POTENTIAL MECHANISM OF ACTINS OF URSOLIC ACID IN THE PREVENTION OF BREAST CANCER

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Ran Yin

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POTENTIAL MECHANISM OF ACTINS OF URSOLIC ACID IN THE PREVENTION OF BREAST CANCER

Ran Yin, Ph.D.

Cornell University 2015

Currently, breast cancer becomes the most frequently diagnosed and the second deadly cancer among women in United States. American Cancer Society reported that an estimated 234,190 new cases of diagnosed and 40,730 new cases of death would occur in United States in 2015. Despite of sex, age, race, pregnancy, breastfeeding and other factors, current therapies, such as breast-conserving surgery (BCS), radiation therapy and systemic therapy (hormone and targeted therapy) seem no longer effectively to control the growth of human breast cancer. Thus, there is an increasing demand to develop effective and alternative cancer treatments to improve the current breast cancer therapies and optimize the recovery procedure.

Phytochemicals from fruits and vegetables are suggested to be associated with reducing the risk of chronic diseases, such as cancer. The advantages of choosing phytochemical as anticancer agents such as easily approachable, large diversity make them great potential to be developed to a new treatment for cancer. For example, EGCG and quercetin, widely distributed in fruits and vegetables, have been reported to exhibit high antioxidant, anti-inflammatory and anti-cancer effects. However, the molecular mechanism of actions of phytochemicals in the prevention of cancer is not fully understood yet.

In my Ph.D. project, ursolic acid (UA), a triterpenoid, has been used for anti-

proliferation and cytotoxicity studies towards MDA-MB-231 human breast cancer cells by methylene blue assay. Results determined that cell proliferation of MDA-MB-231 cells can be effectively inhibited by ursolic acid in a non-cytotoxic concentration range. Thus, UA was chosen as candidate phytochemical in the study of signaling pathways in MDA-MB-231 cells, specifically focused on anti-proliferation and apoptosis pathways. On the anti-proliferation signaling pathway, the expressions of a serious of key proteins, including PCNA, CDK-4, cyclinD1, p21, p-p53, p-p38, p-ASK 1 and TRAF-2, were all measured by immunoblotting assay. Results demonstrated that there was a dose-dependent relationship between UA concentrations and expression of all these proteins. In addition, p38 inhibitor study showed the reversing effect on the expression of PCNA and CyclinD1 inhibited by UA. In the apoptosis signaling pathway, the expressions of Bax, Bxl-2 and cleaved caspase-9 and 3 were also measured by immunoblotting assay. Data suggested that UA induce mitochondria apoptotic pathway in MDA-MB-231 in a dose-dependent manner. Moreover, apoptosis effect induced by UA was also confirmed by TUNEL assay. At last, two-way combination studies on UA and other phytochemicals (EGCG, quercetin, and resveratrol) were performed to test the additive and synergistic effect in MDA-MB-231 cells. Results revealed that combinations of these phytochemicals exhibited the additive and potential synergistic effect in MDA-MB-231 cells. Future work on three- or more way of combination is necessary to elucidate the mechanism of synergy among phytochemicals in fruits and vegetables

In summary, ursolic acid had anti-proliferative effect in MDA-MB-231 human breast cancer cells through p38/MAP kinase pathway by regulating cell cycle cyclinD1/CDK family and PCNA proteins. Meanwhile, ursolic acid induced apoptosis

through mitochondria death pathway by regulating the expression of Bax/Bcl-2 family proteins and caspase family proteins through p38/MAP kinase pathway. Ursolic acid had additive and potential synergistic effect with other phytochemicals. These data provide insights in developing new cancer therapy using ursolic acid and other phytochemicals.

BIOGRAPHICAL SKETCH

Ran Yin was born in May 1984 in Dalian, China. He received his bachelor degree in chemical engineering at Dalian University of Technology (DLUT), Dalian, China, in 2008. Then, he continued pursuing his master degree in chemistry at Rochester Institute of Technology (RIT), Rochester, NY, in 2010, specialized on developing a unique 193 nm photo-resist polymers for semiconductor producing.

After graduated from R.I.T, he joined into the Ph.D. program in the Department of food science at Cornell University, NY. His research was in the area of functional food and nutrients in prevention of cancer, focused on the mechanism of action of ursolic acid, a triterpenoid isolated from apple peels, in the prevention of breast cancer.

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CHAPTER ONE

INTRODUCTION

1.1. Breast cancer

American Cancer Society has reported that breast cancer is the leading cancer being diagnosed and second in cancer death among women in the United States (Siegel et al., 2015). In this report, the estimated numbers of breast cancer in new cases of diagnose and new cases of death are 231,840 and 40,290, respectively, in 2015. The lifetime risk of invasive breast cancer incidence has increased since 1970s, because of the over-use of hormones in skin care products and drugs, along with prolonged life expectancy, changes in reproductive patterns and the development of diagnose technology (mammography) revealed breast cancer in early stages. Risk factors for breast cancer, including family history, obesity, breast-feeding and life-style factors (air/water pollution, smoking, mental stress) are determined, yet not fully applied in cancer prevention and treatment (Yang et al., 2011). Current breast cancer therapies including breast-conserving surgery (BCS), radiation therapy and systemic therapy are performed in various stages and types of breast cancer; however have limitations in eliminating side effects, post-surgical recovery and reoccurrence. Thus, it is of urgency to discover alternative approaches for new breast cancer therapy in both effectiveness and nontoxicity.

Epidemiological studies suggested that high consumption of fruits, vegetables, whole grains and other plant food reduced the risk chronic disease risks such as cancer (Block et al., 1992) and cardiovascular diseases (Dauchet et al., 2006).
In details, dietary pattern of increased intake of fruits and vegetables were determined to have potent effect in reduction of incidence of cancer in many studies (Liu,

2003),(Terry et al., 2001),(Slavin et al., 1997),(Aune et al., 2011). Most of these studies believed that phytochemicals, abundantly distributed in plant world, were considered to be the primary contributors to provide such health benefits by scavenging reactive oxidative species, regulation of cell signaling pathways and enhancing insulin sensitivity (Liu, 2004),(Genkinger et al., 2004),(Adams et al., 2006),(Weickert et al., 2006). By definition, phytochemicals are non-nutrient bioactive compounds that are abundantly distributed in fruits, vegetables, the whole grains and other plant foods (Liu, 2004). They have been determined to exhibit the ability to regulate the expression of key regulatory proteins through multi-signaling pathways against human cancer cells (Yoon and Liu, 2008),(Sun and Liu, 2008). However, the full extent of the roles of phytochemicals in cancer prevention and treatment are still unclear.

Current research focuses on mechanism of actions of phytochemicals against human cancers. For example, studies have shown that phytochemicals in apples, especially from apple peels, has potent antioxidant activity to scavenge free reactive oxidative species and inhibited the cell growth on human lung cancer and colon cancers (Eberhardt et al., 2000a),(Wolfe et al., 2003). Other study showed that berry extracts exhibited high antiproliferative activities on human oral, breast, colon and prostate cancer cells(Seeram et al., 2006a). In addition, lignan and isoflavonoid glycosides, two groups of hormone-like diphenol phyto-estrogens distributed in whole grains, have been found in regular diets among individuals who have low risks of cancer diagnosis (Adlercreutz and Mazur, 1997). The consumption of such whole grains significantly reduced the risk of cancer occurrence influenced by sex-hormone production, protein synthesis and cell proliferation and angiogenesis. Researches on

phytochemical initiated signaling transduction pathways of proliferation inhibition, apoptotic induction, elimination of multi-drug resistance (MDR) and synergistic effects are all important aspects to ascertain the relationship between phytochemicals and cancer prevention and treatments (Tan et al., 2013),(Yang and Liu, 2009).

In this chapter, ursolic acid is discussed, pertaining to its natural source, biosynthesis, absorption and other characteristics. Most importantly, mechanisms of actions for ursolic acid anticancer activities in several human cancer cells are introduced in order to explore novel insights on the development of unique treatment for cancer therapy.

1.2. Ursolic acid

Ursolic acid, or 3-β-hydroxy-urs-12-en-28-oic acid, is a pentacyclic triterpene acid abundantly distributed in fruits and vegetables(He and Liu 2007a) (Yamaguchi et al., 2008),(Waller et al., 1991),(Jayaprakasam et al., 2006),(Lin et al., 2011),(Jager et al., 2009). In different types of plants, ursolic acid content varies from 0.15 to 1.8 g/100 g dry matter. Ursolic acid was isolated firstly from several plant leaves such as *Thymus vuigaris, Verbena stricta, Vinca minor, et.al*. in early 1950's (Rowe et al., 1949a),(King et al., 1950),(Le Men and Pourrat, 1952). Recently, research have been focusing on ursolic acid's anti-cancer, antioxidant and other pharmacological effects in a non-toxic concentration range (Yeh et al., 2010a),(He and Liu, 2007a).

1.2.1 Source

First studies on ursolic acid's natural distribution were reported in the 1950's. Pourrat *et al*. published several papers on ursolic acid content in labiate (Pourrat and Le Men, 1953), apocynaceae (Le Men and Pourrat, 1953), and oleaceae (Pourrat et al.,

1954), yet with no further studies on the roles of pharmaceutical effects. In 1998, Robert *et al*. determined that among all kinds of phytochemicals in six apple cultivars selected from North Carolina, ursolic acid was confirmed to be the largest fragment in all samples based on the data from GC-MS and TLC (Belding et al., 1998). Similar study on ursolic acid content in apple peels was reported in 2008 (Frighetto et al., 2008),(He and Liu, 2008). The average content of ursolic acid found on leaves of four types of apples (Fuji, Gala, Smith and Granny smith) harvested in Brazil, were 0.8, 0.5, 0.8 and 0.2 mg/cm^2 respectively(leaf surface area range: 50-70 cm^2). Controversially, Tony *et al*. discovered that the concentration of ursolic acid in apple peels might not be as high as reported, due to different solvent used for extraction and measurements(McGhie et al., 2012). For other types of fruits and plants, ursolic acid distribution would vary depends on the nature source of species, environments e*tc*. Major edible sources of ursolic acid are summarized in **Table 1.1** (Rowe et al., 1949b),(Vagi et al., 2002),(Fu et al., 2005),(He and Liu, 2007b),(Kowalski, 2007),(Razborsek et al., 2007),(Chen et al., 2012),(Cai and Huang, 2012),(Yin et al., 2012b).

In terms of dietary sources, processing methods significantly affect the content of ursolic acid presented in food (Kondo et al., 2011). Kondo *et al.* suggested that ursolic acid content ranged between 60-100 mg/100 g in fresh whole or sweetened/dried cranberry fruits. However, reduced amount of ursolic acid content was observed in jellied cranberry sauce and commercial cranberry juice. Moreover, ursolic acid was found in higher content in apple peels than in the flesh of apples, which indicated less processed fruits and plant products would reserve the nutrition and health benefits of ursolic acid.

