting tumor-associated stroma to supply them with growth factors [7] [3] [8]. Secondly, cancer cells evade growth suppressors that are present to negatively regulate cell proliferation [7]. RB (retinoblastoma-associated) and TP53 proteins are two prototypical tumor suppressors that operate as central gatekeepers in deciding cell proliferation or activation of senescence and apoptosis [7]. The tumor suppressors are downregulated for cancer cells to thrive.

Thirdly, cancer cells are resistant to any form of cell death. Apoptosis, which is programmed cell death, serves as a barrier to tumorigenesis [7] [8]. Apoptosis has both upstream regulators and downstream effector constituents. The apoptotic regulators receive and process extracellular death-inducing signals that govern the intrinsic and extrinsic apoptotic pathways with ligands such as the Fas ligand/Fas receptor [7] [3]. Proteases such as caspases 8 and 9 are activated along these pathways to enable the activation of a cascade of proteolysis that disassembles damaged cells and enhances phagocytosis [7]. Apoptotic regulators and effectors are controlled by pro- and antiapoptotic members of the Bcl-2 family of regulatory proteins. Bcl-2, Bcl-$x_L$, Bcl-w, Mcl-1, and A1 are antiapoptotic proteins that bind and suppress proapoptotic triggering proteins such as Bak and Bax. If Bak and Bax are not inhibited, they disrupt the mitochondrial membrane's integrity [7] [8]. When DNA breaks, TP53 upregulates the expression of Noxa and Puma BH3-only proteins to activate apoptosis. So, the loss of TP53 tumor suppressor function enables cancer cell survival by avoiding apoptosis [7]. Autophagy is a cell-physiologic response to cellular stress and is similar to apoptosis. Autophagic cellular activation results in the breakdown of key organelles, including ribosomes and mitochondria, with the help of lysosomes [7]. Beclin-1, a member of the BH3-only subfamily, binds to Bcl-2/Bcl-$x_L$ proteins. Stress-sensor-coupled BH3 proteins such as Bid, Bad, and Puma disrupt and release Beclin-1 from Bcl-2/Bcl-$x_L$, which enables

Beclin-1 to trigger autophagy. Necrosis is a form of cell death caused by bloating and the explosion of the necrotic cell [7]. As opposed to apoptosis, necrosis leaves debris after an explosion, which triggers the activation of an immune inflammatory cell to assist in the clearing of the debris [7]. However, activation of these inflammatory cells can promote tumor proliferation by supplying blood vessels to enable the growth of these damaged tissues [7].

Fourthly, cancer cells possess unlimited replicative potential. Cancerous tumors don't enter a state of senescence (a non-proliferative but viable state). Once a crisis occurs in a tissue, cell death is activated [7]. Immortalized cells, including cancer cells, express high levels of telomerase, which extends the end of DNA by adding telomeres. Telomeres prevent the shortening of DNA, leading to the activation of senescence or apoptosis [7] [8]. Moreover, cancer cells require a constant supply of nutrients and oxygen to sustain their high proliferative state. Angiogenesis fulfills this by enabling the formation of new blood vessels from existing ones; thus, blood supply is maximized to the tumors [7] [3]. Examples of prototypical angiogenic inducers and inhibitors include vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (TSP-1) [7] [8].

Finally, cancer cells progress to higher pathological levels of malignancy through invasion and distant metastasis. The expression of E-cadherin, a critical component of cell-to-cell adhesion, is downregulated in carcinomas as it inhibits the invasion and metastatic processes associated with tumors [7] [3]. If novel preventive methods are not found, cancer cells will continue to survive and proliferate by escaping cellular control signals [9]. Thus, sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis are the targeted hallmarks of cancer.

## 1.2 Colorectal Cancer (CRC)

CRC is one of the top five causes of cancer-related deaths in the world [10] [11]. CRC is the third most commonly diagnosed cancer in the West, with approximately 80% of tumors being localized regardless of the rate of spread to nearby lymph nodes [12]. CRC accounts for about 1.2 million new cancer cases and 600,000 cancer deaths per year [10]. The adenoma-carcinoma sequence is the basis. Of CRC formation [13], colon and rectal cancer are the most prominent forms of malignant tumors of adenoepithelial origin [12]. Incidence and mortality rates appear to be high in developing countries but stagnant or low in developed countries. CRC is more common in men than in women [14]. Recently, the right side of the colon has shown an increased incidence of CRC for unclear reasons [13]. The overall incidence of CRC, specifically in the rectal and distal regions, has declined in older populations (50 years and older) [14].

Multiple exogenous and endogenous factors are involved in CRC variation, including risk factor exposure, demographic variations, genetic susceptibility and genetic mutations, prognosis, and treatment response [15]. Epidemiological studies have shown a wide variation of CRC among different populations around the world based on factors such as age, gender, and racial origin [13]. Even though there is a strong association between CRC and hereditary components, the majority of cases are sporadic with gradual development over several years [10]. Screening has extensively reduced CRC incidence and mortality by allowing early detection and removal of precancerous adenomas. About 10–20% of CRC-diagnosed individuals have positive family histories. Germline testing has been used to detect known hereditary markers in ∼5% of these patients [14].

One study depicted the role of mutated oncogenes and tumor suppressor genes in tumorigenesis and the progression of CRC (normal epithelial cells to carcinomas) [16]. Results from this study showed that the frequencies of Kirsten rat sarcoma (KRAS) and p53 mutations were significantly enhanced in patients with CRC metastasis; mutated SMAD4 enabled CRC

distant metastasis. In addition, mutated KRAS provided a tumor growth advantage during the progression from colorectal adenoma to malignant carcinoma [16] [17]. Mutated KRAS is common in CRC, with mutations in about 40% of all CRC patients [16]. This mutation acts as a molecular switch that activates downstream signaling pathways, like cell proliferation and survival, leading to tumorigenesis [11]. CRC patients with KRAS mutations have a dismal prognosis [16] [17]. KRAS mutation testing has recently become a routine clinical practice before treating metastatic cases [11]. This approach has enabled sensitivity and accuracy in treating these patients.

Mutated p53 enhances the later progression of CRC [16]; p53, a stress-inducible transcription factor, is maintained at low levels in the cell [18]; p53 is also a key tumor suppressor gene and an important element of anticancer defense; upon mutation, it enables the progression of CRC through adenoma-carcinoma transition processes [18]. Mutant *p53* occurs at different rates in various parts of the colon: 34% in proximal colon tumors and 45% in distal colon tumors [18]. Mutant p53 is linked to lymphatic invasion in proximal and distal CRC [18]. Mutant p53 in CRC patients shows more chemoresistance and poorer prognosis than wild type p53 [18]. Upon activation, p53 triggers its negative regulator, murine/human double minute 2 (MDM2); MDM2 acts as an E3 ubiquitin-ligase to enable the ubiquitination of p53 for degradation [18]. Mutated p53 contributes to about 40%-50% of sporadic CRC progression and outcome. p53 activates cellular processes including cell cycle arrest, apoptosis, and senescence [18]. Activated p53 can induce both the mitochondrial and death-receptor-induced apoptotic pathways, which leads to the expression of pro-apoptotic B-cell lymphoma-2 (Bcl-2) family proteins, including Bax, Noxa, and PUMA [18]. Moreover, p53 downregulates pro-survival Bcl-2; cytochrome c is released and binds to Apaf-1 in the mitochondria, inducing caspase-3, -6, -7, and -9 [18]; p53 activation also

upregulates the expression of Fas (CD95/APO-1), DR5 (TRAIL-R2), and PIDD (a p53-induced protein that has a death domain). p21, a member of the CDK inhibitor family, is a p53-downstream gene and contains a promoter that is consensus p53-binding; thus, p21 mediates p53-induced growth arrest. In damaged DNA, there is induction of $G_1$ phase arrest, as well as $G_2$/M checkpoint arrest [18]. Certain DNA damage, like dysfunctional telomeres, induces stress on the cell, leading to cellular senescence [18]. Mutated p53 leads to the epithelial-mesenchymal transition (EMT) and enhances CRC cells' invasion and metastasis. Thus, the repression of epithelial markers leads to an increase in mesenchymal marker expression.

TGF-beta signaling induces EMT to regulate CRC onset and progression [16]. Another gatekeeper gene, adenomatous polyposis coli (*APC)*, is a tumor suppressor; however, when mutated, it encourages the growth of CRC by activating canonical Wnt signaling in epithelial cells [19] [20]. The mutated APC gene is responsible for familial adenomatous polyposis (FAP) and plays a rate-limiting role in most sporadic CRC cases [21] [11]. The loss of APC function can result in the formation of colorectal tumors through the adenoma-carcinoma sequence in combination with genetic alterations in CRC-related oncogenes or tumor suppressors [21] [20]. Generally, an intestinal cell needs to comply with two essential requirements to develop into cancer [21]. Inactivation of APC fulfills these two essential credentials (selective advantage and genetic instability) for intestinal cells to develop into cancerous cells [21]. Apart from hereditary factors, a person's dietary intake, nutritional status, and physical activity correlate with the pathogenesis of CRC and poor prognosis in patients with CRC [22].

### 1.3 Apoptosis as a Chemopreventive Target of CRC

Apoptosis occurs in two main pathways: extrinsic and intrinsic apoptotic signaling pathways. The intrinsic (mitochondrial) apoptotic pathway is initiated by DNA damage and growth

factor withdrawal, thereby activating the caspase cascade, which is the primary mediator of apoptosis. Secondly, the extrinsic (death receptor) apoptotic pathway involves transmembrane receptor-mediated interactions [23]. Poly (ADP-ribose) polymerases (PARP) encode poly (ADP-ribose), which is the major signaling molecule for DNA damage response [24]. The pathogenesis and progression of CRC are closely related to PARP, which plays a role in the development and occurrence of tumors [24]. On the other hand, PARP inhibitors are used in the treatment of these tumors [24]. PARP influences molecular pathways such as the NF-κB pathway. In CRC, PARP can influence the activation of TLR4-related signal transduction by mediating NF-κB activity, resulting in NF-κB mobilization and ensuring nuclear conservation [24]. The PARP protein also plays an important role in the Wnt signaling pathway. Inhibition of the Wnt pathway reduces DNA repair ability and significantly suppresses cancer development *in vivo* [24]. Caspase-3 is a crucial executor of apoptosis through the proteolytic cleavage of PARP (Han, 2019).

Bcl-2 family proteins are crucial gatekeepers for both intrinsic and extrinsic apoptotic pathways and the transformation of healthy colon cells into adenomas. Some cancer cells are high in antiapoptotic proteins such as Bcl-2 which inhibit apoptosis and fosters metastasis [25] [26]. Bcl-2 mediates the release of proapoptotic proteins responsible for the induction of caspases by stabilizing the mitochondrial outer membrane. CRC has a distorted balance between the proapoptotic protein Bax and the antiapoptotic protein Bcl-2. The Bax/Bcl-2 ratio is decreased in CRC cancer cells as compared to normal colon cells [26] [27]. Loss of Bcl-2 expression in CRC cells was able to induce apoptosis in these cells [23]. Endoplasmic reticulum (ER) stress is induced by extracellular environmental stress, including reactive oxygen species (ROS), hypoxia, and nutrient deprivation [26] [28] [29]. ER stress is governed by three major UPR signaling pathways, including inositol-requiring enzyme1α (IRE1α), protein kinase RNA (PKR)-like kinase (PERK),

and activating transcription factor 6 (ATF6) [28]. IRE1α restores homeostasis to protect cells and enable apoptosis by inducing Jun-N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK). In addition, activated PERK enables the expression of the pro-apoptotic protein, CHOP, by enhancing the ATF4 transcription factor through phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) [28].

CHOP enhances TRAIL-induced apoptosis through ROS-dependent DR5 expression. ATF6 functions as a sensor and effector of UPR [28]. During ER stress, ATF6 is translocated to the Golgi apparatus and is transformed into an active ATF6, p50 transcription factor, thereby promoting the folding process in the ER [28]. Autophagy, which is "self-eating," is a lysosomal response to degrading organelles [30]. Autophagy is activated via the AMP-activated protein kinase (AMPK) and PI3K/Akt/mTOR signaling pathways [30]. The expression of LC3B protein, which is a double-membrane component of the autophagosome, is downregulated in cancer cells [30] [27]. Oxidative stress, which leads to mitochondrial dysfunction and the induction of apoptosis, is mediated by high levels of reactive oxygen species (a normal by-product of mitochondrial respiration) in the cell [31] [29].

### 1.4 Cell Cycle Arrest as a Chemopreventive Target of CRC

The cell cycle is characterized by checkpoints (G1, S, G2, M) between phases. During the G1 phase, which is the prep phase, cells get ready to enter the cell cycle, and DNA status and cell activities are regulated; the S phase enables DNA replication [32]. p53 and p21 are expressed during the G1 phase, which leads to the regulation of cyclin D1 and CDK2 [32]. The G1-S transition is significant in cancer development and proliferation. The G2 phase follows the S phase, which is made up of a synthesis phase coupled with cellular machinery for mitosis in the M phase [32]. The quality and integrity of the overall cell cycle are mediated by these checkpoints [32].

Cyclins, a protein family, regulate cell cycle progression. For instance, cyclin A has a crucial role in controlling the G2/M transition phase by binding to CDK2, which is a required step for cell progression through the S phase [33]. Defective checkpoint proteins, which can lead to genomic instability, and checkpoint control mechanisms are responsible for tumorigenesis in cancer cells. M-phase checkpoint deficiencies contribute to tumor formation in mammals [34]. For instance, a study indicated that mice with deficiencies in MAD2, BubR1, and Bub3 (M checkpoint proteins) are susceptible to cancer [34]. While a biopsy obtained from 16 CRC patients showed elevated c-MYC expression associated with increased MAD2 levels. c-MYC is known to delay prometaphase by directly transactivating MAD2 and BubR1 [34] [27]. Moreover, proteins implicated in the G1/S phase are impacted by CRC. For example, the p21 waf1/cip1 protein, a p53-inducible inhibitor of cyclin-dependent kinases, as well as cyclins D and E, are key regulators of G1 phase progression [34]. A change in p21 protein and mRNA levels has been seen in CRC. Also, cyclin D1 overproduction can lead to CRC's aggressive biological behavior and has been associated with advanced stages [34].

## 1.5 Treatment of CRC

Cancer cells don’t invent new mechanisms; however, they work with existing molecular and cellular pathways to circumvent tumor suppressor mechanisms [3]. With advancements in technology, the field of cancer has undergone tremendous changes to enable effective and precise treatment options, thereby improving the survival rates of patients [4]. Further, the understanding of cancer hallmarks has led to the discovery of innovative methods for combating the disease. Treatment options vary based on the stage and type of cancer [4]. However, no one method is used on its own; a combination of treatments is used to maximize efficacy [4]. So far, some treatment options that have been used for years include chemotherapy, surgery, radiation therapy, and

hormonal therapy [3]. For instance, chemotherapy is administered to the patient before or after the surgical procedure [4].

Chemotherapy uses anticancer drugs that interfere with tumor growth and destroy cancer cells. It is considered one of the most effective cancer treatment options. However, there are severe side effects as healthy cells can be damaged by these drugs [4] [35]. Immunotherapy, a biological therapy, is a cancer treatment method that stimulates the immune system to fight cancer. The purpose of monoclonal antibodies (a common immunotherapy method) is to block specific protein functions by binding to cancer cells, making it possible for the immune system to recognize and eradicate cancerous cells [4]. When it comes to immunotherapy, certain types of cancer do not always benefit from vaccination [36].