1.2.2 Biosynthesis

In most plants, pentacyclic triterpenoids, such as ursolic acid and oleanic acid derivatives are mainly synthesized by two precursors, dimethylallyl pyrophosphate (DMAPP) and isopentenyl diphosphate (IPP). Evidence suggested that these isomers are produced by their precursors, acetyl-CoA and pyruvate/D-glyceraldehyde 3-phophate through two signaling pathways, mevalonic acid (MVA) and methylerythritol 4-phosphate (MEP)(Chappell, 1995),(Rohmer et al., 1993),(McGarvey and Croteau, 1995). Some studies suggested that there might be a crosstalk between two different biosynthetic pathways(Hemmerlin et al., 2003),(Laule et al., 2003), yet recent data revealed and preferred that cytosolic IPP and DMAPP are the main precursors for ursolic acid and other pentacycli triterpenoids synthesis (Yu et al., 2013). Moreover, it was reported that some systematic enzymes, such as farnesyl pyrophosphate synthase (FPS), squalene synthase (SQS) and squalene epoxidase (SQE) were major catalysts involved in the format of triterpenoids carbon skeletons. These skeletons, including α-amyrin, β-amyrin and cycloarenol were demonstrated to give such unique structure through the isoprenoid pathway and cyclization of 2,3-oxidosqualene(Zhou et al., 2012). Furthermore, it was proposed that multifunctional enzymes of CYP716A subfamily proteins regulated the final step of triterpenoids biosynthesis, led the reaction from α-amyrin, β-amyrin to form ursolic acid derivatives and oleanolic acid derivatives respectively(Fukushima et al., 2011). Specifically, CYP716A12 was found to be solo responsible for ursolic acid synthesis. However, some studies suggested that enzymes involved in triterpenoids carbon skeleton building, such as squalene synthase, and 2,3-oxidosqualene cyclase might have no obvious correlation with the biosynthesis of ursolic acid in some fresh fruits due to the

difficulties in identifying DNA cloning belongings (Zhou et al., 2012). It has been proved that the biosynthesis of ursolic acid and derivatives followed a complicated procedure conjugated with several regulatory proteins and signaling pathways (**Figure 1.1**). Further exploration of this synthetic process would provide novel insights and evidence to increase the ursolic acid content in fruits and vegetables and as a guide to develop unique cancer prevention or treatment therapies using it.

1.2.3 Ursolic acid absorption in vivo

Two groups focused on the ursolic acid plasma concentration in rat models were reported. Liao et al. published that ursolic acid content in plasma rapidly reached the highest absorption peak around 1 hour after oral-administration in Male Wistar rats(Liao et al., 2005). In addition, the level of ursolic acid concentration was lower than the detection limits at 12 hours post dosage. Surprisingly, the concentration was extremely low which suggested that ursolic acid might exhibit either high binding interaction with organs or very low bioavailability. Another study on ursolic acid plasma and tissue distribution were measured by HPLC-MS(Chen et al., 2011a). Chen et al. reported that ursolic acid concentration achieved the peak in plasma at 0.5 hour. Ursolic acid distribution in organs significantly varied, but followed a blood-supplied pattern. From an eight-week long-term study, it was found that the liver and kidney are the preferable organs for the absorption of ursolic acid. Thus, results indicated that these organs were the predominant locations for the ursolic acid antioxidative activities to occur(Yin et al., 2012a). In order to enhance the ursolic acid absorption to match standards for clinical pharmacokinetic and cancer therapeutic developments, modern technology has been involved in developing unique delivery system to achieve higher bioavailability, targeting effect and stability. Zhou et al. developed alternative

phospholipid nanoparticles to improve the accumulation of ursolic acid in most organs with intravenous administration *in vivo*(Zhou et al., 2009). In this study, ursolic acid was extracted from *C. pinnatifida* and mixed with soybean phospholipid and poloxamer 188, a specific tri-block copolymers that used in membrane resealing(Moloughney and Weisleder, 2012). The designed ursolic acid-phospholipid nanopolymer was dispersed and suspended into distilled water, then intravenously administrated into the tail vein in both time and dosage differently. Data revealed that ursolic acid was increasingly accumulated in liver after 4 hours, compared with other organs in an extremely low concentration. In addition to the small size of the nanoparticles (an average diameter of 273.8±2.3 nm) which made it easy for liver cells uptake(Yang et al., 1999), synthesizing phytochemical-nanoparticles is an effective improvement which would assist ursolic acid delivery to target organs, such as the liver. Xia et al. demonstrated that nano-liposomes conjugated with ursolic acid significantly improved the plasma concentration after 4-hour intravenous administration measured by UPLC/MS/MS(Xia et al., 2011). Unfortunately, these evidences were still not sufficient to elucidate the mechanism of action of ursolic acid absorption and organ distribution *in vivo*. Therefore, it is necessary to broaden our knowledge in ursolic acid metabolism in order to further developing conventional cancer therapies using phytochemicals from fruits and vegetables.

1.3. Antioxidant activity

Oxidative stress is described as the imbalance between systemic synthesized reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), or hydroxyl radical ($^{\cdot}OH$), and the antioxidant defense such as the biological

system's ability to remove ROSs or repair the associated damage(Betteridge, 2000). Recently, it is well known that imbalanced ROS production is capable of causing oxidative damage to DNA, RNA and proteins and is associated with incidence of cancer(Jezierska-Drutel et al., 2013), cardiovascular disease(Fearon and Faux, 2009) and other chronic diseases(Ercan et al., 2006),(Mody et al., 2001). Researches on antioxidative activity of phytochemicals have been conducted both in vivo and in vitro Whole apple and whole grain extracts were both determined to exhibit strong antioxidant activities(Eberhardt et al., 2000b),(Miller et al., 2000). Apple extracts also inhibited cell proliferation on human colon cancer Caco-2 and human liver cancer $HepG_2$ cells(McCann et al., 2007),(He and Liu, 2007b). Moreover, berries including blueberry, cranberry and strawberry were demonstrated to have high levels of anthocyanins, a group of flavonoids having high antioxidant activity(Zafra-Stone et al., 2007). Several studies reported that flavonoids and polyphenolics extracted from fruits and vegetables, worked as scavenging free oxidative radicals(Gao et al., 1999),(Sanchez-Moreno et al., 1999) and antioxidant activities of these phytochemicals were measured by DPPH assay(Nanjo et al., 1996). Recently, Wolfe and Liu published cellular antioxidant activity assay quantified the antioxidant activity by measured the protected dichlorofluorescein (DCF) in $HepG_2$ human liver cancer cells (Wolfe and Liu, 2007a). Data revealed that quercetin possessed the highest antioxidant activity among all pure selected phytochemicals. In addition, blueberries possessed the highest antioxidant activity among all selected fruits in this study.

Recently, studies on ursolic acid antioxidant activity were focused on its ability to restore the disturbed oxidative status both in vitro and in vivo. Martin-Aragon et al. (Martin-Aragon et al., 2001) reported that the serum level glutamate-oxalate-

transaminase and glutamate-pyruvate-transaminase, as well as other antioxidant defendant enzymes (superoxide dismutase, catalase *etc*.) in liver, were observed to be significantly reversed by pre-treatment of ursolic acid. In vivo, results revealed that ursolic acid pre-treated at dosage of 1, 2.5 and 5 mmol/kg significantly recovered the CCl_4 intoxicating effect on glutathione peroxidase activities by 36.3, 73.6 and 82.8%, respectively. In vitro, ursolic acid prevented hepatocytes lipid peroxidation by 32.1% at dosage of 500 μM by quantitatively measuring the production of malondialdehyde in primary cultures of rat hepatocytes. Other studies on ursolic acid antioxidant activity and protective effects were evaluated by ultraviolet-B (UVB) radiation induced cytotoxicity, lipid peroxidation and DNA damage in human lymphocytes (Ramachandran and Prasad, 2008). In this study, UVB was used as initiator of overproduced reactive oxidative stress and induced biological responds on cell membrane and cytoplasmic chromophores (Steiling et al., 2007). Pre-treatments of ursolic acid (1, 5, 10 μg/mL) significantly improved cell survival, reduced lipid peroxidation and decreased the level of DNA fragmentation on human lymphocytes (Ramachandran and Prasad, 2008). Moreover, ursolic acid demonstrated significantly reduced the lipid peroxidation products in heart and liver after ethanol-induced oxidative stress introduced *in vivo* (Saravanan and Pugalendi, 2006),(Saravanan et al., 2006). Possible mechanism of antioxidant activity of ursolic acid was proposed to scavenge free radicals at damaged tissues or around tumors(Balanehru and Nagarajan, 1991),(Balanehru and Nagarajan, 1992), yet the exact regulation and metabolism were still unclear. Therefore, it is reasonable to consider ursolic acid and other phytochemicals with high antioxidant activity as potent candidates used in alternative cancer therapies.

1.4. Anti-cancer effects

1.4.1 Inhibition of cell proliferation

Cyclin-CDK protein complexes are indispensable constitute in cell cycle regulation(Serrano et al., 1993). In particular, cyclinD1-CDK4/6 complex play a role in the regulation of G1 phase. Shishodia et al. (Shishodia et al., 2003) reported that ursolic acid suppressed the TNF-induced nuclear factor-κB activation by inhibiting the p65 phosphorylation and IκBα kinases in H1129 cells (human non-small cell lung carcinoma). Cells pretreated with ursolic acid for 8 hours at the dosage of 100 μM, and ursolic acid significantly restrained cyclin D1 levels in TNF-treated H1129 cells, when compared with control group without ursolic acid pretreatment. Moreover, Ursolic acid antiproliferative activity was also identified in A549 human lung cancer cells (Hsu et al., 2004a). In this study, ursolic acid significantly inhibited expression of cyclin D1 and CDK 2 and 4, as long as other proteins at the dosage of 20 μM. Cell cycle analysis revealed that ursolic acid induced G1 arrest on A549 cells after 24 hours treatment. The flow cytometry data revealed that G_0/G_1 ratio was significantly increased in a dose dependent manner by ursolic acid at the concentration from 0 μM to 20 μM. In addition, the mechanism of cell cycle arrest in G1 phase was proposed to go through a p21/WAF1-p53 dependent pathway. Signal transducers and activators of transcription 3 (STAT3) is *STAT3* gene encoded proteins involved in the regulation of cell proliferation and inflammation associated carcinogenesis(Corvinus et al., 2005),(Bollrath and Greten, 2009). Ursolic acid has been determined to suppress the STAT3 activation and inhibit cell proliferation both in a time and dose dependent

manners toward human multiple myeloma cells(Pathak et al., 2007). Results suggested that ursolic acid multi-regulated the inhibitory effect through the DNA binding activity of STAT3, and inducible STAT3 phosphorylation. In addition, down-stream proteins cyclin D1 and Bcl-2 were inhibited in a dose-dependent manner by ursolic acid. Meanwhile, ursolic acid at 50 μM rapidly accumulated cell population in G1 phase measured by flow cytometry, confirmed that ursolic acid induced G1 arrest in cell cycle regulation. For other types of cancers, such as prostate cancer(Shanmugam et al., 2012), colorectal cancer(Prasad et al., 2012) and liver cancer(Jin et al., 2012), ursolic acid were all found to be a key control factor in cell proliferation through cyclinD/CDK complex. Other biomarkers of cell proliferation during cancer progression, such as Na^+-K^+-ATPase, exhibited does-dependent manner with ursolic acid treatment in human lung cancer(Huang et al., 2011). Data revealed that ursolic acid not only inhibited the activity of Na^+-K^+-ATPase, but also affected the expression of vascular endothelial growth factor (VEGF), intercellular adhesion molecule-1 (ICAM-1) and matrix metalloproteinase (MMP) families. All these finding suggests that ursolic acid antiproliferative activity in human cancer cells are achieved through multiple regulatory signal pathways. It is necessary to understand the mechanisms of these effects in order to develop novel cancer therapy using ursolic acid.

1.4.2 Induction of Cell Apoptosis

Apoptosis is self-programmed cell death to maintain cell homeostasis or respond DNA damage(Ghobrial et al., 2005). Signals of induction in cell apoptosis are initiated mainly through either the intrinsic mitochondria death pathway or the extrinsic Fas death receptor pathway. Phytochemicals were reported to induce apoptosis through such signaling pathways and regulated the down-stream proteins, such as Bax, Bcl-2

and caspase family proteins(Yang et al., 1997),(Rosse et al., 1998),(Chen et al., 1996). Phytochemicals exhibited great therapeutic implications on triggering apoptosis in many human cancer cell lines. Kassi et al.(Kassi et al., 2009b) reported that ursolic acid triggered the apoptosis by down regulating the protein expression of Bcl-2, a mitochondria membrane protein in MCF-7 human breast cancer cells. More specifically, it was proposed that ursolic acid (53 μM) significantly inhibited the expression of Bcl-2, caused the release of cytochrome *c* from mitochondria to cytoplasma, which led to the cleavage of procaspase-9. Interestingly, caspase-3, a subsequent protein activated by cleavage caspase-9 and commonly considered as another key regulatory protein for cell apoptosis, observed lack of activity in MCF-7 human breast cancer cells. Moreover, large amount of cleavage of poly-ADP-ribose-polymerase (PARP) were observed by ursolic acid treatment, indicating a compensation effect to this lack of caspase-3 activity phenomenon. Kim et al. (Kim et al., 2011a) found that ursolic acid triggered cell apoptosis in a caspase-dependent pathway in MDA-MB-231 human breast cancer cell line. In this study, it was worthy to note that not only the expression of mitochondrial death pathway associated proteins such as p53, Bax, Bcl-2 and caspase-9 were observed to be correlated with ursolic acid treatment. In addition, ursolic acid also induced the apoptosis via Fas receptor and caspase-8 dependent extrinsic death receptor pathway. At dosage of 40 μM, ursolic acid significantly increased expression of Fas receptor after 1.5 hour treatment on MDA-MB-231 cells. Hsu et al. (Hsu et al., 2004a) also proposed similar mechanism on human lung cancer A549 cells. Ursolic acid up-regulated the expression of Fas/APO1 at various concentrations (10 μM, 20 μM) in various times of treatment (12 h, 24 h, 48 h). Furthermore, ursolic acid regulated cell apoptosis through

multiple signaling pathways in human cancer cell lines, such asp-53/NF-κB pathway in B16F-10 melanoma cell lines(Manu and Kuttan, 2008), PI3K/Akt pathway in HepG2 cell lines(Tang et al., 2009), and mitochondrial death pathways in human colorectal and prostate cancer cell lines(Xavier et al., 2009),(Zhang et al., 2009).

1.4.3 Inhibition of Nuclear Transcription Factor, Migration and Invasion

One of the cancer therapies is to inhibit nuclear transcription factor through NF-κB cytokine signaling pathway. NF-κB represents a family of proteins including c-Rel, RelA (p65), RelB, NF-κB1 (p50 and p105), and NF-κB2 (p52)(Shishodia et al., 2003). These proteins bind to the κB site, a common sequence motif in DNA(Aggarwal and Shishodia, 2006). In resting conditions, the NF-κB proteins remain in the cytoplasm. However, NF-kB can be activated by various agents such as radicals, inflammatory stimuli, tumor necrosis factor (TNF), cytokines, carcinogens, tumor promoters, endotoxins, g-radiation, ultraviolet (UV) light, and X-rays in all cell types and cause pro-inflammatory effects. These circumstance led to the expression of inflammatory genes include cyclooxygenase-2 (COX-2), lipoxygenase-2 (LOX-2), cell-adhesion molecules, inflammatory cytokines, chemokines, and inducible nitric oxide synthase (iNOS)(Aggarwal and Shishodia, 2006),(Yoon and Liu, 2007).. Shishodia et al.(Shishodia et al., 2003) reported that ursolic acid regulated cytokine signaling pathway by suppressing TNF-induced NF-κB activation. Ursolic acid inhibited NF-κB in a dose and time dependent manner with the maximum inhibition at concentration of 100μM. In addition, ursolic acid suppressed the activation of NF-κB induced by TNF through the pathway of inhibition of IKK, IκBα degradation and phosphorylation. Furthermore, the suppression of p65 nuclear translocation ultimately led to the prevention of DNA binding of NF-κB and gene transcription. In addition, down-

regulations of cyclooxygenase 2, matrix metalloproteinase 9 and cyclin D1 were also observed in ursolic acid treatment. Manu et al.(Manu and Kuttan, 2008) suggested that ursolic acid also suppressed the activation of NF-κB transcription factors in B16F-10 melanoma cells. These factors, including NF-κB p65, NF-κB p50 and NF-κB c-Rel, were inhibited by 67.61%, 67.17% and 69.28%, respectively, after treating with ursolic acid at the dosage of 50 μM for 2 hours.

1.4.4 Cytotoxicity

Ursolic acid was considered relatively non-toxic(Liu, 1995). Research demonstrated that ursolic acid exhibited low cytotoxicity against most of human cancer cell lines, such as NTUB1 bladder cancer cells(Tu et al., 2009), hepatoma cell line HepG2, gastric carcinoma cell line AGS, colorectal carcinoma cell line HT-29, prostatic carcinoma cell line PC-3(Bai et al., 2012b), ovarian cancer cell line SK-OV3 and A2780(Song et al., 2012). According to the data, the IC_{50} of cytotoxicity for ursolic acid towards cancer cell lines varies between the concentration range of 20.6 μM to 65.0 μM. To explore the relationship between structure and cytotoxicity, ursolic acid derivatives was modified and applied on human cancer cells, such as colon cancer HT29 cells(Bai et al., 2012a). In this study, ursolic acid showed the most cytotoxic on HT 29 cells compared with other derivatives. In addition, C-3 position acylation and C-28 glycosylation on ursolic acid derivatives showed reduced or no cytotoxicity on HT 29 cells, indicating these structural modifications are potential contributors of cytotoxicity. Moreover, Bai *et al.* (Bai et al., 2012b) reported that IC_{50} was significantly decreased by esterification of hydroxyl group and carboxyl group of ursolic acid; negatively and positively charged UA derivatives would significantly affect the cytotoxicity value in different cancer cells.

1.5. Anti-inflammatory effects

Ursolic acid anti-inflammatory effect *in vitro* were initially reported by Najid *et al* in 1992(Najid et al., 1992). In this study, ursolic acid was extracted from *Calluna vulgaris*, demonstrated to significantly inhibit the formation of the lipoxygenase (12-LOX and 15-LOX) and cyclooxygenase productions on peritoneal macrophages, platelets cell lines and leukemic HL60 cell lines by 80, 60 and 38%, respectively. Other studies reported that ursolic acid had anti-inflammatory effect on rat adrenal gland pheochromocytoma PC12 cell line(Tsai and Yin, 2008). Ursolic acid had minor effect on the expression of IL-6 and TNF-α (pro-inflammatory indicators) on normal PC12 cells. However, after H_2O_2 and MPP^+ introduced, pre-treatment of ursolic acid at 40 μM significantly inhibited the inflammatory induced expression of IL-6 and TNF-α by 52.8% and 65.8%, respectively. Takada *et al*. suggested that ursolic acid suppressed TNF-α induced inflammatory injury by the inhibition of NF-κB activation in cultured umbilical vein endothelial cells (Takada et al., 2010). Wang *et al.* proposed that ursolic acid inhibited LPS induced IκBα phosphrolation and degradation on mouse brain cell, decreased the translocation of NF-κB and p38 activation, reduced the expression most of pro-inflammatory markers COX-2, iNOS, TNF-α, IL-1β, IL-6 *in vivo*(Wang et al., 2011). Lu *et al*. reported that ursolic acid inhibited the damage on d-galactose induced inflammatory on mouse prefrontal cortex. In this study, ursolic acid inhibited COX-2 and iNOS through AGEs/RAGE/NF-κB signaling pathways (Lu et al., 2010). Other inflammatory indicators, like VCAM-1, LOX-1, ICAM, IL-17 were also found to be inhibited by ursolic acid on human umbilical vein endothelial cells (HUVECs)(Schwaiger et al., 2011),(Lv et al., 2012) and T-helper 17 cells (T_h17)(Xu et al., 2011). However, some studies determined that ursolic acid might have pro-

inflammatory effect on non-induced murine peritoneal macrophages (Ikeda et al., 2007),(Ikeda et al., 2008). It is proposed that aggregated ursolic acid would bind cell membrane protein CD36 and generate ROS, activate the p38 MAPK pathway, release the elevated mount of IL-1β which would eventually lead to the inflammatory damage.

1.6. Conclusions and Perspectives

There are growing interests on the relationships between health benefits and specific phytochemicals presenting in fruits and vegetables. The underlying mechanisms still remains unclear. However, it does provide plenty of insights for developing new therapeutic treatments by using these bioactive compounds. Ursolic acid has been considered as an anti-cancer and anti-inflammatory phytochemical. Current research proposed several molecular mechanisms and explore possible interaction initiated by ursolic acid. Therefore, the development of drugs and therapies based on ursolic acid bioactive properties would not only target the pharmaceutical industry but also improve human health for the future.

Figure 1.1 Proposed biosynthesis pathways of ursolic acid derivatives

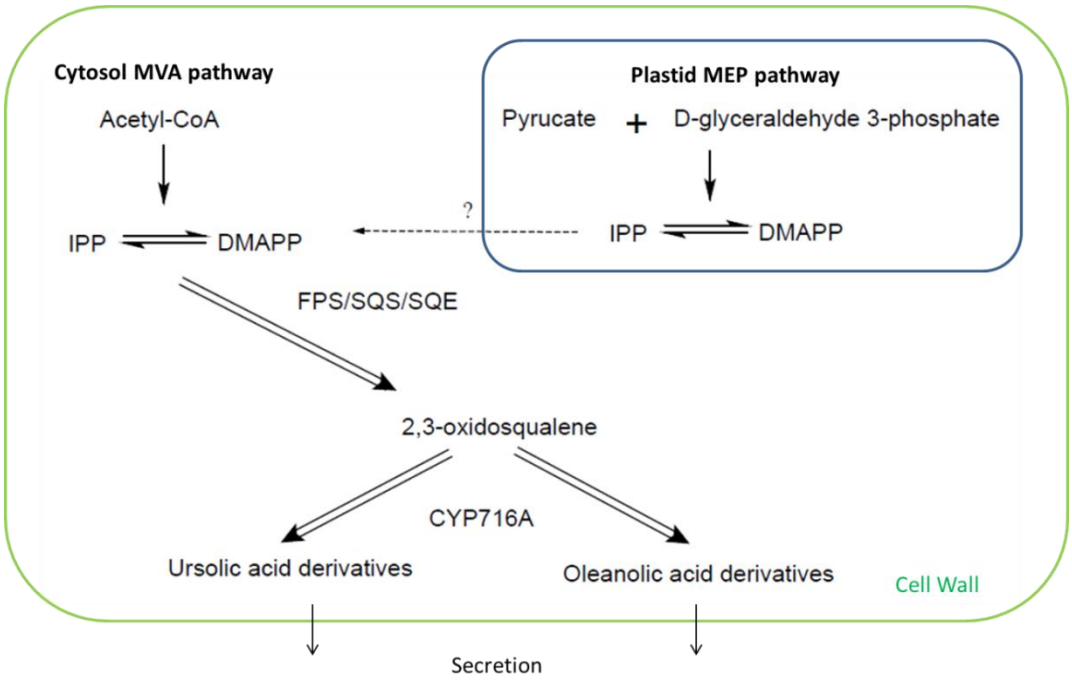


Table 1.1 Major edible sources containing ursolic acid and levels presented in mg/100g