Even though previous studies show that cancer patients with solid tumors normally develop a robust immune response after vaccination, regardless of their treatment status [36], hematological cancer sufferers, especially individuals with B-cell malignancies (chronic lymphocytic leukemia), don't fall under this category. Most cancer patients without B cells still generate vaccine-induced T-cell responses [36]. Furthermore, evidence has indicated that the gut microbiota greatly contributes to the relationship between diet and cancer [37]. Diet and energy modulate the gut microbiota composition and function. Gut microbiota can produce oncometabolites or tumor-suppressive metabolites depending on the host's diet and digestive components of the GI tract. Gut microbiotas influence the immune cells in the lamina propria, which affect inflammation and potentially CRC. Aberrant epigenetic markers accumulate, and epimutation drives tumorigenesis during CRC [38].

## 1.6 Pharmacological Chemopreventive of CRC: Aspirin and NSIAD

Aspirin acts as a protective agent against the formation and progression of CRC. Currently, aspirin is recommended as a primary risk reduction for CRC in all patients aged 50 to 59 years old in the U.S. [39] [29]. Long-term aspirin consumption decreases the risk of colorectal carcinoma by about 40%. On the other hand, non-steroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenase (COX) in adenoma formation [40] [29] [41]. Upregulation of the *COX-2* gene is observed in 40–50% of human colorectal adenomas and 80–90% of hepatocellular carcinomas.

In the colonic mucosa, COX-2 is localized mostly in tumor tissue, including epithelial cells. Also, upregulated COX-2 is linked to phenotypic changes such as increased cell adhesion, resistance to apoptosis, tumor angiogenesis, as well as enhancement of prostaglandin production [40] [41]. Prostaglandin levels in CRC tissue are 3–4 folds higher than in normal tissue. For instance, beneficial outcomes were observed for a COX-2-selective inhibitor (celecoxib) in familial adenomatous polyposis, implying that COX-2 is a crucial factor in the pathophysiology of colorectal carcinoma [40] [41]. Inflammation and tumorigenesis are affected by aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs). Aspirin and non-aspirin NSAIDs inhibit CRC by blocking COX-2-derived $PGE_2$ formation, interrupting β-catenin signaling, and restoring apoptosis [40].

## 1.7 Nutritional Chemopreventive of CRC

For years, nutrition has been a significant contributor to the risk of developing cancer [42]. Generally, the diet has a crucial role in the formation of neoplasia and the progression of CRC [41]. For instance, a higher adherence to the Mediterranean diet was able to reduce approximately 30% and 45% of the CRC risk in men and women, respectively. Foods rich in fiber, calcium,

cruciferous vegetables, and garlic possess protective elements to reduce CRC [43]. Phytochemical, a secondary metabolite, is abundantly common in fruits and vegetables [44]. Its non-nutritive bioactive chemical compound is synthesized only in specific parts of a plant [45].

Recently, a total of 10,000 phytochemicals, including prebiotics and probiotics, polyphenols, carotenoids, steroids, and thiosulfates, have been identified. Phytochemicals are either metabolized in the liver or by gut bacteria; hydrolyzation is an essential step in the absorption of these phytochemicals. This active ingredient in the plant is a modulator of important cellular signaling pathways for disorders such as cancer, cardiovascular diseases, neurodegenerative diseases, and obesity [45]. Also, plants with higher concentrations of phytochemicals possess protective abilities against free radicals, enhancing their antioxidant and anti-inflammatory activities [45]. Emerging evidence on the promising potential of active compounds in plants has prompted more attention to their anti-carcinogenic action. The anti-carcinogenic action of photochemical agents involves the induction of apoptosis, inhibition of mitosis, and enhancement of carcinogenic excretion [45]. These active ingredients have promising anti-cancer abilities because of their high efficiency and low toxicity. It is believed that they down- and up-regulate important anti-cancer mechanisms including programmed cell death (apoptosis, pyroptosis, and autophagy), migration, and senescence-related signaling pathways of cancer through reactive oxygen species (ROS), mitogen-activated protein kinase (MAPK) pathways, nuclear factor κ light-chain-enhancer of activated B cell (NF-κB) pathways, and glycolytic enzymes [46].

There are several phytochemicals with anti-carcinogenic effects against CRC, such as Carotenoid, a pigment found in plants, which has antioxidant properties, controls cellular growth, activates immune response, and modulates genome expression in cells to suppress the growth of cancer cells [47]. Lutein, a fat-soluble xanthophyll, is found in yellow and green vegetables; it

regulates apoptosis and cell proliferation by reducing the concentration of β-catenin in CRC and reduces K-ras and AKT concentration resulting in cell cycle arrest [45]. Lycopene, a lipophilic pigment found in red-colored fruits and vegetables, mainly inhibits cytochrome P450 2E1, reduces pro-caspase 3, 8, and 9, enhances Bcl-2-associated X protein (BAX) expression, reduces Hmg Co-A reductase expression, enhances Ras translocation, and inhibits the expression of NF-κB and c-Jun N-terminal kinases (JNK). β-carotene, mostly found in carrots, induces apoptosis and cell cycle arrest by increasing the levels of BAX and P53 expression, reducing Bcl-2 levels, and suppressing the MAPK/ERK and PI3L/AKT pathways. Quercetin is abundant in cranberries; it inhibits cell proliferation and enhances apoptosis through inhibition of the PI3K, AKT, and COX-2 signaling pathways and induction of the AMPK pathway. Allicin, which is found in garlic, suppresses the action of NF-κB by inhibiting phosphorylated P65 translocation. Asparagusic acid reduces cellular viability and inhibits cell migration and invasion through the Rho GTPase signaling pathway; activation of the TRAIL death receptor pathway leads to upregulation of caspase-8 and caspase-3 in colon cancer; sulforaphane is from cruciferous vegetables like cabbage. They induce acute oxidative stress in the cell by inhibiting P38 MAPK, leading to the prevention of Nrf2-Keap-1 dissociation. Curcumin is found in the ginger family; curcumin possesses anti-cancer effects by inducing apoptosis through the intrinsic and extrinsic pathways, preventing cell proliferation, and inducing cell cycle arrest [45].

When migrants move from low-risk to high-risk countries, changes in the environment, which involve poor dietary changes, increase their risk for cancer. For instance, populations with high intakes of red meat showed high rates of CRC. In addition, animal models in experimental conditions with restricted energy intake showed a reduced risk of cancer [42]. Moreover, diets rich in animal or caloric content, refined sugars, and alcohol contribute to about 30–35% of all cancers

[48]. Most research used factor analysis components to derive a combination of dietary patterns [48]. In addition, fiber consumption correlates with reduced CRC risk. The results of a study revealed that participants who consumed red and processed meat at the highest levels consumed an average of 76 grams per day; although these individuals met the current recommendations, they still demonstrated a 20% higher risk of developing colon cancer compared to individuals who consumed 21 grams per day on average [49]. In conclusion, regular dietary intake of fiber, fruit, and vegetables such as cruciferous vegetables have a protective role against cancer onset [43].

## 1.8 Health Benefits of Hops

The hop plant (*Hamulus lupulus* L.), the key ingredient in beer, has biological benefits. *In vitro* and *in vivo* studies have confirmed the hop compounds as novel anticancer agents because they exert important and diverse beneficial activities [50]. Hop belongs to the Cannabaceae family and is widely located in the Northern Hemisphere, Europe, Asia, and North America [51]. Approximately 4-14% of polyphenols are present in hops' dry weight. Xanthohumol (XN), being the most abundant prenylated flavonoid, takes up about 85% of total content; XN continues to be of great interest because of its biological roles (anti-inflammatory, neuroprotective, anti-microbial, and anti-carcinogenic) [42] [51] [52]. Isoxanthohumol (IXN), 8-prenylnaringenin (8-PN), and 6-prenylnaringenin (6-PN) are very common isomers of XN [53]. One-way XN can be extracted from the hop plant is by using supercritical carbon dioxide ($CO_2$). This method is done under high pressure to improve the solubility of the compound; 115 mg/kg of XN is the yield from this technique compared to the yield extracted from 1% carbon extraction from the hop cones. One study used this method to isolate 10–30% of XN from 50 liters of spent hop extract [51].

Moreover, another study showed that a higher content of XN was obtained from hop ethanolic extract ($3.75 \pm 0.05$ g per 100 g). Another technique used high-speed counter-current

chromatography (HSCCC) to obtain a higher yield of 93.60% XN (95% pure) from a 607 mg crude extract of Humulus lupulus L. using the solvents n-hexane, ethyl acetate, methanol, and water (5:5:4:3 ratio). The most efficient method used different choline chloride-based deep eutectic solvents (DES), and the precipitates formed were extracted with DES, yielding a higher XN content (2.30 mg/g of spent hops) [51]. XN is consumed mainly in beer; its intake level is very low because XN is converted into its isomeric form, IXN, by thermal processes (brewing or stomach hydrochloric acid) [52, 54]. XN bioavailability is very low because about 80% of the dose administered orally is lost in feces and urine [54]. IXN can be further converted to 8-PN, the most potent phytoestrogen. Both *in vitro* and *in vivo* showed XN isomerization into IXN. The gut microbiome and the host's hepatic cytochrome P450 enzymes continue the process by metabolizing IXN into 8-PN [52].

Recently, α, β-Dihydroxanthohumol (DXN), and Tetrahydroxanthohumol (TXN) were found to be the non-estrogenic derivatives of XN [52]. These two derivatives lack the α, β-unsaturated ketone found in XN; thus, there is no metabolic interconversion to XN or IX. This loss of interconversion prevents the formation of 8-PN [52]. There are several metabolic conditions that can be prevented using XN and its derivatives, including diabetes, obesity, and cancer.

## 1.9 Hop and Metabolic Diseases

Hop treatment prevents postmenopausal symptoms by regulating the blood lipid profile, fat accumulation, blood estrogen, bone resorption factors, and dermal blood flow. For example, oral hop administration has been shown to improve ovariectomy-induced overweight, osteoporosis, and hot flashes [55]. In one study, ovariectomy in rats markedly increased TG, TC, LDL, and the LDL/HDL ratio, which are major risk factors for obesity and cardiovascular diseases; treatment of these rats with hops balanced such lipid abnormalities [55]. Also, mice fed with a

high-fat diet long-term were treated with hops, and its effects on obesity and oral glucose tolerance were determined. Results showed a decrease in obesity, adipose tissue weight, and adipocyte hyperplasia [56].

In addition, after 19 weeks of hop extract treatment, there was an inhibition in PPARγ protein expression in the adipose tissues and an improvement in glucose intolerance caused by the consumption of a high-fat diet in mice [56]. Hop extract also exerts other biological properties, like antimicrobial and antibiofilm properties. Multidrug resistance has led to new findings on the prevention of microbial proliferation and infection. For instance, one study analyzed hop extracts' effect on staphylococci strains and C. acnes [57]. Its ability to adhere to the cell wall and membrane and bind to PBP inside the wall obstructed biofilm formation [57]. Furthermore, $CO_2$ hop extract with 50% humulone and lupulone was used against P. acnes to assess the antioxidant, anti-inflammatory, and antibacterial potential. The susceptibility of P. acnes and S. aureus to hop extract was analyzed using broth microdilution technique.

The results showed that hop extracts had an antioxidative effect at the half maximal inhibitory concentration ($IC_{50}$) of 29.43 µg/mL and an anti-inflammatory effect by decreasing IL-6 expression ($IC_{50}$: 0.8 µg/mL) [58]. Another article discovered that hop extracts might be an alternative treatment for acne-prone skin because of their anti-oxidative and anti-inflammatory effects [59]. In addition, hop extracts are weak scavengers of ROS, although they might function indirectly as antioxidants by enabling GSH synthesis and other antioxidative enzymes. For example, in XN, the Keap1-Nrf2 activator (Michael acceptor) induces detoxification enzymes such as NQO1 and GST, as well as alkylates IKK and NF-κB, resulting in anti-inflammatory activity. XN may have pro-oxidant effects at higher concentrations, resulting in the induction of apoptosis

and the inhibition of cell proliferation. Finally, XN activates AMPK, which indirectly prevents obesity and glycemic effects [60].

## 1.10 Anticancer Activity of Xanthohumol

Cancer's overwhelming global burden brought more challenges; however, it provided researchers with opportunities to develop anti-cancer therapies. Phytochemical, a synergistic compound, has promising anti-cancer effects as it is used to supplement chemo- and immune-therapeutic procedures [45] [50]. The synergistic anti-cancer effects of phytochemicals derived from flavonoids such as quercetin, apigenin, kaempferol, hesperidin, and emodin have been investigated. XN has demonstrated key anti-proliferative activities both at the cellular and molecular level i*n vitro* and *in vivo* using animal models for xenograft tumor studies [61]. XN has therapeutic potential to contribute to the prevention and treatment of several diseases [51]. For example, in cancers, XN prevents cell proliferation and metastasis in melanoma and hepatocellular carcinoma. It also inhibits the proliferation, differentiation, and overproduction of cardiac fibroblasts activated by TGF-β1 through modulation of the phosphatase and tensin homolog (PTEN)/Akt/mammalian target of rapamycin (mTOR) pathway. It inhibits the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway and FOS-related antigen 1 (Fra1) leading to the inhibition of activator protein-1 (AP-1) transcription and lowering cyclin D1 in non-small cell lung cancer (NSCLC) cells.

XN blocks cyclin B1 and Ras/methyl ethyl ketone (MEK)/ERK signaling pathways, and enables G2/M cell cycle arrest by inducing caspase-3 and 9 and upregulating B-cell lymphoma protein 2 (Bcl-2) linked to X protein (Bax)/Bcl-2 ratio in CRC cells; uterine grafts with endometrial lesions in mice showed reduced tumor size when treated with XN [51]. XN possesses enormous therapeutic benefits against different tumors and exhibits potential as a multi-targeted anti-cancer

agent with low adverse effects. Moreover, XN modulates multiple signaling pathways and proteins, such as Akt, AMPK, ERK, IGFBP2, NF-κB, STAT3, Notch1, caspases, MMPs, Bcl-2, cyclin D1, oxidative stress markers, tumor-suppressor proteins, and miRNAs. Thus, XN functions to inhibit the growth and proliferation of cancer cells [51]. To add, other articles showed that XN significantly improved the activity of caspase 3, enabling the expression of cleaved-caspase 3 and -PARP. Results from flow cytometry and immunofluorescence staining indicated a dose-dependent apoptosis induction in HCT116 cells by XN [51]. Furthermore, XN's chemo preventive or therapeutic potential can be seen in its ability to induce apoptosis through the downregulation of Bcl-2 and activation of the caspase cascade [62].

The investigation of the anti-cancer effect of XN against CRC cells revealed the induction of G2/M cell cycle arrest and apoptosis through the activation of caspases, Bax/Bcl-2 ratio, and PARP cleavage [51]. To add, when HT-29 cells were treated with XN, cell cycle arrest was induced, which resulted in the suppression of cyclin B1 and the Ras/MEK/ERK pathway [51]. Also, hexokinase-2 (Hk-2) is often overexpressed in colon cancer, and suppression of HK-2 may inhibit cancer survival and proliferation. Results from one study showed that in vivo treatment of CRC xenograft and in vitro treatment of FHC, CCD841 CoN, HT29, SW480, LOVO, HCT116, and SW620 cells with XN prevented glycolysis and cell proliferation by reducing Hk-2 [51]. Other mechanisms *in vitro* cancer cell mechanisms were affected after treatment with XN. For instance, XN induced apoptosis by releasing cytochrome c and suppressing the EGFR/Akt signaling pathway; XN decreased cell viability, induced ROS, and suppressed the OXPHOS complexes and Sirtuin 1 in SW620 cell lines. XN induced cytotoxicity by inhibiting DNA topoisomerase I and genes involved in drug effluxes such as ABCB1 (MDR1), ABCC1 (MRP1), ABCC2 (MRP2), and ABCC3 (MRP3) in HCT-15 cells [51].

### 1.11 Anticancer Activity of Tetrahydroxanthohumol

To our knowledge, only one paper published data on TXN effects against CRC. Based on their results, XN and its derivatives, like TXN, prevent cancer cell proliferation through a molecular mechanism that involves caspase-mediated apoptosis. Also, TXN was the only XN derivative that caused the induction of cell cycle arrest in the $G_1$ phase [52]. Therefore, the purpose of our study is to elucidate the detailed molecular mechanisms through which XN and TXN prevent and/or mitigate CRC.

## Chapter 2: Material and Methods

### 2.1 Cell culture and reagents

Human colorectal adenocarcinoma cells (HCT116, SW480, SW620) were purchased from American Type Culture Collection (ATCC; Manassas, VA) and grown in Dulbecco's modified Eagle medium (DMEM/F12) supplemented with 10% FBS (Fetal Bovine Serum). The cells were maintained in a humidified atmosphere (37 ºC and 5% $CO_2$). XN(Xanthohumol), IXN (Isoxanthohumol), and 8-PN (8-prenylnaringenin) were purchased from the Cayman chemical company (Ann Arbor, Michigan, USA). TXN (tretrahydroxanthohumol)was a gift from Hopsteiner Inc. (New York, USA). Propidium iodide (PI)/ribonuclease A (RNAse A) staining buffer was purchased from BD Biosciences (San Jose, CA, USA). TACS™ annexin V-FITC apoptosis detection kit was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Protease and phosphatase inhibitor cocktail was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Primary antibodies for poly (ADP-ribose) polymerase (PARP - # 9542), inositol-requiring enzyme 1α (IRE1α - # 3294), β-actin (# 5125), and anti-rabbit immunoglobulin G

(IgG) (# 7074) was purchased from Cell Signaling Technology, Inc (Danvers, MA, USA). ROS-Glo™ hydrogen peroxide ($H_2O_2$) assay kit was purchased from Promega Corporation (Madison, WI, USA). All cell culture and transfection reagents and other chemicals were purchased from Fisher Scientific International Inc. (Pittsburgh, PA, USA) and Santa Cruz (10410 Finnell Street, Dallas, TX, United States, 75220) unless otherwise specified.

## 2.2. Preparation of Hop compounds

XN, TXN, IXN, and 8-PN (Figure 1) were dissolved in dimethyl sulfoxide (DMSO) and stored in a -80-degree freezer until use. DMSO is used as a vehicle in the control groups and the final concentrations of DMSO did not exceed 0.1% (v/v).

## 2.3 Cell proliferation Assays

Cell proliferation was determined by the MTT [(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] (Sigma, St. Lous, MO) assay. Human colorectal adenocarcinoma cells (HCT116, SW480, and SW620) were plated in a 96-well plate ($8x10^3$ per well) with three replicates and incubated for 24-h. The following day, the cells were treated with different concentrations (0 µM, 3 µM, 6 µM,12 µM, 25 µM, and 50 µM) of XN, TXN, IXN, and 8-PN in media supplemented with 1% Fetal Bovine Serum (FBS) for 24-h. The next day, cells were incubated with 100 µL of MTT solution for 2 h in humidified conditions (37ºC and 5% $CO_2$). After 3 h, the cells' optic density was recorded at 490 nm using an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments Inc., Winooski, VT).

## 2.4 Apoptosis assay

HCT116 cells were seeded in 6 well plates ($5x10^4$ per well) and incubated for 24 h. The next day, the cells were treated with XN and TXN of various concentrations (0 µM, 12 µM, and 18 µM) for 24 h. Both attached and floating cells were harvested by trypsinization and washing twice with ice-cold phosphate-buffered saline (PBS). Cells were resuspended in Annexin V-FITC and PI staining buffer according to the manufacturer's protocol. After 15 min incubation, resuspended cells were diluted according to the manufacturer's protocol. FACS analysis was used to count apoptotic cells using a BD LSRFortessa™ system (BD Biosciences, San Jose, CA, USA).

## 2.5 Cell cycle assay

HCT116 cells were seeded in 6 well plates ($5x10^4$ per well) and incubated for 24-h. The cells were treated with XN and TXN of various concentrations (0 µM, 12 µM, and 18 µM) for 24-h. Both attached and floating cells were harvested by trypsinization and fixed with 70% ethanol in phosphate-buffered saline (PBS) overnight at -80 °C. The cells were washed serially with 50% ethanol/PBS, 20% ethanol/PBS, and PBS and then stained with PI/RNase staining buffer. Cell cycle distribution was analyzed using a BD LSRFortessa™ system (BD Biosciences, San Jose, CA, USA).

## 2.6 JC-1 staining assay

HCT116 cells were seeded in 6 well plates ($5x10^4$ per well) and incubated for 24 h. The cells were treated with XN and TXN of various concentrations (0 µM, 12 µM, and 18 µM) for 24 h.

Mitochondrial membrane potential was assessed by staining the cells with 1 µM of JC-1 for 1 h at 37 °C. The cells were then harvested by trypsinization, resuspended in PBS, and quantified using FACS analysis through the BD LSRFortessa™ system (BD Biosciences, San Jose, CA, USA).

## 2.7 ROS Assay

HCT116 cells were plated in 96-well plates as described in the cell viability assay. The cells were treated with XN and TXN of various concentrations (0 µM, 12 µM, and 18 µM) for 18 h. A mixture of $H_2O_2$ substrate dilution buffer and $H_2O_2$ substrate were added to the plate and then incubated at 37 C for 6 h and the mixture including Luciferin detection solution, D cysteine, signal enhancer solution was added to each well and then incubated at room temperature for 20 min. Analysis was done as described in the cell viability assay.

## 2.8 SDS-PAGE and Western Blot

The cells were washed twice with ice-cold 1x phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) buffer (Boston Bioproduct Inc, Ashland, MA) containing protease and phosphatase inhibitor cocktail (Sigma Aldrich). Protein concentration was determined using bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Equal amounts of proteins (about 30 µg of cell lysate) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane (Osmonics, Minnetonka, MN). The membranes were blocked with 5% non-fat milk diluted Tris-buffered saline tween-20 (TBST) for 1 hour at room temperature to block out non-specific antibodies. Then incubated with primary antibodies at 4ºC overnight. Three washing was done using TBST followed by probing in horse radish peroxidase (HRP)-conjugated immunoglobulin

G (IgG) for 1 hour at room temperature. Three washing were done, and the target proteins were activated using Chemiluminescence (ECL) detection solution and hydrogen peroxide(H2O2). Then visualization and photographs were done by Chemidoc MP Imaging system (Bio-Rad, Hercules, CA, USA).

### 2.9 Statistical analysis

Data are represented as the mean ± standard deviation (SD) from triplicates. Statistical variations were determined using the two-tailed Student's *t-test*. The significance of data was recorded using $*P < .05$; $**P < .01$; $***P < .001$; $****P < .0001$ as depicted in the result section. Also, one-way analysis of variance (ANOVA), proceeded by Duncun posthoc tests were used for intergroup comparisons (SPSS, version 21; SPSS Inc., Chicago, IL, USA).

## CHAPTER 3: Results

### 3.1 Xanthohumol and its analogue

The four hop compounds are prenylated, phenolic compounds that possess diverse biological functions. Xanthohumol (XN) is the most abundant and main form which can be converted into diverse analogues. Isoxanthohumol (IXN) has an additional ring compared with XN and is obtained when heating XN during the brewing process or exposing XN to low pH environment such as the stomach after consumption. IXN can be converted to either 8-prenylnaringrenin (8-PN) or tetrahydroxanthohumol (TXN), which is a one-way process. 8-PN is most similar to IXN with three rings while TXN is more similar to XN with two rings. The main difference between XN and TXN is that TXN has two fewer double bonds than XN (Fig. 1).

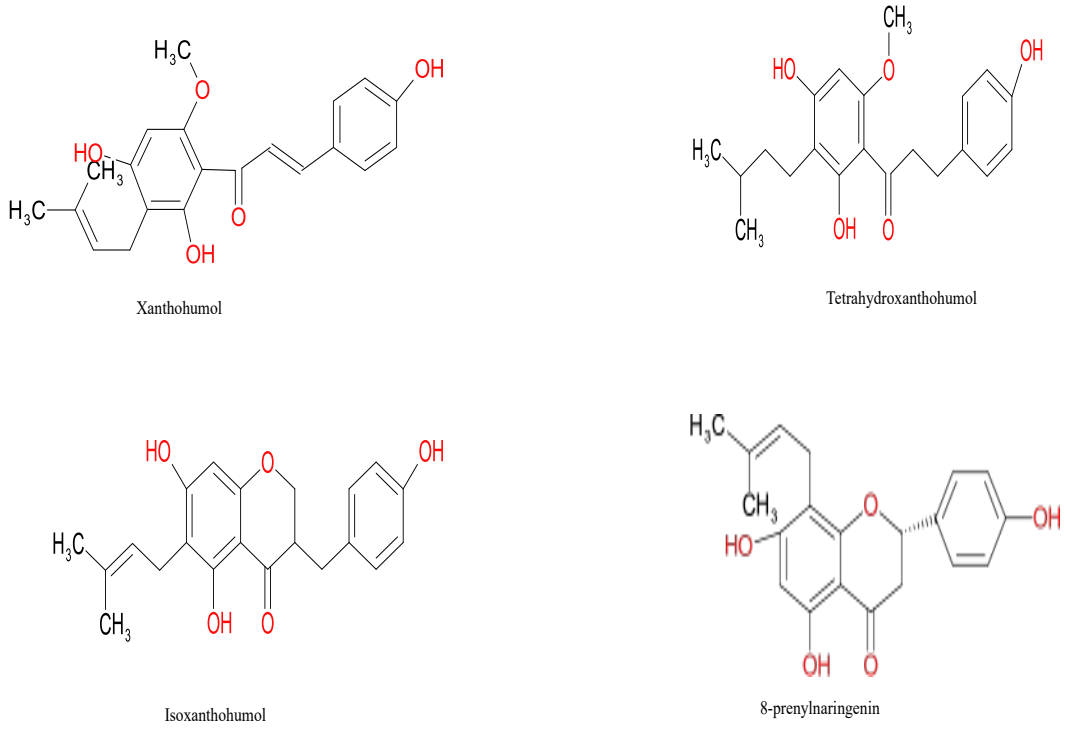


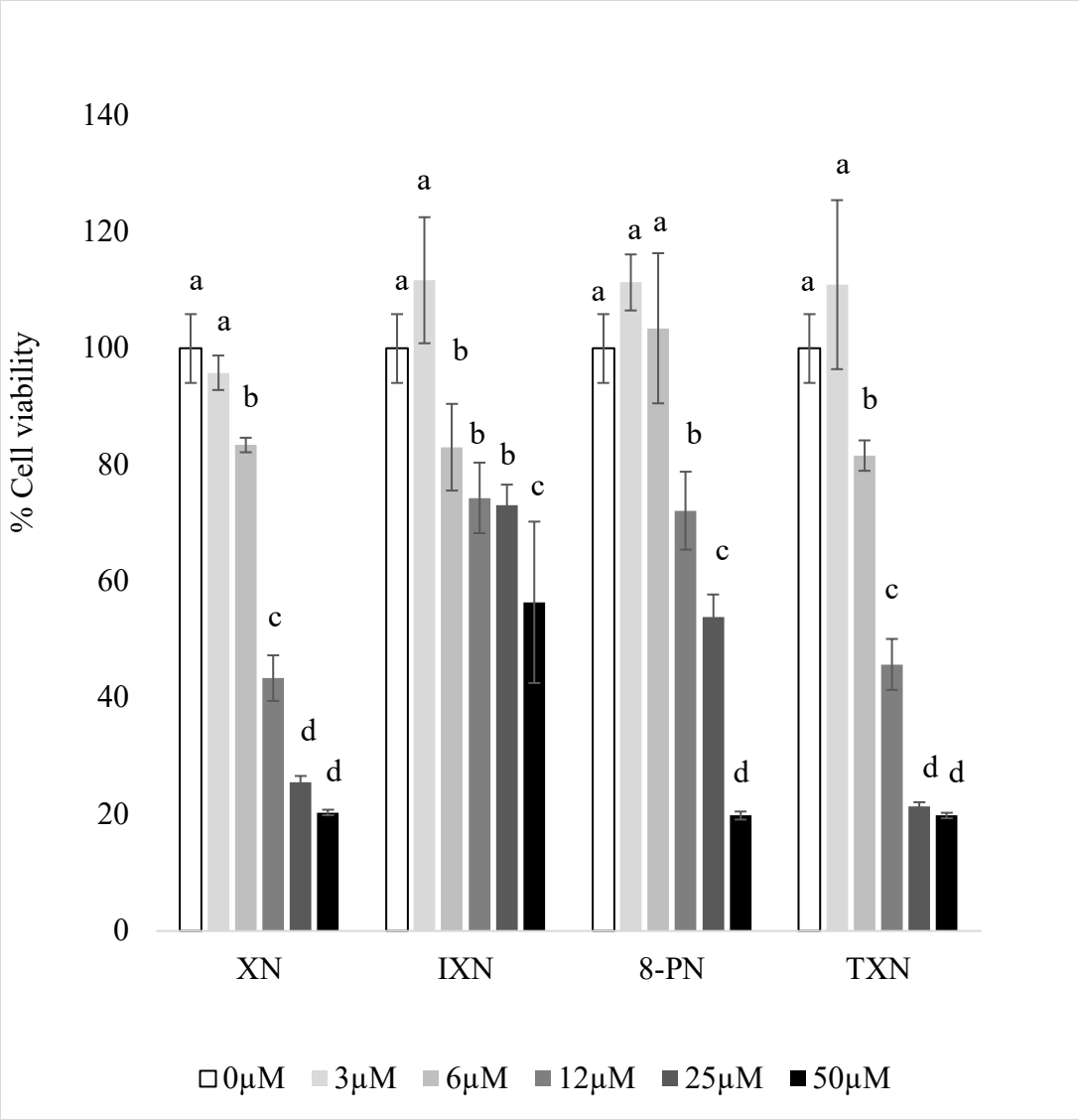

Figure 1: Chemical structure of four hop compounds. XN, the parent chalcone, is commonly isomerized to IXN during the brewing process of beer or in the stomach by acid or any form of heat. IXN differs from XN because of its extra ring and loss of double bond. IXN can then be converted to 8-PN. 8-PN differs from the parent form by an extra ring and loss of carbon chain. 8-PN being a phytoestrogen cannot be converted to TXN and vice versa. However, XN can be converted to TXN through IXN. TXN has fewer double bonds than XN, but it is most similar to XN.