Sources		Botanical Name	Levels (mg/100g)	Reference
Dietary Sources	Apple Peel	*Malus pumila*	152	(He&Liu, 2007)
	Balsam Pear	*Momordica charantia*	42±5	(Yin et al, 2012)
	Brown Mustard	*Brassica juncea*	14±5	(Yin et al, 2012)
	Star Fruit	*Averrhoa carambola*	13±4	(Yin et al, 2012)
	Guava	*Psidium guajava*	12±2	(Yin et al, 2012)
Herb and Spices	Thyme	*Thymus vulgaris* L.	940	(Rowe et al, 1949)
	Marjoram	*Origanum marjorana*	660	(Vagi et al, 2002)
	Sage	*Salvia officinalis* L.	415	(Razborsek et al, 2007)
	Rosemary	*Rosmarinus officinalis L.*	189	(Razborsek et al, 2007)
	Holy Basil	*Ocimum sanctum* L.	17±6	(Yin et al, 2012)
	Lavender	*Lavandula augustifolia Mill.*	1050-1590	(Gotfredsen, 2009)
	Rosinweeds	*Silphium integrifolium*	1498-1550	(Kowalski, 2007)
	Oleander	*Nerium oleander*	1270	(Fu et al, 2005)
	Bearberry Leaves	*Arctostaphylos uva-ursi*	1240	(Gotfredsen, 2009; Duke, 2009)
	Winter Savory	*Satureja montana*	667	(Razborsek et al, 2007)
	Hawthorn	*Crataegus*	520	(Liu et al, 2009)
	Damnacanthus	*Damnacanthus*	19.2-192	(Cai&Huang,

	indicus		2012)
Self Heal	*Prunella vulgaris* L.	98-177	(Chen et al, 2012)
Mahogany	*Swietenia macrophylla*	57±8	(Yin et al, 2012)
Daylily	*Hemerocallis sp*	19±5	(Yin et al, 2012)

CHAPTER TWO

URSOLIC ACID, A TRITERPENOID ISOLATED FROM APPLE PEELS, INHIBITED MDA-MB-231 HUMAN BREAST CANCER CELL PROLIFERATION THROUGH p38/MAP KINASE PATHWAY

2.1 Introduction

Breast cancer is becoming the most frequently diagnosed (29%) and the second death rate (15%) cancer among women in United States in 2014 with an estimated 232,670 new cases and 40,000 death(Siegel et al., 2014). Siegel *et al*. reported that incidence and mortality rates of breast cancer and can be distinguished by sex, age, race and ethnicity. Between 2006 and 2010, non-Hispanic white has the highest incidence (127.3 per 100,000) with mortality rate (22.7 per 100,000) and Asian/Pacific Islander has the lowest incidence (84.7 per 100,000) with mortality rate (11.5 per 100,000) between 2006 and 2010. Despite of early detection and treatment, current breast cancer therapies, including BCS (breast-conserving surgery), radiation therapy and systemic therapy (hormone and targeted therapy), cannot effectively control the growth of human breast cancer. Thus, there is a urgent need to find an alternative treatment to improve therapy efficiency with low side effects .

Scientific evidence have suggested that high consumption of fruits and vegetables are related with reducing the risk of chronic diseases including cancer, cardiovascular diseases *etc*. (Liu, 2003),(Boyer and Liu, 2004). Phytochemicals from fruits and vegetables are considered as be primarily responsible for these health

benefits. Therefore, most of studies have been focusing on the function of phytochemicals in cancer treatment and prevention. Sartippour *et al.* reported that green tea extracts, majorly including epigallocatechin-3-gallate (EGCG), epicatechin (EC) and epigallocatechin (EGC), enhanced the inhibitory effect of tamoxifen on three estrogen receptor positive (ER^+) human breast cancer cells, MCF-7, ZR75 and T47D(Sartippour et al., 2006). In addition, the combination of green tea extracts and tamoxifen synergistically induced the cell apoptosis and suppressed the angiogenesis in treated xenografts *in vivo*. These results implied that phytochemicals in green tea are highly associated with regulation of estrogen related signaling pathways in ER^+ cancer, and then enhanced the inhibitory effect on tamoxifen tradition therapy.

Some epidemiology studies suggested that overall protective effect of fruits and vegetables might be more significant on specific types of breast cancer than others(Thomson and Thompson, 2013). Jung *et al*. reported that a significant protective effect was observed on ER^-, but not ER^+ breast cancer by high intake of fruits and vegetables(Jung et al., 2013). In another 20 cohort studies, a statistically significant inverse-association between ER^- negative breast cancer and vegetables consumption were observed in a large pooled analyses. Similar conclusion was also found in a case-only study designed for building risk factors correlating with human breast cancer subtypes(Martinez et al., 2010). Moreover, Adams *et al*. proposed that the expression of phosphatidylinositol 3-kinase (PI3K)/AKT, metalloproteinase-9 and caspase-3 were regulated by blueberry extracts on the triple negative cancer MDA-MB-231 cells. In addition, data of scratch motility assay and migration assay suggested that blueberry extracts significantly inhibit the MDA-MB-231 cell motility and migration ability *in vitro*. *In vivo*, blueberry-pretreated mice showed significantly

decreased in tumor size and MDA-MB-231 cell proliferative ability when compared to control on xenograft model. Immunological analysis showed that the activity of AKT and p65 NF-κB from tumor from blueberry-treatment mice was found to be significantly decreased. All these finding indicated that phytochemicals from fruits and vegetables exhibit a great potential on the regulation of breast cancers in various types. However, mechanism of action of specific phytochemicals on breast cancer is not well understood yet.

In this paper, our objective is to determine the mechanism of actions of ursolic acid antiproliferative effect on human breast cancer MDA-MB-231. Ursolic acid is a triterpenoids distributed in apples peels and other fruits, and has been reported to exhibit significant anticancer effect on human breast cancer MCF-7(Kassi et al., 2009a),(Wang et al., 2012) and MDA-MB-231(Yeh et al., 2010b). It is critical to fully understand about regulatory signaling transduction pathway by ursolic acid for further exploration of unique therapy against breast cancer.

2.2. Materials and methods

2.2.1 Cell Culture

MDA-MB-231 human breast cancer cells were obtained from Animal Tissue Culture Collection (ATCC, Rockville, MD), maintained in Minimum Essential Media α (α-MEM) (Invitrogen, Carlsbad, CA) at 37°C and 5% CO_2.Working medium contained 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Inc, Lawrenceville, GA), 10 mM Hepes (Sigma Aldrich, St. Louis, MO), 50 unites /ml pencilin and 50 μg/ml Streptomycin(Gibco BRL Life Technologies, Grand Island, NY, USA).

2.2.2 Chemicals

Ursolic acid was isolated and purified from our laboratory as reported previously(He and Liu, 2007a) with the purity is >99%. Dimethyl sulfoxide (DMSO) was purchased from VWR (Radnor, PA). DPBS was obtained from Thermo Scientific (Waltham, MA), and polyoxyethylene (tween 20) sorbitan monolaurate was obtained from Calbiochem (Billerica, MA)

2.2.3 Antibodies and inhibitor

Primary antibody against PCNA was obtained from Calbiochem (Billerica, MA). Antibodies against CDK-4, Cyclin D1, p21, p-p53, p-ASK-1 and TRAF-2 were purchased from Santa Cruz Biotechnolgy, Inc. (Santa Cruz, CA). Antibody against p-p38 was from Cell Signaling Technolgy, Inc. (Danvers, MA). Antibodies against β-actin and α-tublin, and HRP-conjugated secondary antibodies (anti-mouse IgG and anti-rabbit IgG) were obtained from Sigma-Aldrich Inc. (St. Louis, MO). SB203580 (p38 MAPK inhibitor) was obtained from Cell Signaling Technology, Inc. (Danvers, MA).

2.2.4 Measurement of inhibition in cell proliferation of ursolic acid

The proliferation inhibition of ursolic acid was analyzed by methylene blue assay as described previously (Felice et al., 2009). In brief, ursolic acid was dissolved in dimethyl sulfoxide to the range from 1mM to 10 mM. MDA-MB-231 human breast cancer cells were plated in 96-well plate at a density of $2.5x10^4$ cells per well and were incubated for 6 hours at 37 ^oC and 5% CO_2. Then cell were treated at designed concentrations of ursolic acid for another 72 hours with control containing 1% DMSO. Cell were rinsed with phosphate buffered saline (PBS) twice and stained with methylene blue solution containing 1.25% glutaldehyde and 0.6% methylene blue at

37 ^{o}C and 5% CO_2 for 1 hour. Methylene blue stain was eluted by 49% (v/v) PBS, 50% (v/v) ethanol and 1% (v/v) acetic acid elution buffer at room temperature for 30 mins. The absorbance was measured at 570 nm by using MRX II Dynex plate reader (Dynex Technologies, Inc., Chantilly, VA). Data were collected in triplicate for statistical analysis.

2.2.5 Measurement of cytotoxicity activity of ursolic acid

The cytotoxicity of ursolic acid on MDA-MB-231 cells was measured by methylene blue assay as reported previously(Wolfe and Liu, 2007b). Briefly, $4x10^4$ cells per well of MDA-MB-231 cells were plated in 96-well plate and incubated for 24 hours at 37 ^{o}C and 5% CO_2. Then, serious dosages of ursolic acid were treated with cell for another 24 hours. After that, cell were rinsed by PBS and stained in methylene blue solution containing 1.25% glutaldehyde and 0.6% methylene blue at 37 ^{o}C and 5% CO_2 for 1 hour, and then eluted by 49% (v/v) PBS, 50% (v/v) ethanol and 1% (v/v) acetic acid elution buffer at room temperature for 30 mins. Absorbance was measured at 570 nm as described above. Data were collected in triplicate for statistical analysis.

2.2.6 Determination of specific proteins expression by western blot

For protein analysis and western blotting, methods were followed by our previous work(Liu et al., 1997) with modification(Yoon and Liu, 2008). In brief, MDA-MB-231 cells were plated in 6-well plates at 37 ^{o}C and 5% CO_2 for 6 hours. Ursolic acid was treated at the dosage of 20, 35 and 45 μM with control group containing 1% DMSO only. After 24 hours treatment, cells were rinsed and collected by PBS buffer solution, lysed by RIPA buffer (50 mM Tris Base, 1% Igepal, 150 mM sodium chloride, 1 mM EDTA, pH=7.4) with protease inhibitors (1 mg/mL aprotinine,

1 mg/mL leupeptin, 1 mg/mL pepstain, 200 mM sodium orthovanadate). Suspensions were vortexed periodically for 30 mins, centrifuged at 12000g for 15 mins at 4 ^{o}C and then supernatant was collected as the cytoplasmic protein and stored at -20 ^{o}C. Certain amount of proteins was subjected to 10% or 12% (w/v) SDS-polyacrylamide gel for electrophoresis where 100 V were applied for 1.0 to 1.5 hours. After gel electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membrane in a 10% methanol transfer buffer solution at 100 V for 40 mins. Then, PVDF membrane was blocked by 5 % non-fat milk dissolved in Tris-buffered saline with Tween-20 (TBST) at the room temperature for 2 hours. Primary antibody were applied at a dilution of 1:1000 for overnight incubation at 4 ^{o}C, followed by the corresponding secondary antibody in 5% non-fat milk incubation for 2 hours at room temperature. Western Blot results were revealed by LumiGLO chemoluminescent substrate (Cell Signaling, Beverly, MA) followed by Phototope-HRP detection assay. Protein expression was quantified on Kodak Biomax MR Film (Kodak, Rochester, NY) by developing and fixing process. The band analysis was produced by ImageJ software 1.43u.