## 3.2 Viability of Human CRC cell lines treated with four different type of hop compounds.

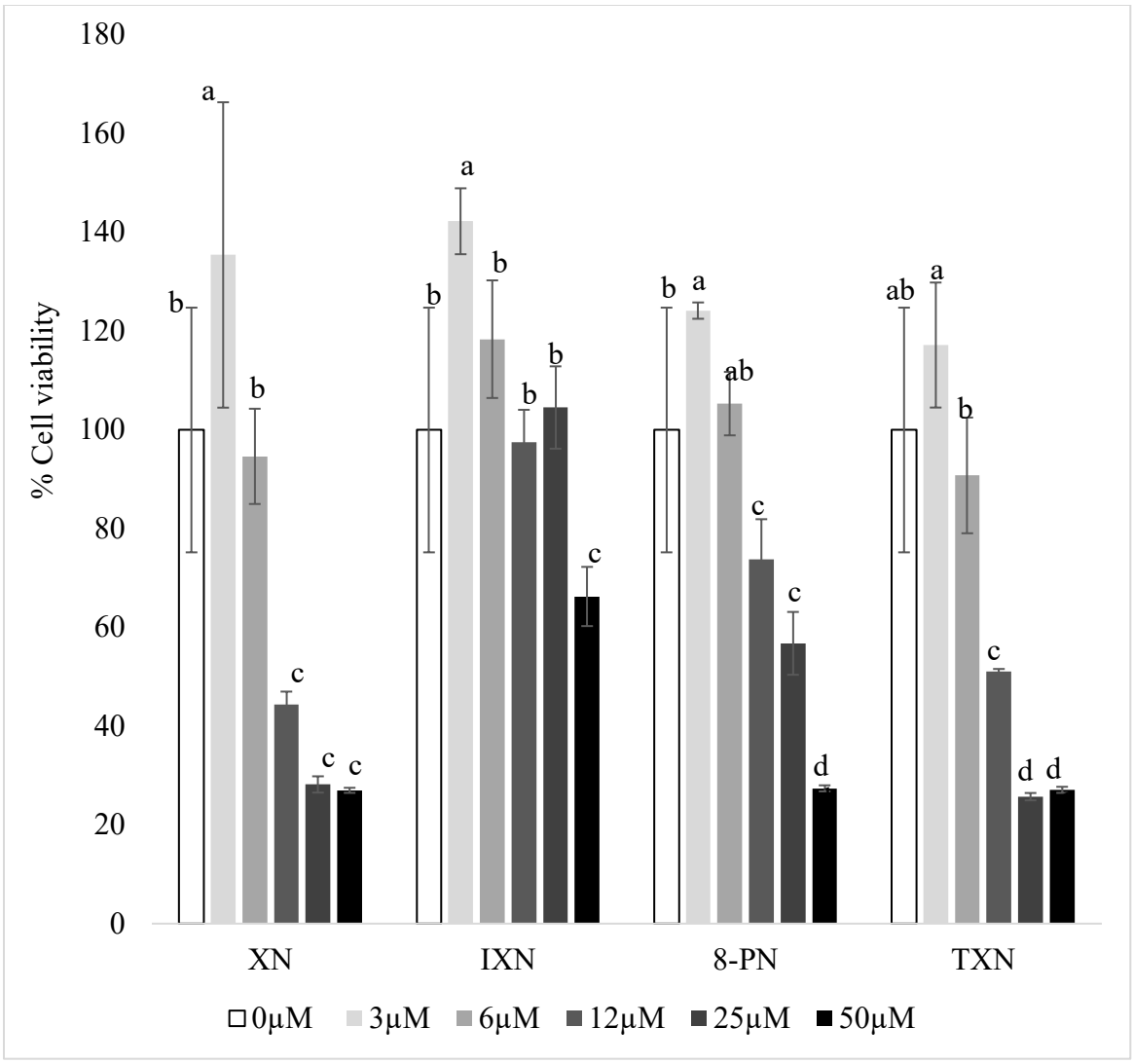
The effect of XN, IXN, 8-PN, and TXN at different concentrations (0, 3, 6, 12, 25, and 50 µM) on cell proliferation of SW480, SW620, and HCT116 CRC cell lines was determined using an MTT assay. As indicated in Fig. 2, the viability of SW480 cells was 100, 95.8, 83.4,

43.4, 25.5, and 20.3% for XN; 100, 111.7, 83.0, 74.3, 73.1, and 56.4% for IXN; 100, 111.3, 103.4, 72.1, 53.8, and 19.8% for 8-PN; 100, 110.9, 81.6, 45.7, 21.4, and 19.8% for TXN after treatment of 0, 3, 6, 12, 25, and 50 μM each compound for 24 hours, respectively. The viability of SW620 cells was 100, 135.4, 94.6, 44.4, 28.1, and 27.0% for XN; 100, 142.2, 118.3, 97.5, 104.5, and 66.2% for IXN; 100, 124.1, 105.3, 73.7, 56.7, and 27.3% for 8-PN; 100, 117.2, 90.8, 51.1, 25.7, and 27.1% for TXN after treatment of 0, 3, 6, 12, 25, and 50 μM of each compound for 24 hours, respectively (Fig. 2). The viability of HCT116 cells was 100, 108.7, 100.3, 36.3, 16.8, and 15.4% for XN; 100, 115.9, 116.0, 112.1, 79.8, and 60.1 for IXN; 100, 115.5, 105.6, 99.1, 57.1, and 15.4% for 8-PN; 100, 126.8, 115.6, 67.5, 15.4, and 15.3% for TXN (Fig. 2). As depicted in Fig. 2, XN, 8-PN, and TXN showed a significant decrease at a dose of 12 μM, 25 μM, and 50 μM for three cell lines (HCT116, SW620, and SW480). In SW480 cells, we observed a significant decrease ($p<0.01$) from XN-treated cells (6, 12, 25, and 50 μM), IXN-treated cells (12, 25, and 50 μM), 8-PN-treated cells (12, 25, and 50 μM) and TXN-treated cells (6, 12, 25 and 50 μM) (Fig. 2). In addition, XN-treated SW620 cells at dose of 25 and 50 μM, 8-PN-treated SW620 cells at dose of 50 μM, and TXN-treated SW620 cells at dose of 25 and 50 μM showed a significant difference (Fig. 2). Finally, a significant difference was observed in HCT116 treated cells at XN (12, 25 and 50 μM), IXN (50 μM), 8-PN (25 and 50 μM), and TXN (25 and 50 μM)-treated cells (Fig. 2).

**SW480**


% Cell viability
140
120
100
80
60
40
20
0
a a b c d d
a a b b b c
a a a b c d
a a b c d d
XN
IXN
8-PN
TXN
0μM
3μM
6μM
12μM
25μM
50μM

**SW620**


% Cell viability
180
160
140
120
100
80
60
40
20
0
XN
IXN
8-PN
TXN
0μM
3μM
6μM
12μM
25μM
50μM

**HCT116**

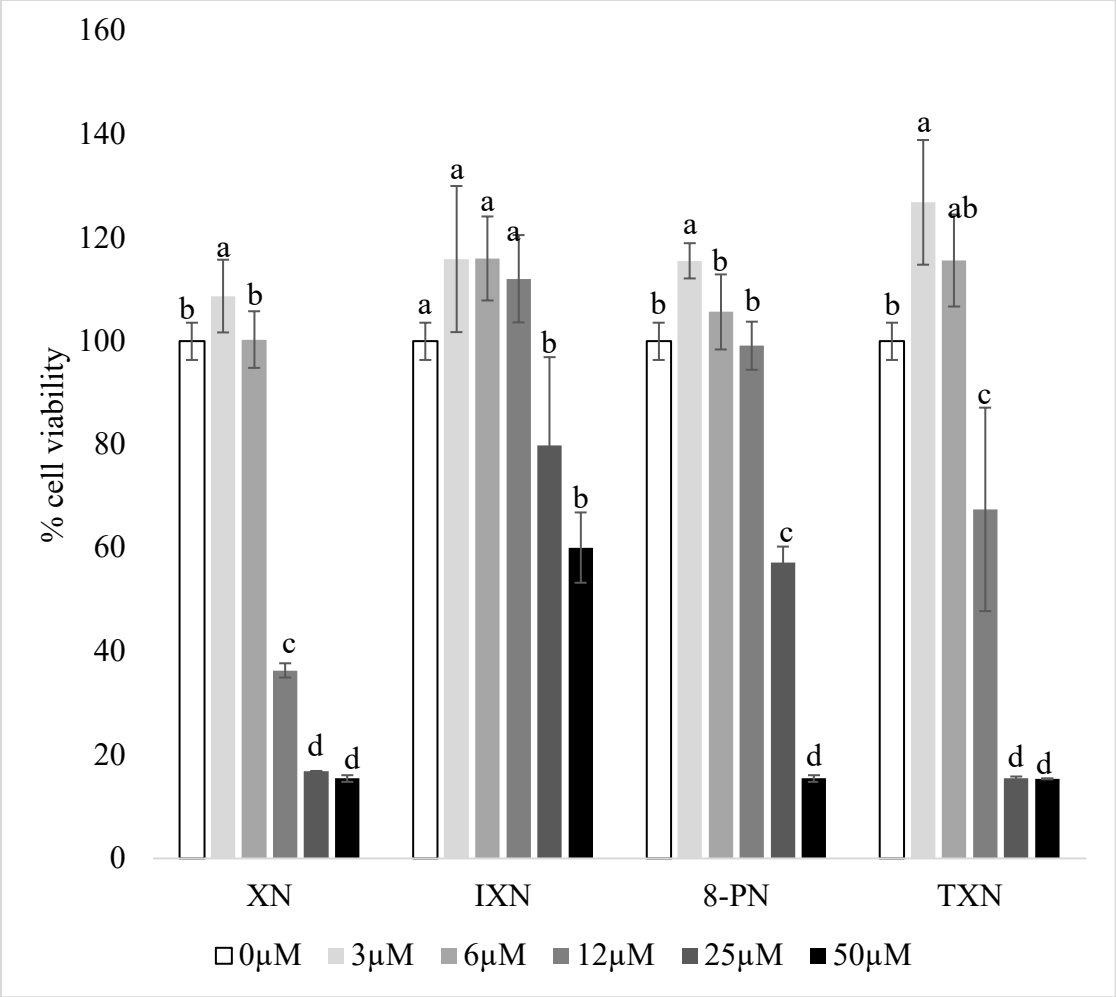
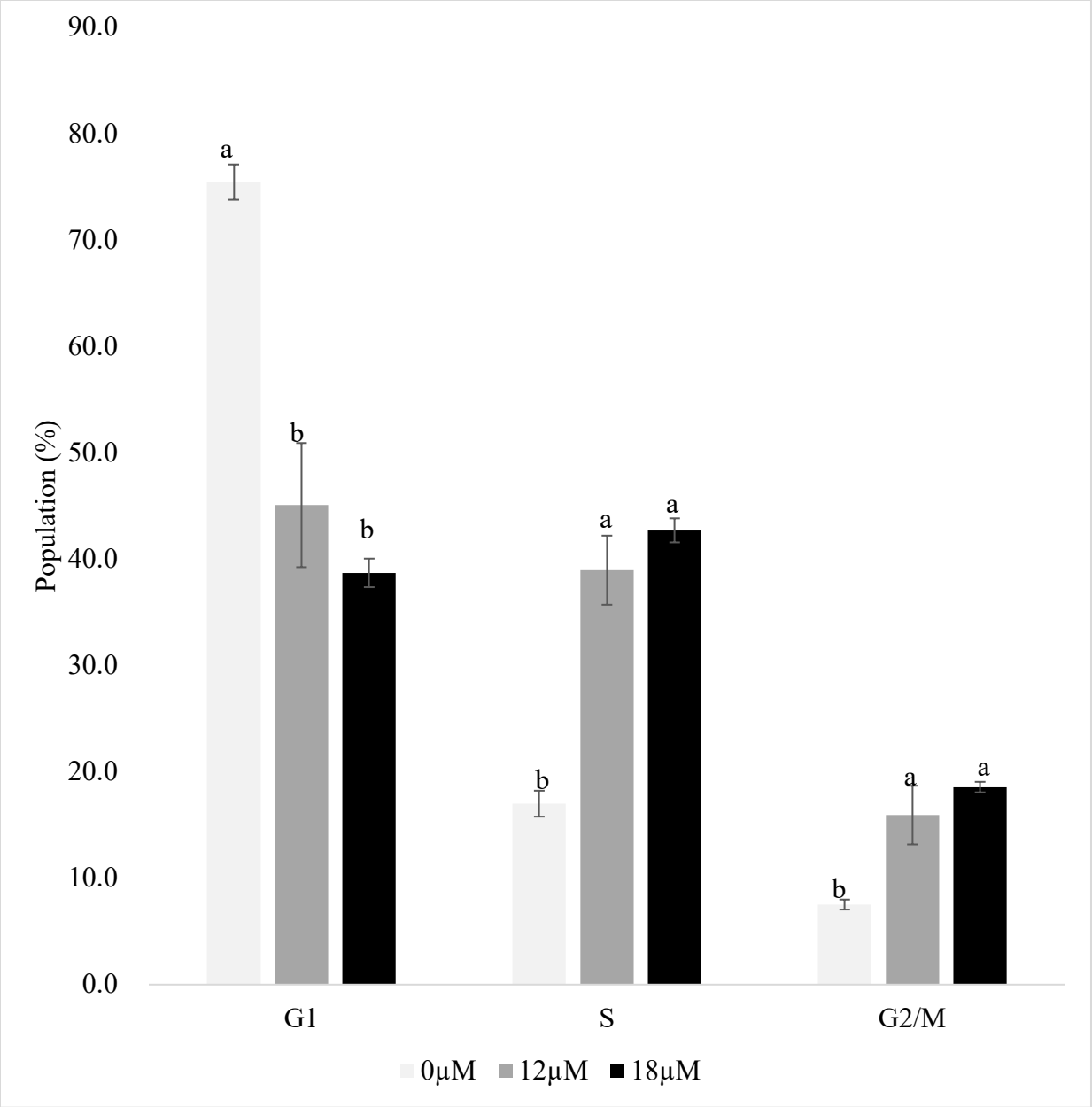



Figure 2: Four hop compounds inhibit viability of three CRC cell lines SW480, SW620 , and HCT116 Cells were plated onto a 96-well plate and treated with different concentrations of XN, IXN,8-PN, and TXN (0, 3, 6, 12, 25, and 50 µM) supplemented with 1% FBS for 24 hours at 37ºC under 5% $CO_2$. Cell viability was assessed by the MTT [(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide]. Values are means ± SD (n = 3). Different letters indicate significant difference at $P < 0.05$.

### 3.3 Distribution of cell cycle phases in HCT116 CRC cell line treated with XN and TXN

Since cell cycle arrest is one of factors to affect cell proliferation, we performed flow cytometry to analyze the distributions of cell cycle phases using HCT116 cells treated with 12 μM and 18 μM of the XN and TXN. As shown in Fig. 3, proportions of each cell cycle phase in HCT116 cells treated with 0, 12, and 18 μM of XN were as follows: G1 phase was 75.5, 45.1, and 38.7%; S phase was 17.0, 39.0, and 42.7%; G2/M phase was 7.5, 15.9, and 18.6%. Proportions of each phase in the cells treated with 0, 12 and 18 μM of TXN were as follows: G1 phase was 75.5, 54.0, and 44.8%; S phase was 17.0, 32.0 and 40.8%; G2/M phase was 7.5, 14.0, and 14.3% (Fig. 3). Statistical analysis indicated the significant S phase arrest and G2/M phase arrest in the cells treated with 12 μM and 18 μM of XN and TXN. However, no significant difference was observed between the two treatments. Treatment of 12 μM and 18 μM of XN showed a 2.2 and 2.5-fold increase of S phase, and treatment of TXN led to an S phase induction of 1.8 and 2.3-fold, respectively. XN and TXN induced the G2/M phase of the cell cycle with a 2.4 and 1.9-fold increase at concentrations of 18 μM of XN and TXN-treated HCT116 cells, respectively. All taken together, we found that decreased viability of CRC cells treated with XN and TXN might be associated with cell cycle arrest at S and G2/M phase.