2.2.7 Inhibition of p38/MAPK pathway

SB203580, p38 specific inhibitor, was pretreated with MDA-MB-231 cells at dosage of 50 nM, 1 μM and 20 μM for 1.5 hours, then replaced by growth medium containing ursolic acid working concentration at 45 μM for 24 hours. Fresh medium with 1% DMSO (v/v) was used as negative control. Growth medium with 20 μM of SB203580 and growth medium with 45 μM of ursolic acid were selected as positive controls. Protein analysis and western blot assay were followed the protocol as

described above. Protein expression of PCNA and Cyclin D1 were evaluated for mechanism of p38/MAP kinase pathway regulation by ursolic acid.

2.2.8 Statistical analysis

All data were collected as mean ± SD for triplicate. Statistical results was performed by SigmaPlot software 11.0 (Systat Software, Inc. Chicago, IL) and all dose-effect analysis was performed by Calcusyn Software version 2.0 (Biosoft, Cambridge, UK). The significant difference in western blot results was performed by T-test with a p-value of 0.05. Results were analysis by JMP software 10.0.0 for ANOVA method (SAS institute, Inc.)

2.3. Results

2.3.1 Inhibition of cell proliferation and cytotoxicity by ursolic acid in MDA-MB-231 human breast cancer cells

The antiproliferative activity of ursolic acid in MDA-MB-231 human breast cancer cells was evaluated and shown in **Figure 2.1**. Ursolic acid significantly inhibited MDA-MB-231 cell proliferation at the dose of 10 μM and above ($p < 0.05$); The EC_{50} (half maximal effective concentration) of ursolic acid in inhibition of MDA-MB-231 cell proliferation was observed at 25.26 ± 0.28 μM. At the dosage of 50 μM, ursolic acid inhibited cell proliferation of MDA-MB-231 by approximate 90%.There was no cytotoxicity (<10%) was observed at the concentration below 50 μM.

2.3.2 Regulation of PCNA expression by ursolic acid in MDA-MB-231 cells

Ursolic acid down-regulated the expression of PCNA in MDA-MB-231 human breast cancer cells in a dose-dependent manner (**Figure 2.2**). At the dosage of 45 μM,

ursolic acid significantly decreased the expression of PCNA by 45.51 ± 3.65%. At and above 35 μM, ursolic acid significantly inhibited the proliferation of MDA-MB-231 human breast cancer cells when compared with the control.

2.3.3 Regulation of Cyclin D1 expression by ursolic acid in MDA-MB-231 cells

Ursolic acid down-regulated the expression of Cyclin D1 in MDA-MB-231 human breast cancer cells in a dose-dependent manner (**Figure 2.3**). At the dosage of 45 μM, ursolic acid significantly decreased the expression of PCNA by 50.27 ± 9.68%. At and above 35 μM, ursolic acid significantly inhibited the expression of Cyclin D1 in MDA-MB-231 cells when compared with the control.

2.3.4 Regulation of CDK-4 expression by ursolic acid in MDA-MB-231 human breast cancer cells

Ursolic acid down-regulated the expression of CDK-4 in MDA-MB-231 human breast cancer cells in a dose-dependent manner (**Figure 2.4**). At the dosage of 45 μM, ursolic acid significantly decreased the expression of PCNA by 35.84 ± 11.3%. At and above 35 μM, ursolic acid inhibition effect was significantly different when compared with the control.

2.3.5 Regulation of p21 expression by ursolic acid in MDA-MB-231 human breast cancer cells

Ursolic acid up-regulated the expression of p21 in MDA-MB-231 human breast cancer cells in a dose-dependent manner (**Figure 2.5**). At the dosage of 45 μM, ursolic acid significantly increased the expression of p21 by 176.97 ± 18.77%. At and above 45 μM, ursolic acid inhibition effect was significantly different when compared with the control.

2.3.6 Regulation of p-p53 expression by ursolic acid in MDA-MB-231 human breast cancer cells

Ursolic acid up-regulated the expression of p-p53 in MDA-MB-231 cells in a dose-dependent manner (**Figure 2.6**). At the dosage of 45 μM, ursolic acid significantly increased the expression of p-p53 by 50.61 ± 11.00%. ANOVA data suggested that at and above 20 μM, ursolic acid significantly increased the expression of p-p53 when compared with the control.

2.3.7 Regulation of p-p38 expression by ursolic acid in MDA-MB-231 human breast cancer cells

Ursolic acid up-regulated the expression of p-p38 in MDA-MB-231 human breast cancer cells in a dose-dependent manner (**Figure 2.7**). At the dosage of 45 μM, ursolic acid significantly increased the expression of p-p38 by 225.46 ± 19.94%. At and above 35 μM, ursolic acid significantly increased the expression of p-p38 when compared with the control.

2.3.8 Regulation of p-ASK1 expression by ursolic acid in MDA-MB-231 human breast cancer cells

Ursolic acid up-regulated the expression of p-ASK1 in MDA-MB-231 human breast cancer cells in a dose-dependent manner (**Figure 2.8**). The expression of p-ASK1 were significantly increased by 147.76 ± 10.11% at the concentration of 45 μM. ANOVA data suggested that compared with control group, ursolic acid inhibition effect was significantly different at and above the concentration of 35 μM.

2.3.9 Ursolic acid regulation of TRAF-2 expression in MDA-MB-231 cells

Ursolic acid down-regulated the expression of TRAF-2 in MDA-MB-231 human

breast cancer cells in a dose-dependent manner (**Figure 2.9**). The expression of TRAF-2 were significantly increased by 68.45 ± 2.10% at the concentration of 45 μM. ANOVA data suggested that compared with control group, ursolic acid inhibition effect was significantly different at and above the concentration of 35 μM.

2.3.10 Specific inhibitor of p38/MAPK effect on ursolic acid regulation of PCNA expression

SB203580, a specific inhibitor for p38, was used to determine whether ursolic acid regulated the cell proliferation through p38/MAPK pathway. Results were shown that at dosages of 20 μM, SB203580 was not observed any interference in the protein expression in MDA-MB-231 cells. Ursolic acid alone at the dosage of 45 μM inhibited the expression by 64.36% ± 8.72. With a UA dosage of 45 μM, SB203580 inhibited the protein expression in a dose-dependent manner when compared with control group: at the dosages of 50 nM, 1 μM and 20 μM, SB203580 reversed the UA antiproliferative effect by 2.7%, 24.3% and 41.2%, respectively. At last, SB203580 efficiently inhibited the UA antiproliferative effect on MDA-MB-231 at the dosage of 20 μM by 105.54% ± 6.27. All data were shown in **Figure 2.10**.

2.3.11 Specific inhibitor of p38/MAPK effect on ursolic acid regulation of Cyclin D1 expression

SB203580 at the dosage of 20 μM was not observed much effect in the protein expression of Cyclin D1 in MDA-MB-231 cells. Ursolic acid alone at the dosage of 45 μM inhibited the protein expression by 65.43% ± 8.94. With a UA dosage of 45 μM, SB203580 inhibited the expression of protein in a dose-dependent manner when compared with control group: at the dosages of 50 nM, 1 μM and 20 μM, SB203580

reversed the UA antiproliferative effect by 0.3%, 16.4% and 23.1%, respectively. At last, SB203580 inhibited the UA antiproliferative effect on MDA-MB-231 at the dosage of 20 μM by 88.5% ± 3.26. All data were shown in **Figure 2.11**.

2.4. Discussion

This is the first time to report that ursolic acid inhibits MDA-MB-231 human breast cancer cell proliferation through p38/MAP kinase pathway. In our study, cyclin D1 and CDK4 has been determined to be regulated by ursolic acid. Both cyclin D and CDK families are key regulatory proteins in cell cycle checkpoint system, and ursolic acid is strongly responsible for these anti-proliferation effects. Huang *et al*. reported that combined treatment of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (5GG) and quercetin induced sub-G1 phase and S phase accumulation in MDA-MB-231 human breast cancer cells in flow cytometric analysis, and immunochemical detection of cyclinD1 and CDK4 expression were both reduced *in vitro*(Huang et al., 2013). Other phytochemicals mixture, such as fruits extracts, is also revealed to exhibit antiproliferation effect in MDA-MB-231 cells(Sun and Liu, 2008). Sun *et al.* proposed that phenolics from apples extracts modulated the expression of CDK4 and cyclin D1 to achieve the effect of anti-proliferation in MDA-MB-231 cells. DNA flow cytometric analysis also showed that apple extracts could significantly induced G1 arrest in MDA-MB-231 cells. All these results implied the regulation of UA antiproliferation effect might be through cell cycle system in MDA-MB-231 cells. PCNA is considered to be a critical protein for cell proliferation. Paunesku et al. suggested that PCNA was highly associated with cell proliferation in most mammalian cells(Paunesku et al., 2001). Most recently, Malkas *et al*. developed a unique antibody

which can specifically detect one PCNA isomer (caPCNA) associated with growth in human breast cancer cells and tissues(Malkas et al., 2006). Thus, PCNA was chosen as an important biomarker to determine the UA antiproliferative effect in this paper.

p53 is commonly considered as tumor-suppression gene. Controversially, Li et al. reported that genistein, an isoflavonoid distributed mostly in soy products, induced the down-regulation of p53 expression in MDA-MB-231 cells. They proposed that genistein might affect the cell proliferation in a p53-independent signal pathway due to the p53 mutant status in MDA-MB-231 cells. Di et al. proposed that in the breast tumor cell with mutant p53 (MDA-MB-231), induction of p21 by Adriamycin, a drug used in cancer chemotherapy, highly occurred without the function of p53. Our immunological data showed that ursolic acid modulated the similar effect on down-regulation of the expression of p53, suggesting the ursolic acid might go through a p53 independent pathway in order to induce the expression of p21. Further studies are required to elucidate the function of p53 in p53 mutant status of cancer cells.

Most significantly, p38 specific inhibitor is applied with ursolic acid in order to determine the role p38/MAP kinase in regulation of proliferation by UA in MDA-MB-231 human breast cancer cells. Du *et al*. reported that evodiamine, a quinolone alkaloid, treated in MDA-MB-231 human breast cancer cells with SB203580 significantly increased the cell migration rate by 20% when compared with evodiamine treatment alone. This result suggested that p38 MAP kinase pathway plays an important role in the regulation of cell migration in MDA-MB-231 cells. Our data revealed that SB203580 effectively reversed the expression of PCNA and cyclin D1 reduced by ursolic acid by blocking the function of p38. All these evidence suggest that ursolic acid control the cell proliferation on MDA-MB-231 through p38/MAP

kinase pathway.

Developing a unique therapy for breast cancer treatment is one of the most promising researches in the last decades. Most women with breast cancer will perform surgery, combined with other treatments such as chemotherapy, radiation, and hormone therapies. Estrogen, as well as progesterone and progestin, were found out to promote breast cancer cell growth. Women who have positive testing results on estrogen or progesterone receptors (ER^+) can be given hormone therapy to low down the level of estrogen, in order to block the growth effect on breast cancer cells. Tamoxifen is well used as known anticancer agent by modulating circulating estrogen level in breast tissues, inhibiting the tumor growth and proliferation since early 1980s(Reddel et al., 1983). Interestingly, phytochemicals, such as EGCG and CG., might enhance the anticancer effect combined with tamoxifen treatment. These new insights provide strong evidence to support that phytochemicals in fruits and vegetables have great potential in design and development of new therap-iesy to improve the outcome of human breast cancer.