**XN**


90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Population (%)
a
b
b
b
a
a
b
a
a
G1
S
G2/M
0μM
12μM
18μM

**TXN**

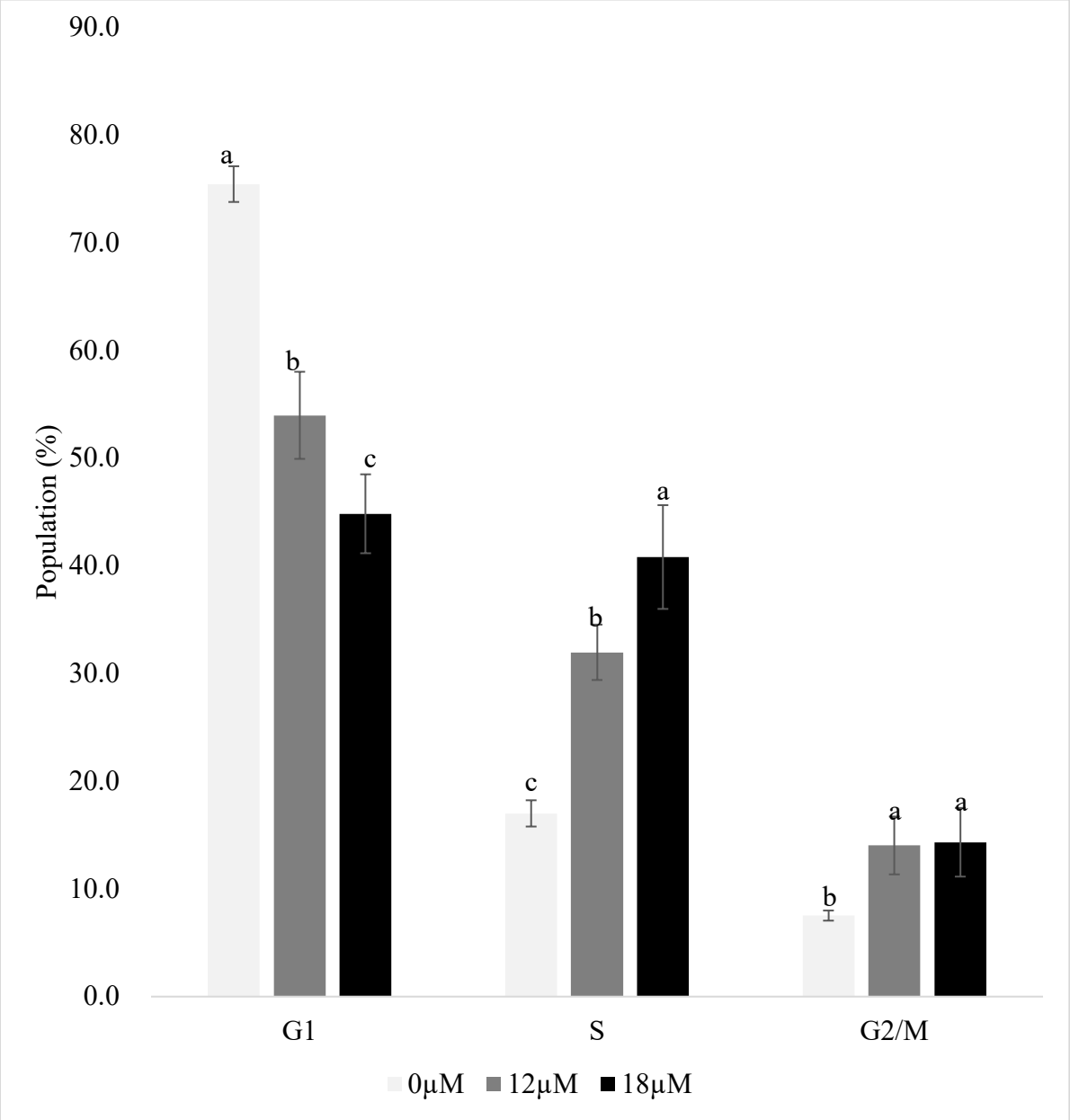
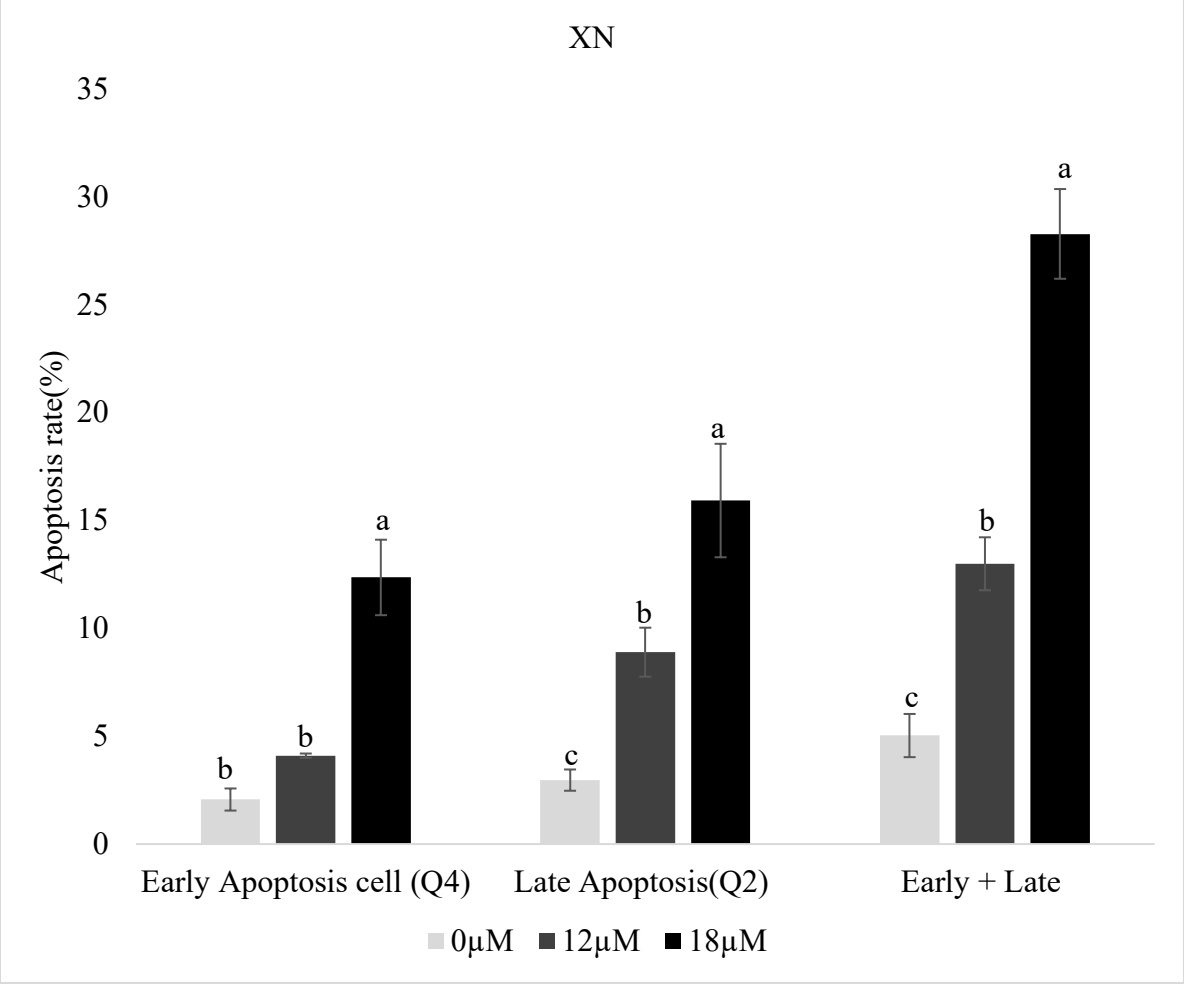



Figure 3: Cell cycle distribution based on population percentage in HCT116 cells treated with XN and TXN (0 µM,12 µM and 18 µM). XN and TXN significantly increase the S phase and G2/M phase in the HCT116 CRC cell line. Values are means ± SD (n = 3). Different letters indicate significant difference at $P < 0.05$.

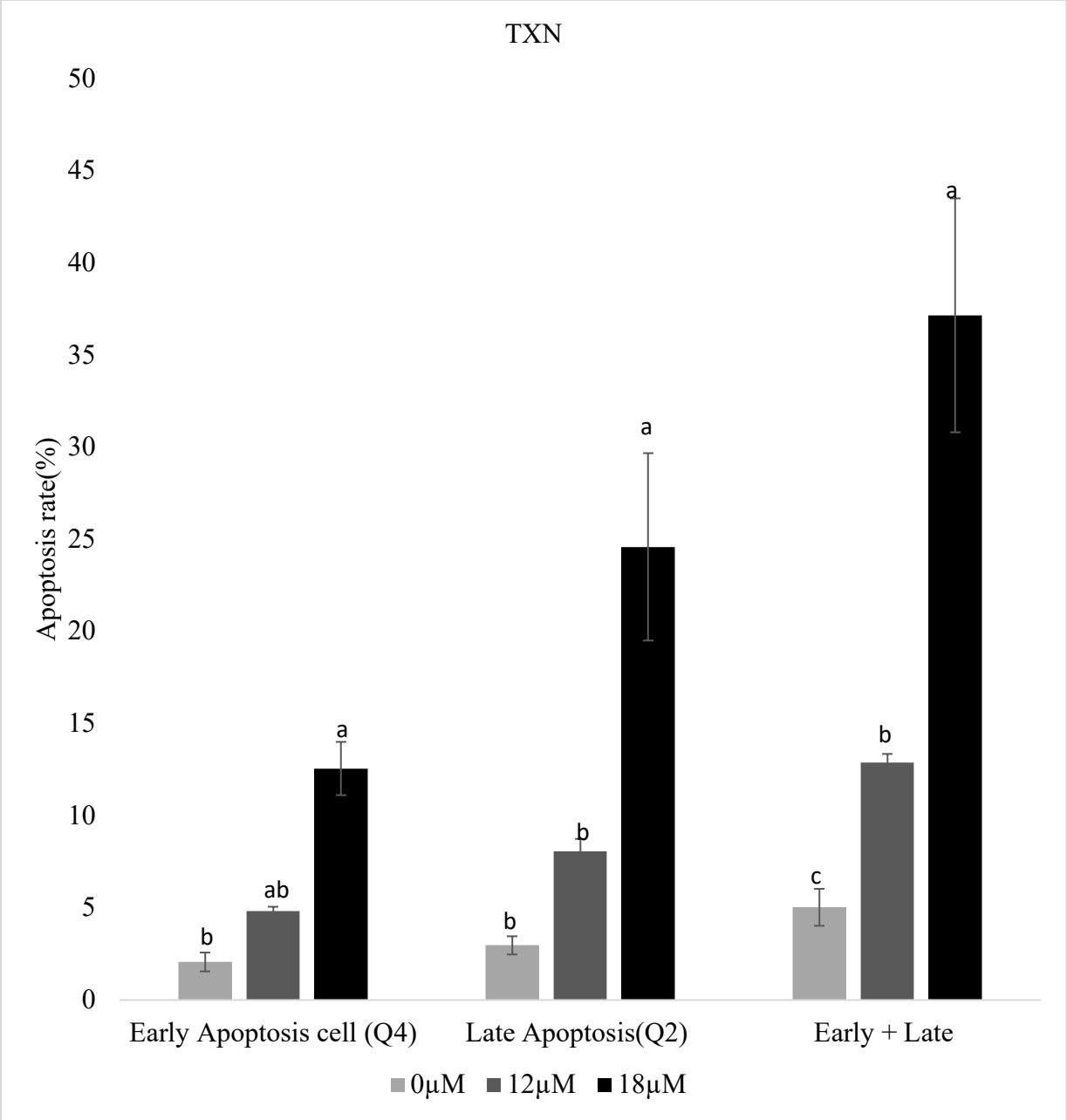
### *3.4 Analysis of apoptosis in HCT116 CRC cell line treated with XN and TXN*

We performed flow cytometry to examine if apoptosis is associated with reduced cell viability. As shown in Fig. 4, there was a 1.9 and 5.9- fold increase of early apoptosis in the cells treated with 12 and 18 µM of XN and a 2.3 and 6.0-fold increase in the cells treated with 12 and 18 µM of TXN compared to the control. Moreover, there was 3.0- and 5.3-fold increase of late apoptosis in the cells treated with 12 and 18 µM of XN and a 2.7 and 8.2-fold increase in the cells treated with 12 and 18 µM of TXN as compared to the control.

**XN**




**TXN**


TXN
Apoptosis rate(%)
50
45
40
35
30
25
20
15
10
5
0
b
ab
a
b
b
a
c
b
a
Early Apoptosis cell (Q4)
Late Apoptosis(Q2)
Early + Late
0μM
12μM
18μM

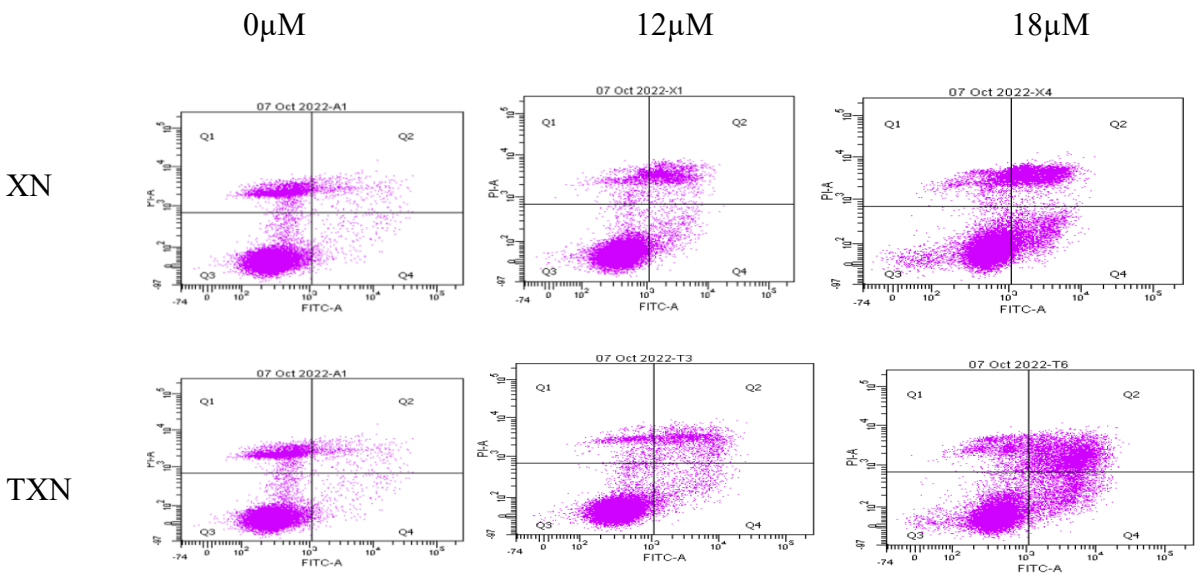



Figure 4: Induction of early and late apoptosis as depicted by an increase in Q2 (Early apoptosis cell) and Q4 (Late apoptosis cell) phases. Induction of early and late apoptosis is depicted by an increase in Q2 (Early apoptosis cell) and Q4 (Late apoptosis cell) phases. Both graph and snapshot showed an increase of apoptosis in HCT116 cells treated with with XN and TXN. Values are means ± SD (n = 3). Different letters indicate significant difference at $P < 0.05$.

## 3.5 Analysis of apoptosis marker protein in HCT116 CRC cell line treated with XN and TXN

PARP cleavage is a molecular marker of cell apoptosis. A western blot was performed to see if treatment of XN and TXN affect cleavage of PARP. As observed in Fig. 5, there was a significant induction of cleaved PARP in the HCT116 cells treated with 12 µM and 18 µM of XN and TXN as compared to the control. A 5.0 and 6.2-fold increase in cleaved PARP was seen at concentrations of 12 and 18 µM XN; a 3.2 and 4.2-fold increase in cleaved PARP was seen at 12 and 18 µM of TXN compared to the control, with no difference between 12 µM and 18 µM in both XN and TXN treated groups.

**XN and TXN**

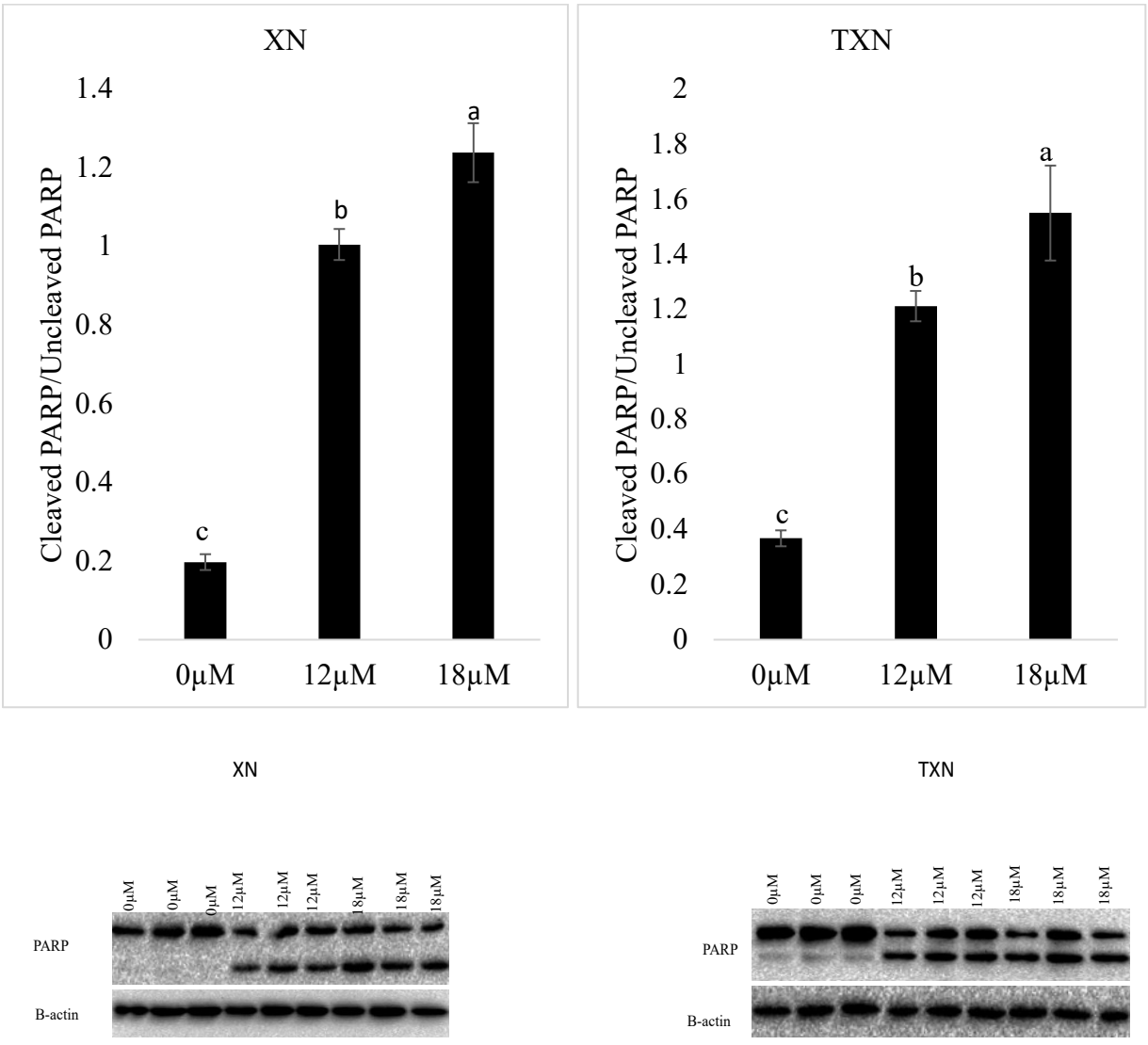
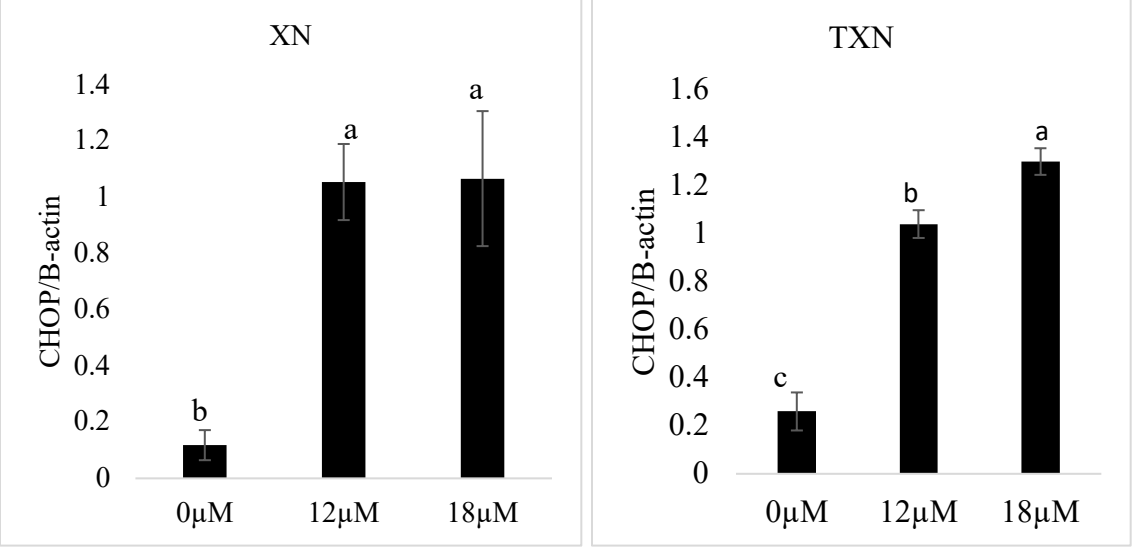



Figure 5: Expression of an apoptotic marker, cleaved PARP in HCT116 cells treated with different concentrations (0, 12, and 18 μM) of XN and TXN. Values are means ± SD (n = 3). Different letters indicate significant difference at $P < 0.05$.

## 3.6 Expression of ER stress marker proteins in HCT116 CRC cell line treated with XN and TXN

ER, stress-mediated apoptotic cell death was analyzed by the presence of ER stress markers in Western blot imaging. As indicated by Fig. 6, CHOP expression showed 8.9 and 9.0-fold increase in 12 and 18 μM XN-treated cells and 4.0- and 5.0-fold increase in 12 and 18 μM TXN-treated cells compared to the control. Another ER stress marker, ATF-4 was highly expressed in XN and TXN-treated HCT116 cells with a 2.2 and 1.4- fold increase at a concentration of 12 μM of XN and TXN; And 4.3 and 1.5-fold increase in expression for 18 μM of XN and TXN compared to the control. In addition, IRE1α had a 1.8 and 2.4- fold increase at concentrations of 12 μM of XN and TXN; a 2.3 and 2.5-fold increase in expression for 18 μM of XN and TXN as compared to the control. Finally, PERK showed no significant expression in HCT116 cells treated with TXN and XN at concentrations of 12 μM and 18 μM.



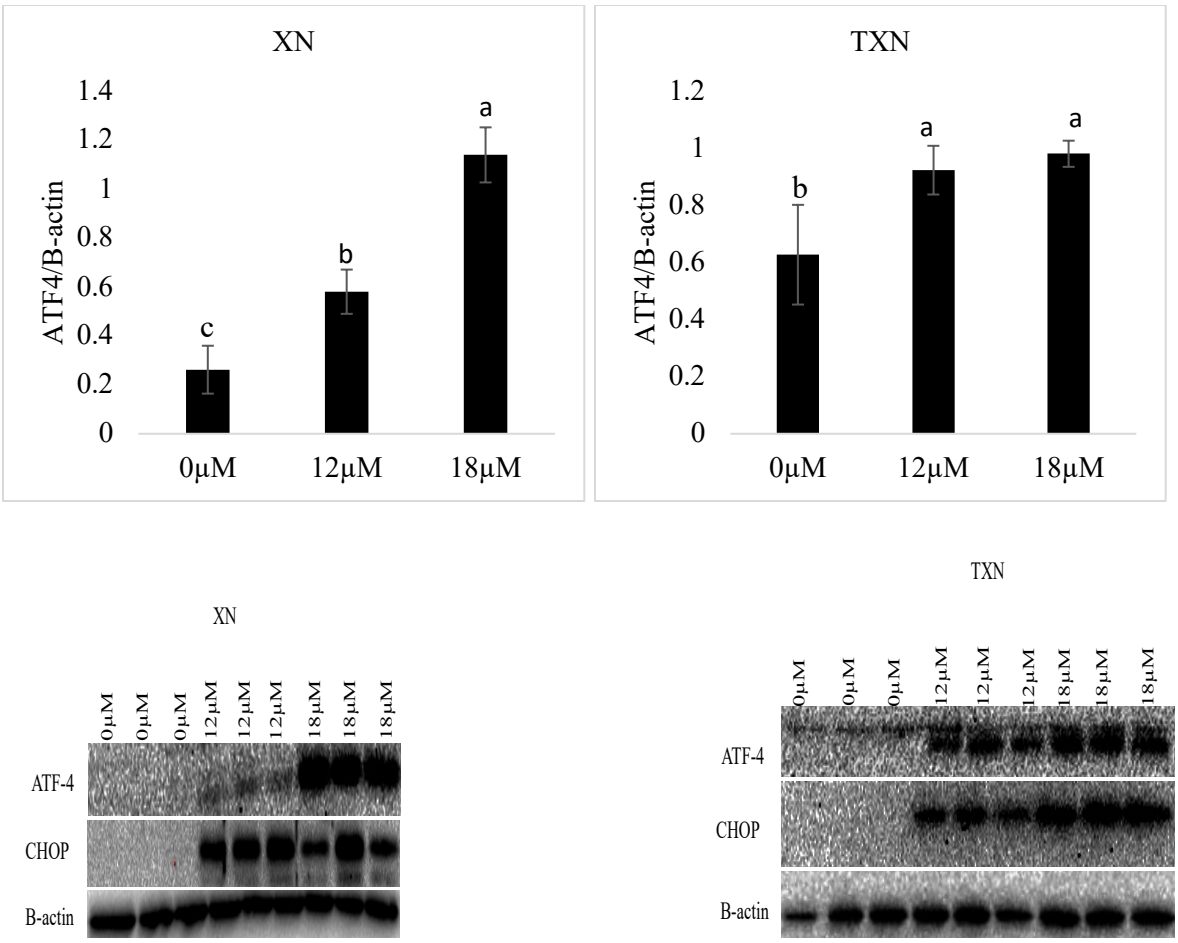
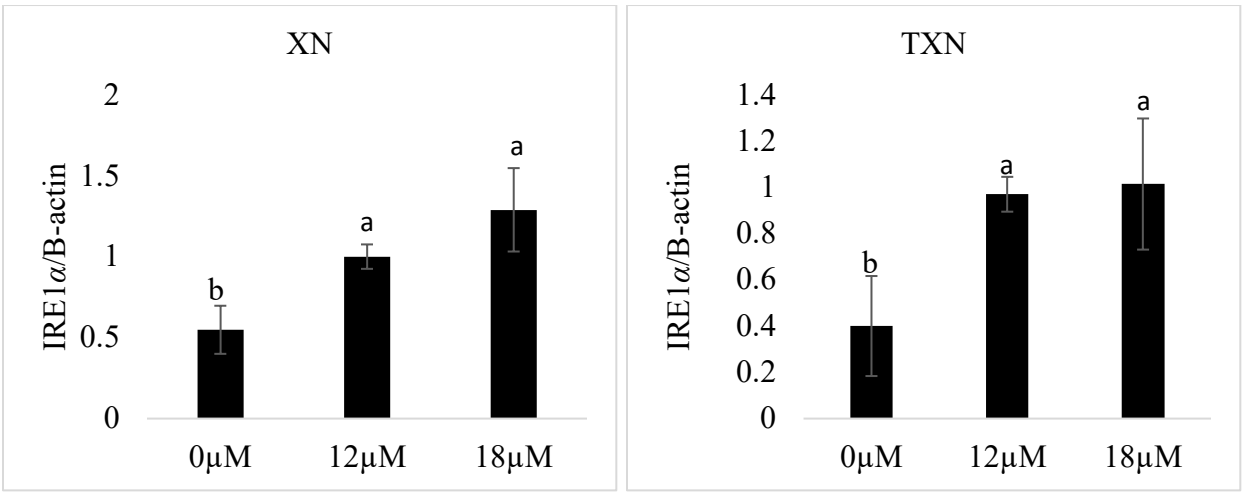



Figure 6 : Expression of ER stress marker proteins, CHOP and ATF-4, in HCT116 cells treated with different concentrations (0, 12, and 18 μM) of XN and TXN. Values are means ± SD (n = 3). Different letters indicate significant difference at $P < 0.05$.