In this study, ursolic acid, a tertripenoid widely distributed in fruits and vegetables, exhibits high anticancer effect in MDA-MB-231 human breast cancer cells and can be potentially used in treatment and prevention of breast cancer. Kim *et al*. reported that ursolic acid induced apoptosis through mitochondrial death pathway by regulating proteins of Bax/Bcl-2 and caspase families towards MDA-MB-231 human breast cancer cells (Kim et al., 2011a). Our previous work suggested that ursolic acid, isolated from apple peels(He and Liu, 2007a), had the ability to inhibit cell proliferation in a dose-dependent manner through p38/MAP kinase signaling transduction pathway (**Figure 2.12**). Hypothetically, ursolic acid has great possibility

in regulation of TNF-α and associated receptors. TNF-α is a cytokine initially expressed as a transmembrane precursor, pro-TNF-α, which will be cleaved by TNF-converting enzyme in various cells(Black et al., 1997). After TNF-α is activated, it binds either TNF receotpr-1 (TNFR-1) or TNF receptor-2 (TNFR-2)(Gaur and Aggarwal, 2003). Upon the binding between TNF-α and TNFR-1, the initiated signaling would transmit to TNFR-associated death domain (TRADD), which binds to one of adaptation proteins TRAF-2 (TNFR-associated factor 2)(Wertz and Dixit, 2008).

In summary, results demonstrated that ursolic acid inhibited the cell proliferation towards MDA-MB-231 human breast cancer through p38/MAP kinase signaling transduction pathway. UA inhibition of cell proliferation was regulated by the down-regulation of PCNA, cyclinD1, CDK 4, p-p53, TRAF-2 expression, as well as the up-regulation of p21, p-p38 and p-ASK1. Specific inhibitor of p38/MAP kinase was applied to block the expression of p38 in order to evaluate the role of p38/MAP kinase in the UA regulation of cell proliferation in MDA-MB-231 human breast cancer. Protein expression of PCNA and cyclinD1 were significantly recovered by SB203580 treatment at high dosage of ursolic acid, indicating UA cell proliferation is modulated through p38/MAP kinase. Future work on mechanism of actions of ursolic acid on cell apoptosis or cell membrane receptors interaction would be essential to fully understand the function of ursolic acid in the prevention of human breast cancers.

Figure 2.1. Effects of ursolic acid on cell proliferation and cytotoxicity in MDA-MB-231 human breast cancer cells. Each value represents the mean ± SD with triplicates.

* indicates significant difference from the proliferation control.

# indicates significant difference from the cytotoxicity control.

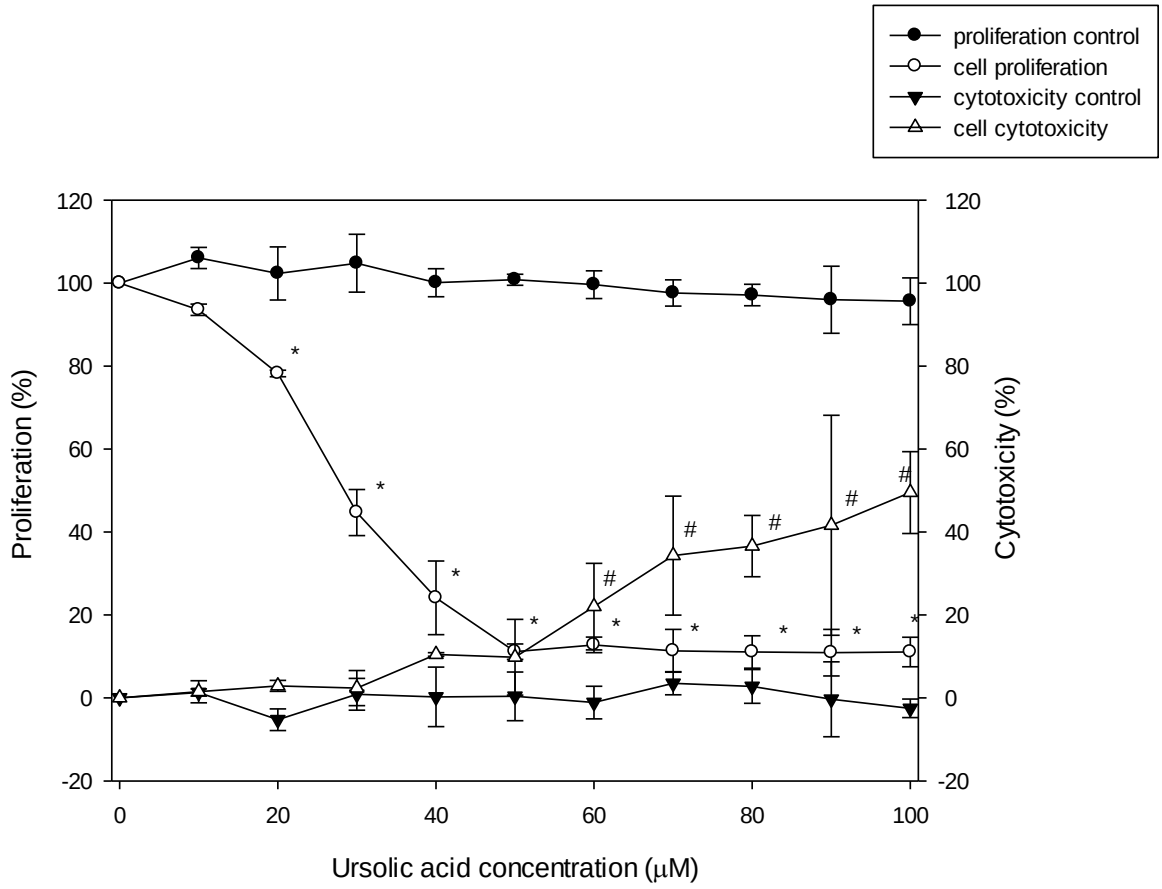


Figure 2.2. Effects of ursolic acid on PCNA expression in MDA-MB-231 human breast cancer cells. Data were expressed as mean ± SD, n=3. Bar with no letters in common are significantly different ($p<0.05$).

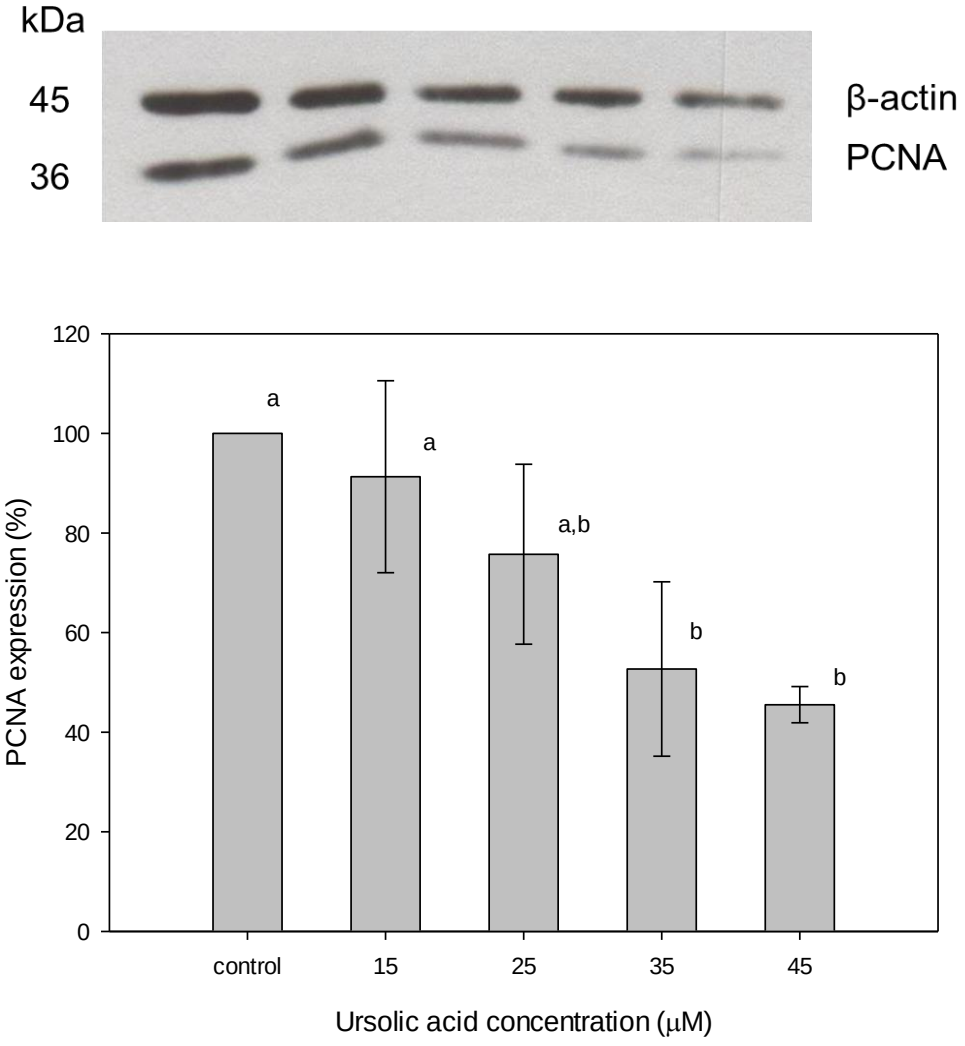
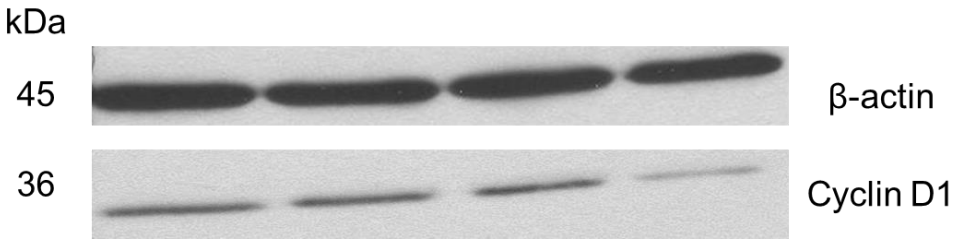


Figure 2.3. Effects of ursolic acid on Cyclin D1 expression in MDA-MB-231 human breast cancer Cells. Data were expressed as mean ± SD, n=3. Bar with no letters in common are significantly different ($p<0.05$).



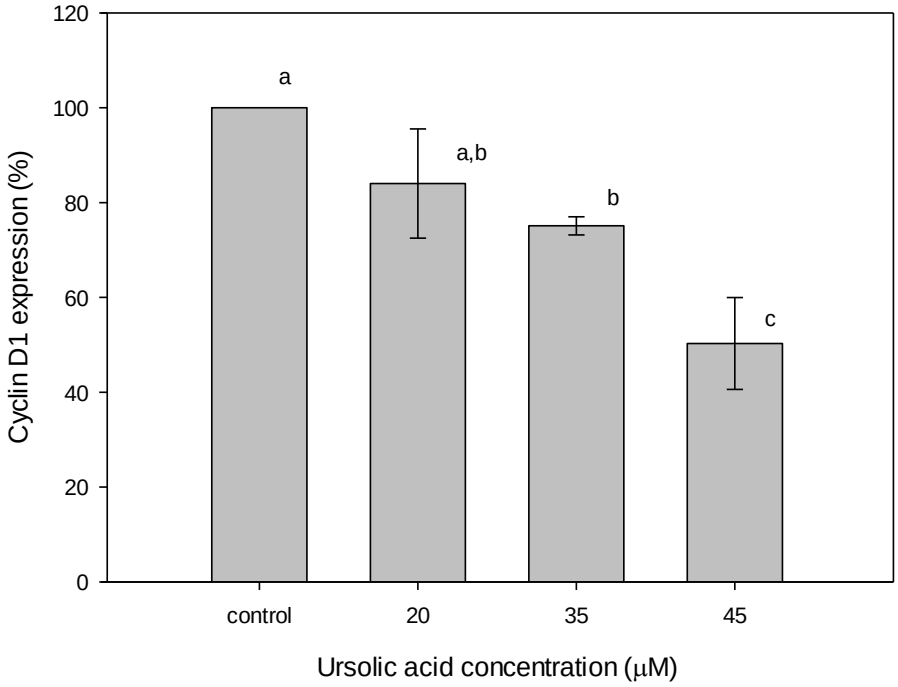
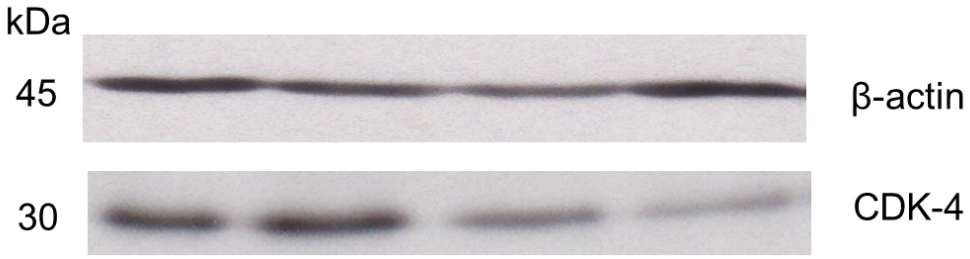


Figure 2.4. Effects of ursolic acid on CDK4 expression in MDA-MB-231 human breast cancer cells. Data were expressed as mean ± SD, n=3. Bar with no letters in common are significantly different ($p<0.05$).