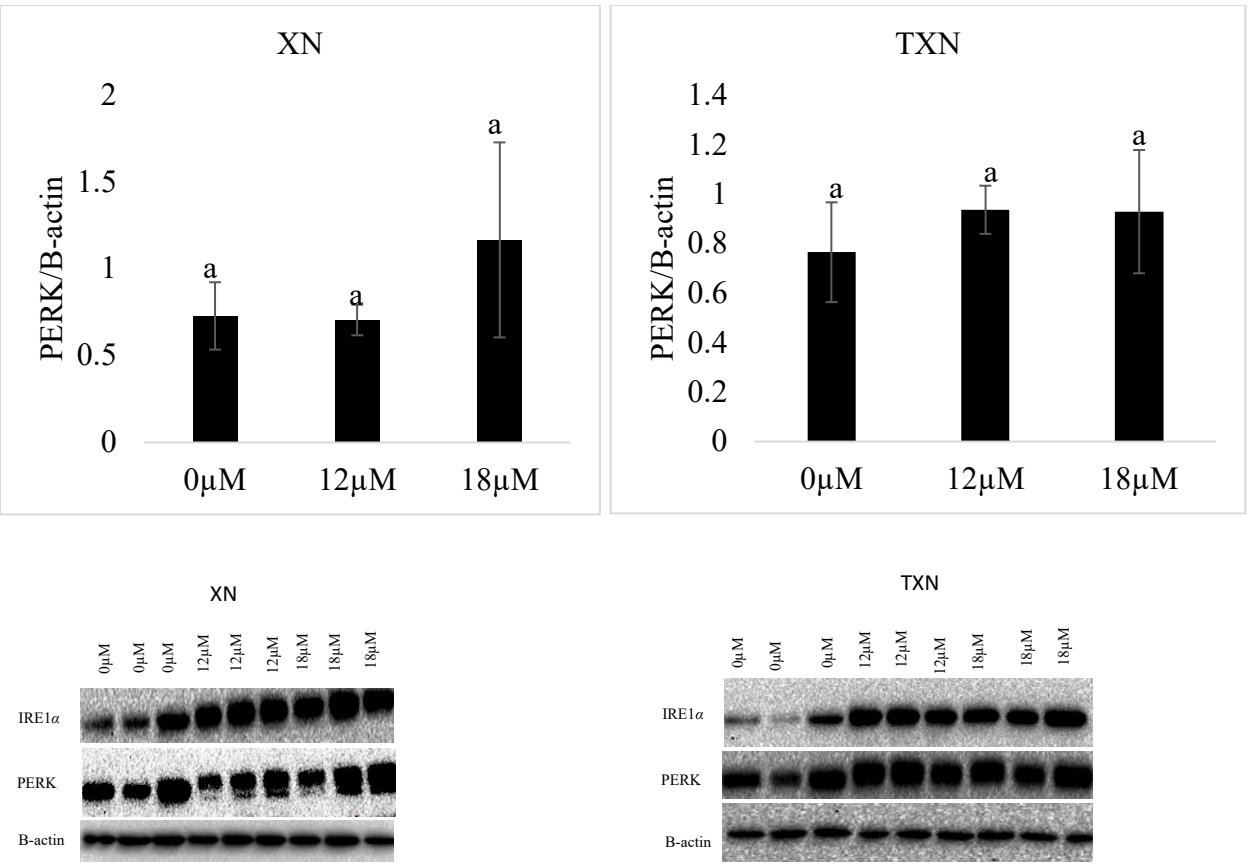
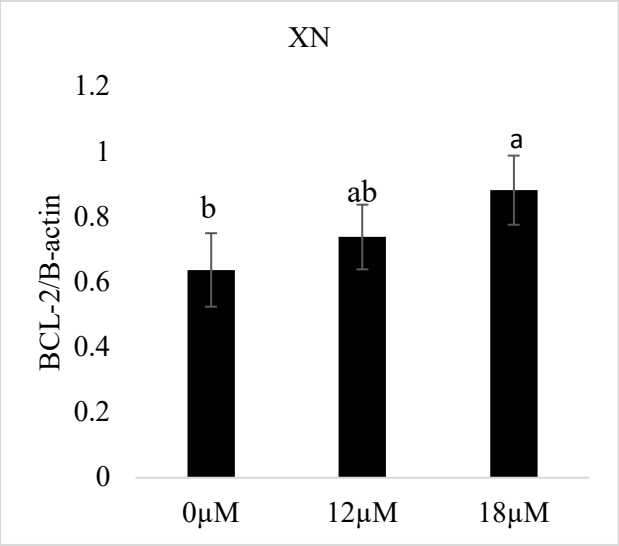


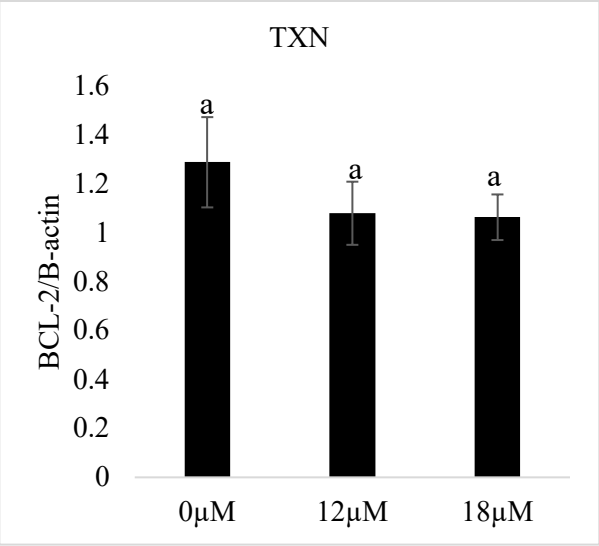

Figure 6: Expression of ER stress marker proteins, IRE1α and PERK, in HCT116 cells treated with different concentrations (0, 12, and 18 μM) of XN and TXN. Values are means ± SD (n = 3). Different letters indicate significant difference at $P < 0.05$.

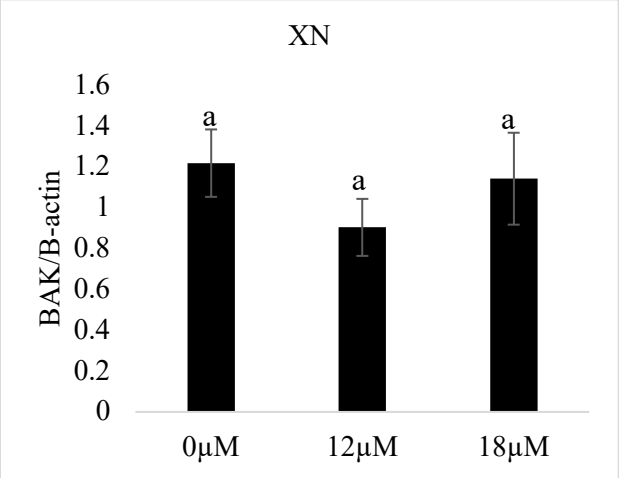
### 3.7 Expression of other apoptotic marker proteins in HCT116 CRC cell line treated with XN and TXN

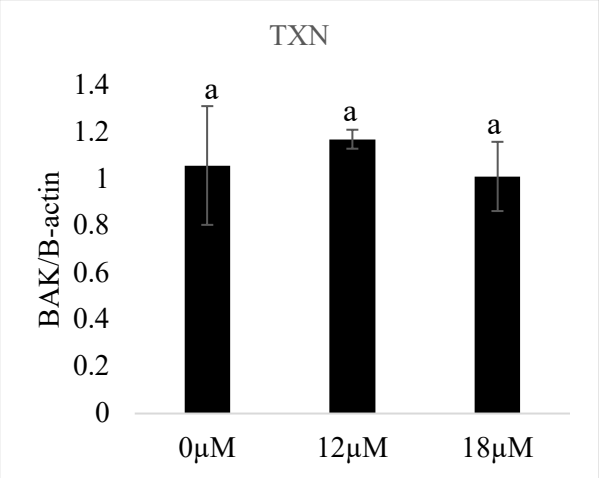
Apoptosis is regulated by two pathways, intrinsic (mitochondria-dependent) and extrinsic (mitochondria-independent) pathways. Since BCL-2 family proteins control mitochondria-dependent apoptotic pathway, we measured expression of two mitochondrial membrane proteins, pro-apoptotic BAK and anti-apoptotic BCL-2. As shown in Fig. 7 below, there was no significant changes in the expression of these two proteins in the cells treated with XN and TXN.

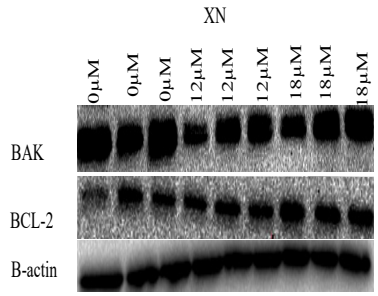
These data indicate that induced apoptosis by XN and TXN is not associated with mitochondria-dependent apoptotic pathway.



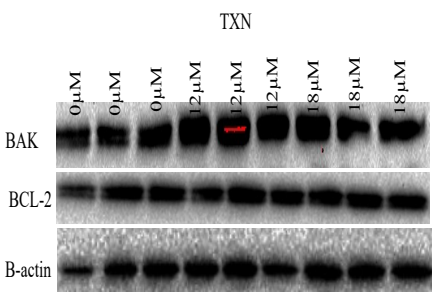
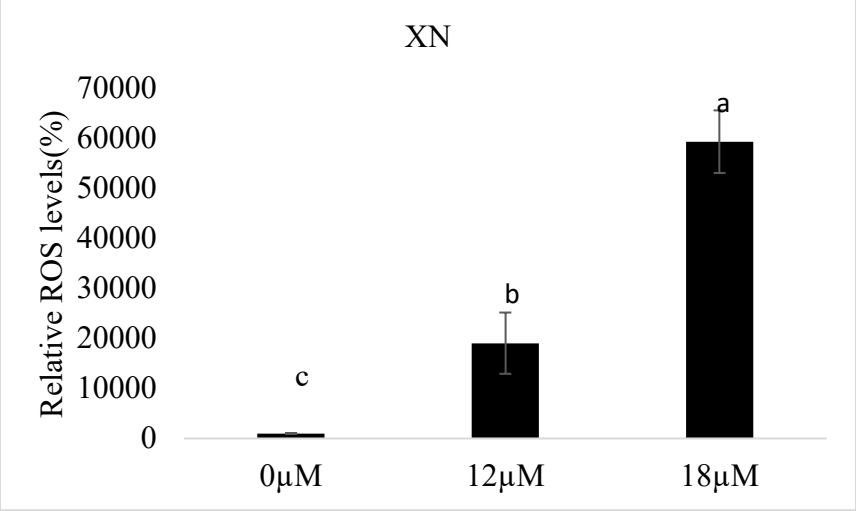



Figure 7: Suppression and Induction of BCL-2 family protein marker, BCL-2, and BAX, in HCT116 cells treated with different concentrations (0, 12, and 18 µM) of XN and TXN. Values are means ± SD (n = 3). Different letters indicate significant difference at $P < 0.05$.

### 3.8 ROS release in HCT116 CRC cell line treated with XN and TXN

Excess cellular level of ROS is one of proposed proapoptotic mechanism of many anti-cancer drugs and compounds. To investigate if ROS is associated with induced apoptosis, HCT116 cells were treated with XN and TXN and ROS production was compared. As indicated in Fig. 8, treatment of cells with 12 µM XN and TXN led to a 19.0 and 25.7-fold increase of ROS production. And treatment of 18 µM XN and TXN led to a 59.3 and 53.9-fold increase in ROS production compared to the control group, respectively. With a significance of $p<0.001$ at 18 µM- XN and TXN-treated HCT116 cells.

**XN**




**TXN**

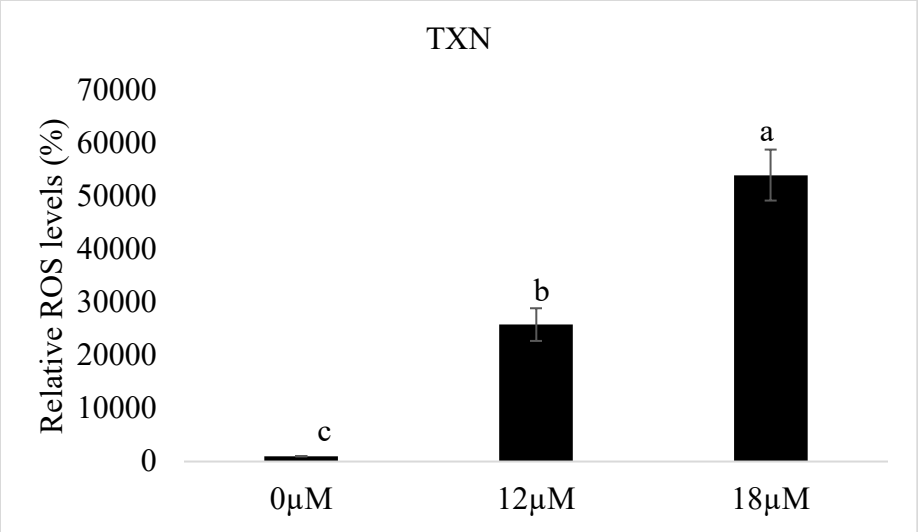


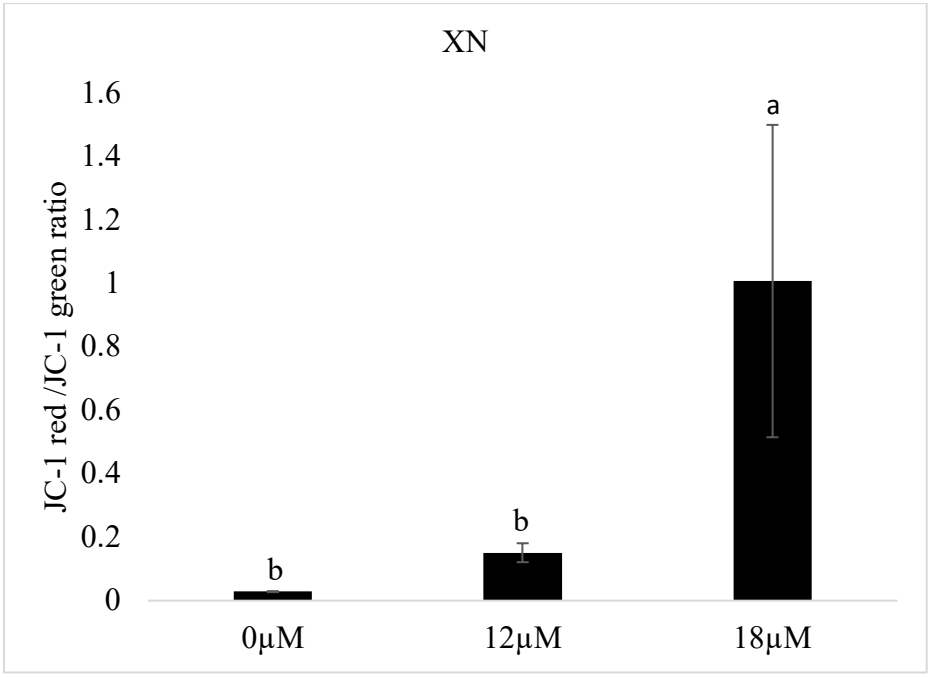

Figure 8: Overproduction of H2O2 in treated HCT116 CRC cell lines by XN and TXN. This increase signifies that the levels of mitochondria ROS are high which leads to apoptosis. Values are means ± SD (n = 3). Different letters indicate significant difference at $P < 0.05$.

### 3.9 Mitochondria membrane potential in HCT116 CRC cell line treated with XN and TXN

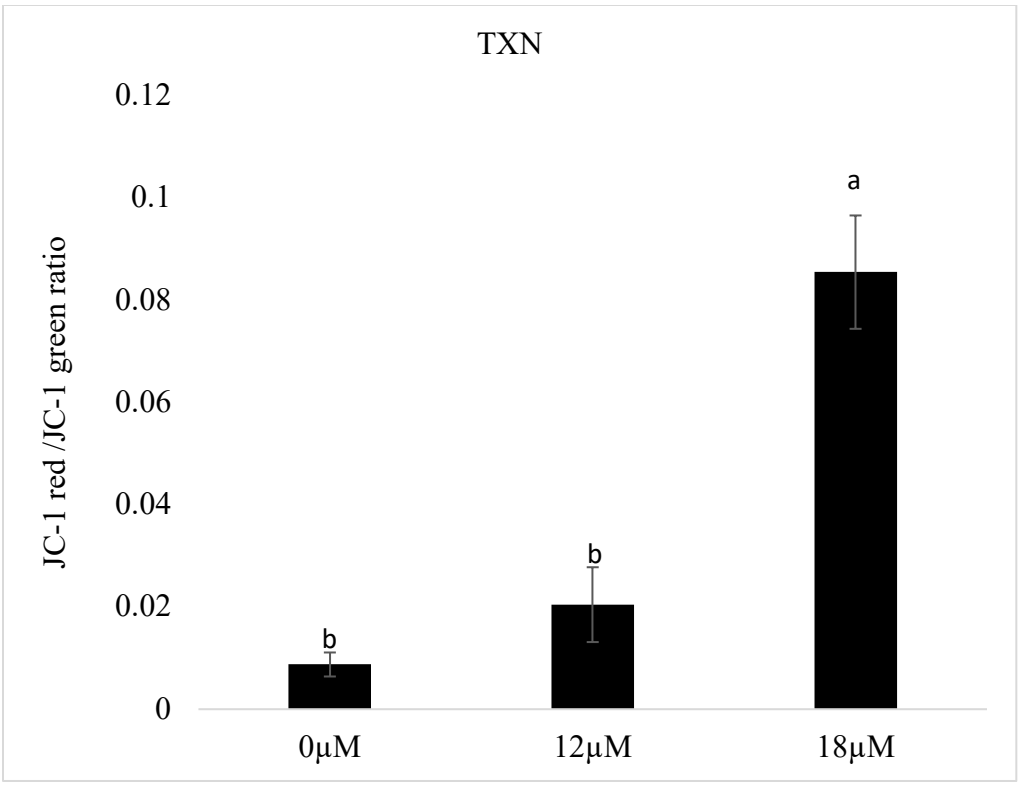
Next, to determine if XN and TXN-stimulated ROS production is associated with dysfunction of mitochondria, we measured mitochondria membrane potential using JC-1 staining from HCT116 CRC cell line treated with different doses of XN and TXN. As shown in Fig. 9, an increase in loss of mitochondria membrane potential was detected in HCT116 cells subjected to XN and TXN treatment. The Q2 (red) quadrant showing an aggregation of healthy mitochondria cells decreases as concentration increase from 0 μM to 18 μM while the Q4 (green) quadrant representing the unhealthy status of the mitochondria cells increased as the concentration of XN increased from 0 μM to 18 μM (Fig. 9). Thus, the ratio (0.02, 0.15, and 1.00) of JC-1 red/ JC-1 green increases based on the aggregation of cells in these quadrants. A 5.3 and 35.4-fold increase was observed at 12 μM and 18 μM of XN compared to the control.

For HCT116 TXN-treated cells a 2.3- and 9.7-fold increase was noticed. The enormous loss of mitochondria membrane potential signifies an induction of apoptosis by XN and TXN.

**XN**




**TXN**


TXN
JC-1 red /JC-1 green ratio
0.12
0.1
0.08
0.06
0.04
0.02
0
a
b
b
0μM
12μM
18μM

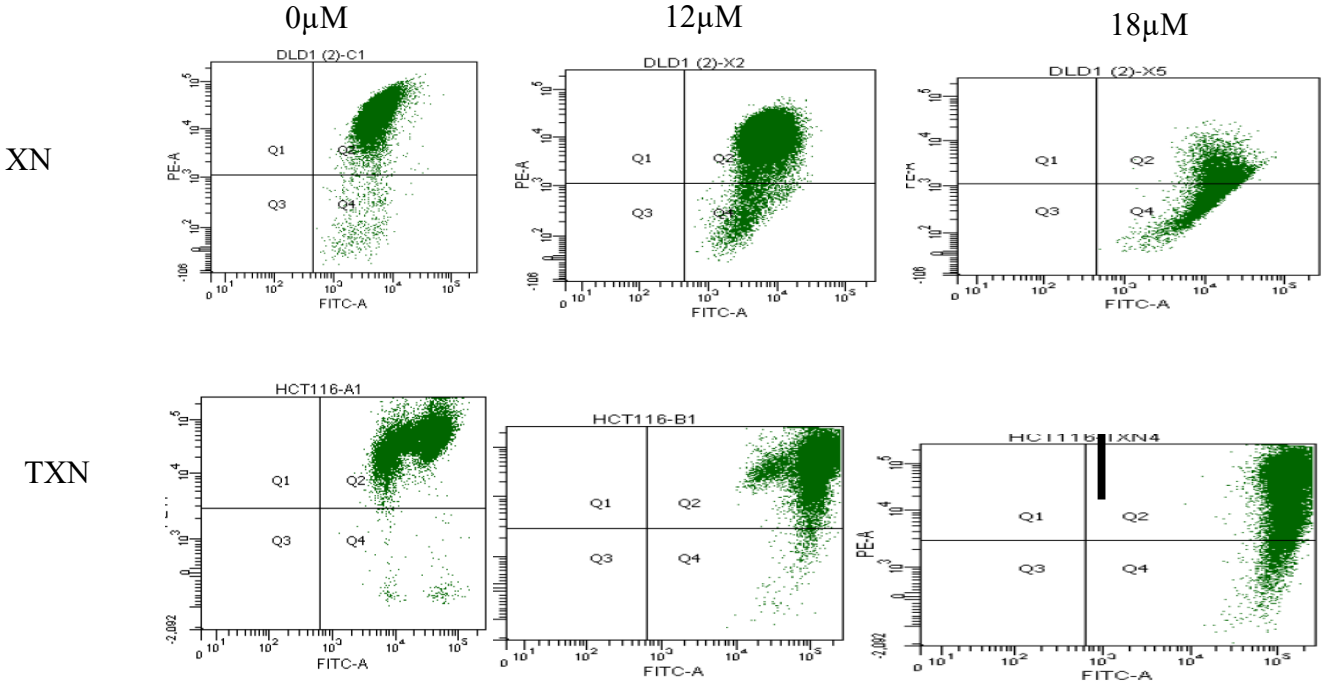

0μM
12μM
18μM
XN
TXN
DLD1 (2)-C1
DLD1 (2)-X2
DLD1 (2)-X5
HCT116-A1
HCT116-B1
PE-A
FITC-A
Q1
Q2
Q3
Q4

Figure 9: Loss of mitochondria membrane potential was well shown in the graph and snapshot of XN and TXN by an increase in the Q4 (unhealthy mitochondria) phase and a decrease in Q2 (healthy mitochondria) phase. Values are means ± SD (n = 3). Different letters indicate significant difference at $P < 0.05$.

# CHAPTER 4: Discussion

## 4.1 Discussion

Globally, cancer is a major cause of death and the second-leading cause of death in the United States [1]. Colorectal cancer is the third most commonly diagnosed cancer in the West [12]. CRC accounts for about 1.2 million new cancer cases and 600,000 cancer deaths per year [10]. Adenoma-carcinoma sequence is the basis for the development of CRC [13], with colon and rectal cancer being the most prominent forms of malignant tumors of adeno-epithelial origin [12]. Researchers continue to work earnestly in pursuit of novel treatment options for cancer patients. Nutritional chemoprevention is a promising area in the management of cancer. Phytochemicals, which are active ingredients in plant-based diets, have protective effects against cancer. Hop, commonly used for beer production, contains active ingredients with potential health benefits. XN, the prenylated and most common flavonoid of the hop plant, has been noted to demonstrate positive effects when utilized for the treatment of various chronic conditions, including cancer.

Our study used three CRC cell lines, SW480, SW620, and HCT116, and subjected them to four hop treatments: XN, IXN, 8-PN, and TXN. As depicted in Fig. 2, XN, 8-PN, and TXN showed a significant decrease at a dose of 12 μM, 25 μM, and 50 μM for three cell lines (HCT116, SW620, and SW480). In SW480 cells, we observed a significant decrease ($p<0.01$) from XN-treated cells (6, 12, 25, and 50 μM), IXN-treated cells (12, 25, and 50 μM), 8-PN-treated cells (12, 25, and 50 μM), and TXN-treated cells (6, 12, 25, and 50 μM) (Fig. 2). In addition, XN-treated SW620 cells at doses of 25 and 50 μM, 8-PN-treated SW620 cells at doses of 50 μM, and TXN-treated SW620 cells at doses of 25 and 50 μM showed a significant difference (Fig. 2). Finally, a significant difference was observed in HCT116-treated cells at XN (12, 25, and 50 μM), IXN (50 μM), 8-PN (25 and 50 μM), and TXN (25 and 50 μM)-treated cells (Fig. 2). Previous studies examined the anti-cancer effects of XN and its analogues like DXN (dihydroxanthohumol) and TXN against CRC cell lines. XN and its derivatives inhibited cell proliferation in CRC cell lines HCT116 and HT29 and liver carcinoma cell lines HepG2 and Huh7 [52]. The results from our cell viability evaluation thus supported XN and its analogues' capability of inhibiting cell proliferation in CRC cell lines (SW480, SW620, and HCT116). Also, the HCT116 CRC cell line was most sensitive to XN, 8-PN, and TXN (Fig. 2) compared to SW480 (Fig. 2), which was the least sensitive to these treatments. Moreover, 8-PN might be more detrimental than beneficial to a cell because it is a potent phytoestrogen. Their structural differences (Fig. 1) could be the proposed reason why these hop compounds show varying biological functionalities. We continued to work with XN and TXN because XN has been used over the years because of its effective anti-cancer ability. However, TXN has not been fully explored for its protective role against CRC. The results of the investigation on HCT116 cell lines showed a significant decrease in cell viability when exposed to XN and TXN concentrations between 6 μM to 50 μM (Fig. 2). Thus, apoptosis and cell cycle

arrest were two peculiar pathways we targeted in our study of XN and TXN against the HCT116 CRC cell line.

Cell cycle distribution above (Fig. 3) depicts a great induction of the S-phase in the HCT116 CRC cell line after treatment with XN and TXN. Three concentrations (0, 12, and 18 μM) were used, and the highest S-phase arrest was seen at both 12 μM and 18 μM with no significant difference between the two treatments. In addition, 12 μM and 18 μM of XN-treated HCT116 cells showed a 2.2- and 2.5-fold increase, and concentrations of 12 μM and 18 μM of TXN treatment led to an S-phase induction of 1.8- and 2.3-fold in HCT116 cells, respectively. Also, XN and TXN induced the G2/M phase of the cell cycle with a 2.4- and 1.9-fold increase at concentrations of 18 μM of XN and TXN-treated HCT116 cells, respectively. Other studies claim that the anti-cancer activity of XN against CRC cells is a result of the induction of G2/M cell cycle arrest [51]. Also, the induction of the sub-$G_1$ population when cancerous cells were subjected to a high dosage of XN (50 μM) was observed; TXN also inhibited the $G_1$ phase [52]. All taken together, we found that decreased viability of CRC cells treated with XN and TXN might be associated with cell cycle arrest at the S and G2/M phases.

We performed a FACS analysis to evaluate the apoptosis induction in HCT116 cells that had been treated with XN and TXN. Apoptosis, which is programmed cell death, has two pathways: intrinsic (mitochondria) and extrinsic (cell death receptor) apoptotic signaling pathways. The intrinsic pathway is primarily mediated by caspase activity, with initiation occurring through events like DNA damage and growth factor withdrawal [23]. Mitochondria dysfunction occurs, leading to the up or downregulation of proteins from the Bcl-2 family. On the other hand, the death receptor pathway is initiated by transmembrane-receptor-mediated interactions, which are members of the tumor necrosis factor (TNF) receptor gene superfamily.

These pathways are of particular interest to researchers because dysregulation most often leads to cancer [23]. We carried out flow cytometry to examine if apoptosis is associated with reduced cell viability. The combined graph of early and late apoptosis depicted a tremendous induction of overall apoptosis. The most significant cell death was observed at concentrations of 18 μM-XN and TXN-treated HCT116 cells. However, TXN had a greater increase in the Q2 phase compared to XN. XN and TXN both induce early and late apoptosis in a concentration-dependent manner in HCT 116 cell lines. Previous studies showed that XN and its derivatives, like TXN, inhibit cancer cell proliferation through a molecular mechanism that involves caspase-mediated apoptosis [52]. Cell cycle arrest and apoptosis could be possible mechanisms by which these hop compounds execute cell death in HCT116 CRC cells.

In order to further confirm apoptosis as the mechanism of XN and TXN reducing the viability of CRC cells, Western blotting was utilized to detect proteins associated with these pathways. As observed in Fig. 5 above, there was a significant induction of cleaved PARP in HCT116 cells treated with 12 μM and 18 μM of XN and TXN, respectively, as compared to the control. Cleaved PARP confirmed the caspase-mediated cascade pathway in XN and TXN-HCT116-treated cells. Bcl-2 family proteins, which are crucial for the intrinsic apoptotic pathway, were observed using western blotting. The two mitochondrial membrane proteins, pro-apoptotic Bak and anti-apoptotic Bcl-2 (Fig. 7 above) showed no significant changes in expression in HCT116 cells treated with XN and TXN. The data suggests that XN and TXN induced apoptosis is not associated with a mitochondrial-dependent pathway. Furthermore, ER stress-mediated apoptotic cell death was analyzed by measuring the expression of ER stress markers proteins including CHOP, ATF4, IRE1α, and PERK (Fig. 6). CHOP, a C/EBP homologous protein and transcription factor, is activated at multiple levels in ER-stressed cells [63]. PERK, an ER stress-

activated kinase, phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α), thereby decreasing eIF2 activity, which leads to the activation of translation in ATF4 mRNA [63]. The ATF4 protein binds and activates the CHOP promoter, and deregulated CHOP activity affects cell viability. CHOP sensitizes cells to apoptosis via ER stress-mediated death by the direct regulation of target genes in the nucleus [63]. The concentration of 12 μM of XN and TXN had an 8.9 and 4.0-fold increase compared to control, respectively. Another ER stress marker, ATF-4 (Fig. 6), was highly expressed in XN- and TXN-treated HCT116 cells. In addition, IRE1α (Fig. 6) had an increase at concentrations of 12 μM of XN and 18 μM of XN and TXN as compared to the control. Finally, PERK (Fig. 6) showed no significant expression in HCT116 cells treated with TXN and XN at concentrations of 12 μM and 18 μM. Expression of these important protein markers strongly signifies that apoptosis is a possible mechanism by which these two compounds (XN and TXN) suppress cell viability in HCT116 CRC cells.

To elaborate on the cause of the extrinsic apoptosis pathway, we measured ROS production in mitochondria. Mitochondria are a source of reactive oxygen species (ROS) that may be associated with the initiation and progression of cancer. ROS leads to the induction of genomic instability. Thus, scientists targeted different processes in this organelle for the treatment of cancer [64]. Our data in Fig. 7 showed a huge increase in cellular ROS levels when HCT116 cells were treated with XN and TXN. The highest ROS levels were detected at 18 μM of XN and TXN.

We also examined the mitochondrial membrane potential of HCT116 cells after treatment with XN and TXN. Mitochondrial membrane potential is a valuable indicator of a cell's health and functional activity. Cyanine dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide) provides discrimination between energized and de-energized mitochondria. Generally, the green fluorescent dye forms red fluorescent aggregates when put in the energized

mitochondria in response to the mitochondria's higher membrane potential [65]. The JC-1 staining in Fig. 9 above depicts an increase in the loss of mitochondrial membrane potential when HCT116 cells are subjected to XN and TXN treatments (Fig. 9). The Q2 (red) quadrant, which showed aggregation of healthy mitochondrial cells, decreased as concentration increased from 0 μM to 18 μM. While the Q4 (green) quadrant, which represents the unhealthy status of mitochondrial cells, increased as concentration increased from 0 μM to 18 μM. Thus, the ratio of JC-1 red to JC-1 green increases based on the aggregation of cells in these quadrants (Fig. 9). The enormous loss of mitochondrial membrane potential signifies the induction of apoptosis by XN and TXN.

We hope that future research can continue to investigate these hop compounds and discover more mechanisms that will benefit cancer research.

## 4.2 Conclusion

Our studies demonstrate that XN and TXN suppress HCT116 CRC cells by inducing cell cycle arrest and apoptosis, which are associated with induced ROS.

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