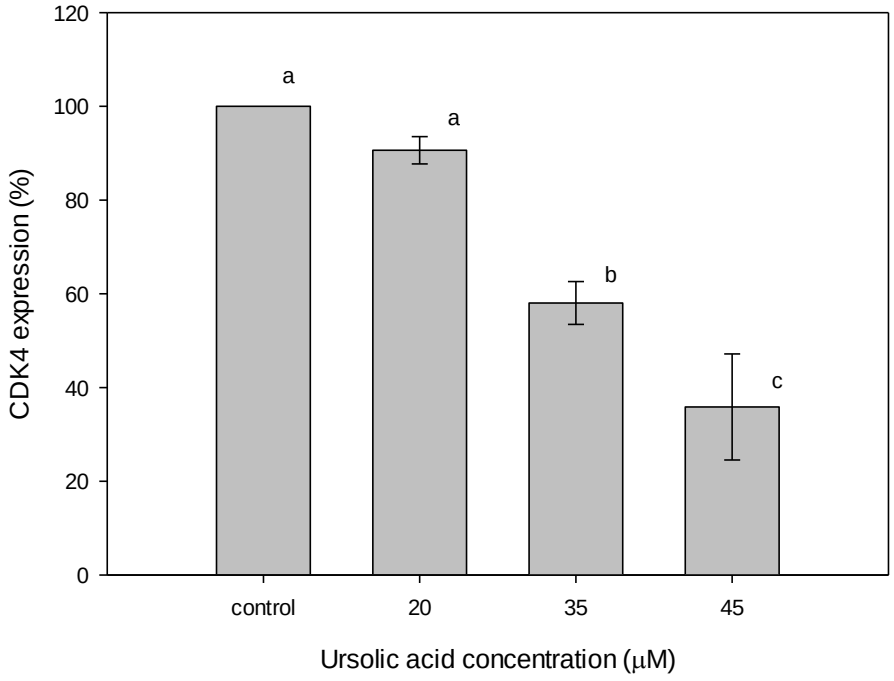
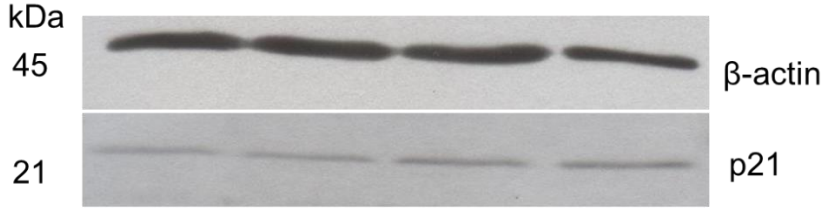


Figure 2.5. Effects of ursolic acid on p21 expression in MDA-MB-231 human breast cancer cells. Data were expressed as mean ± SD, n=3. Bar with no letters in common are significantly different ($p<0.05$).



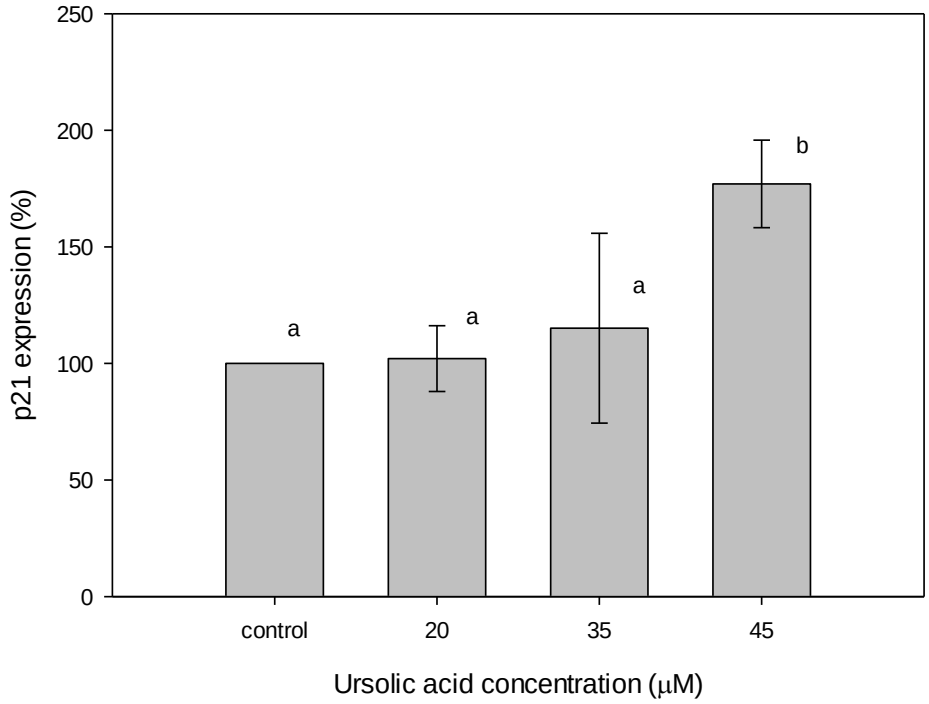


Figure 2.6. Effects of ursolic acid on p-p53 expression in MDA-MB-231 human breast cancer cells. Data were expressed as mean ± SD, n=3. Bar with no letters in common are significantly different ($p<0.05$).

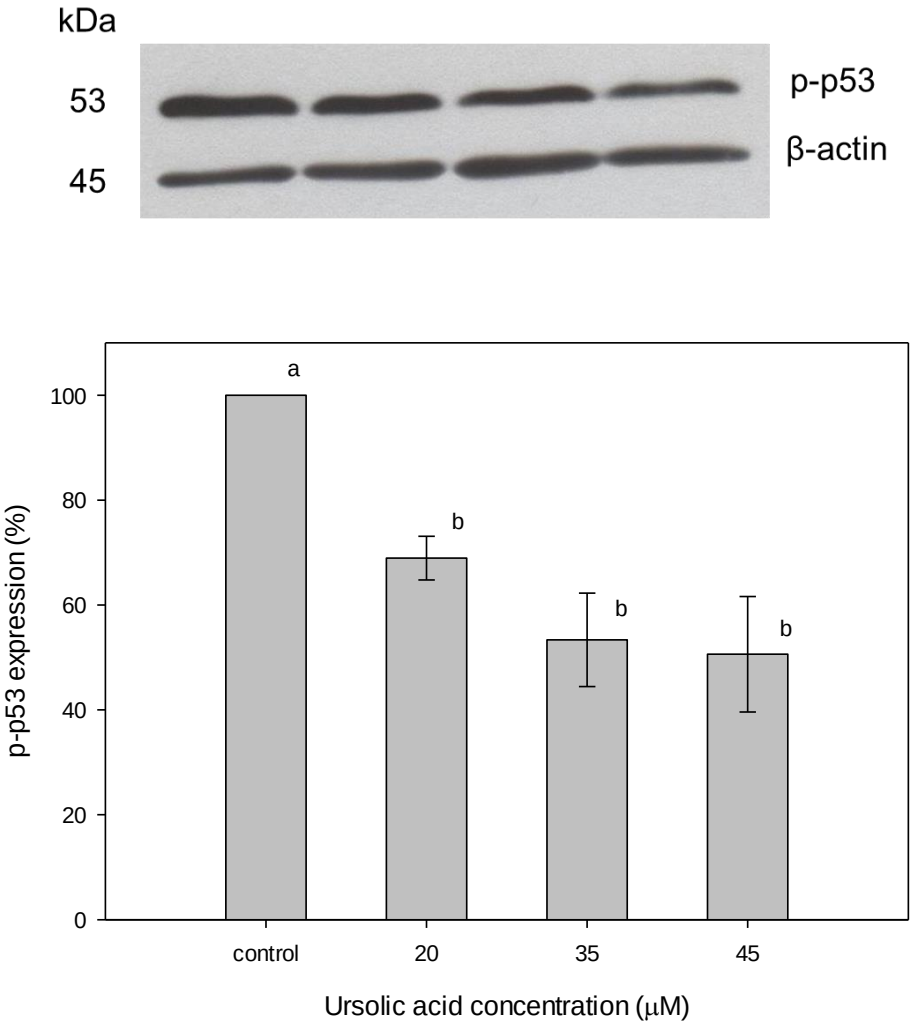
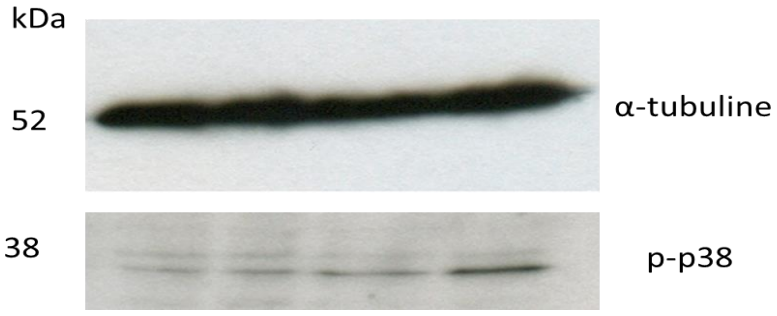


Figure 2.7. Effects of ursolic acid on p-p38 expression in MDA-MB-231 human breast cancer cells. Data were expressed as mean ± SD, n=3. Bar with no letters in common are significantly different ($p<0.05$).



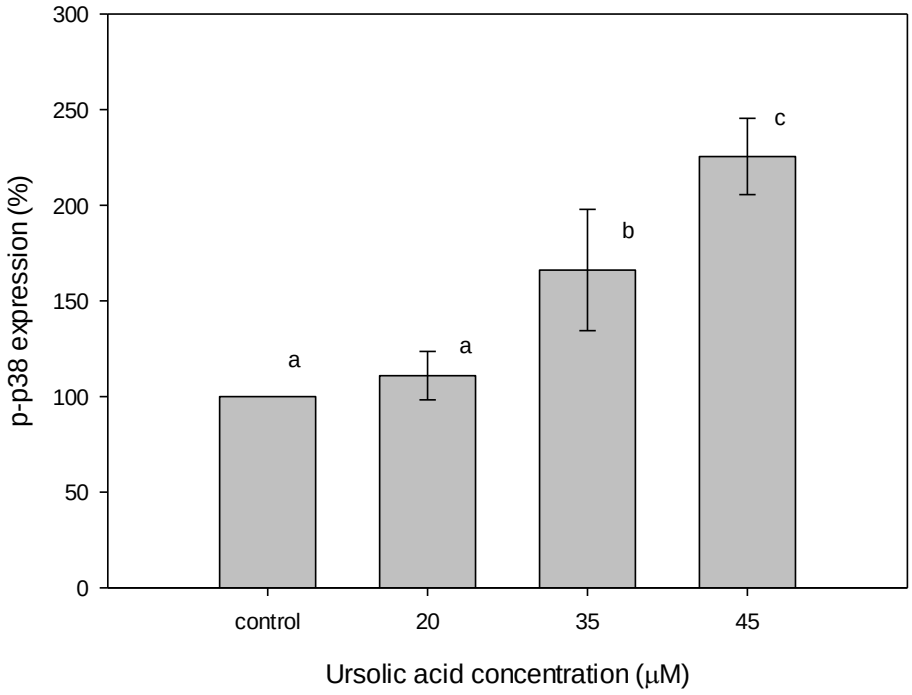


Figure 2.8. Effects of ursolic acid on p-ASK 1 expression in MDA-MB-231 human breast cancer cells. Data were expressed as mean ± SD, n=3. Bar with no letters in common are significantly different ($p<0.05$).

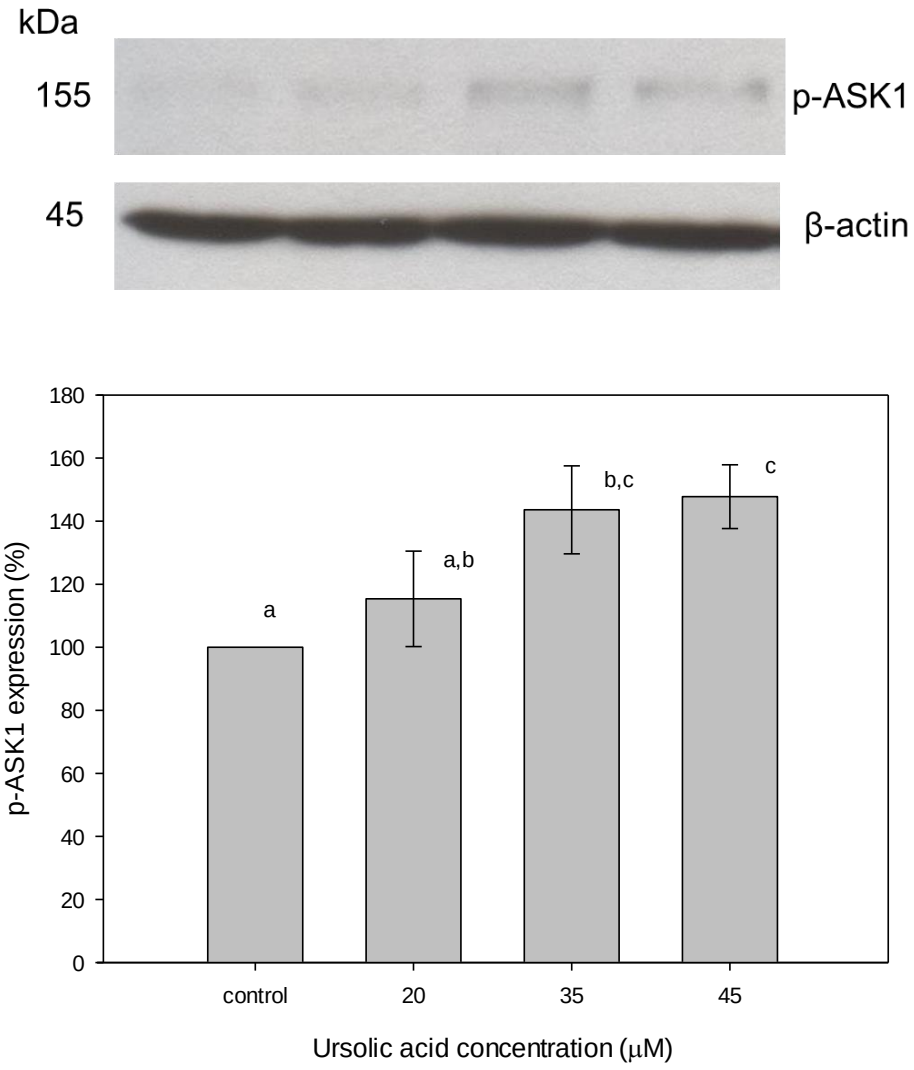


Figure 2.9. Effects of ursolic acid on TRAF-2 expression MDA-MB-231 in human breast cancer cells. Data were expressed as mean ± SD, n=3. Bar with no letters in common are significantly different ($p<0.05$).

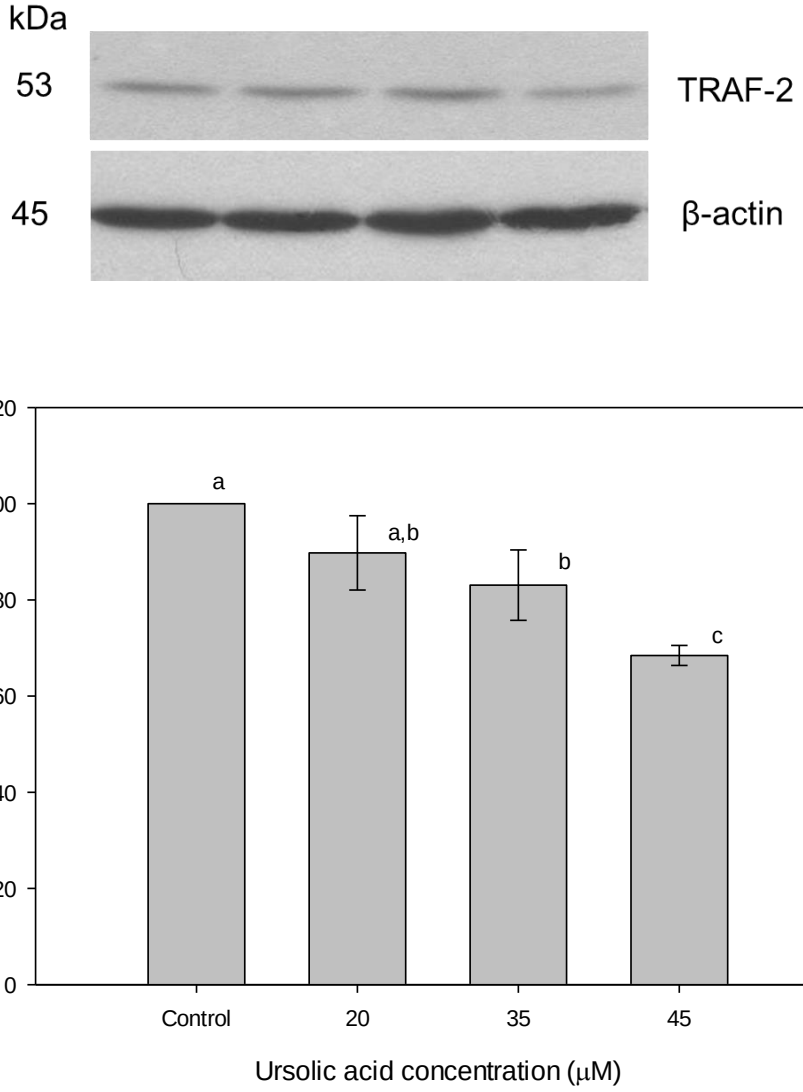
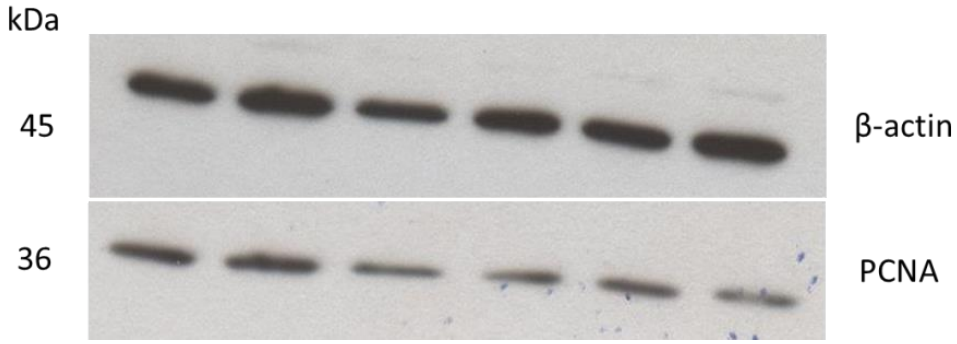


Figure 2.10. Inhibitive Effects of Ursolic Acid on PCNA expression in MDA-MB-231 human breast cancer cells. Data were expressed as mean ± SD, n=3. Bar with no letters in common are significantly different ($p<0.05$).



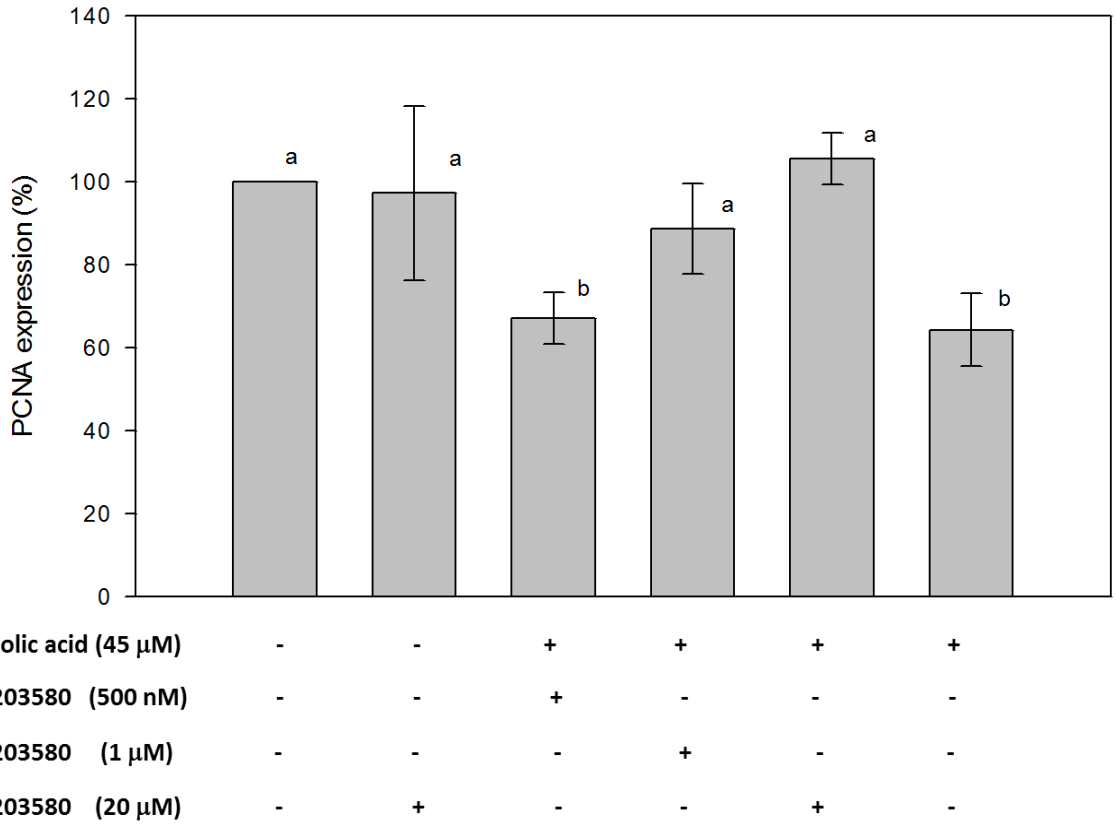
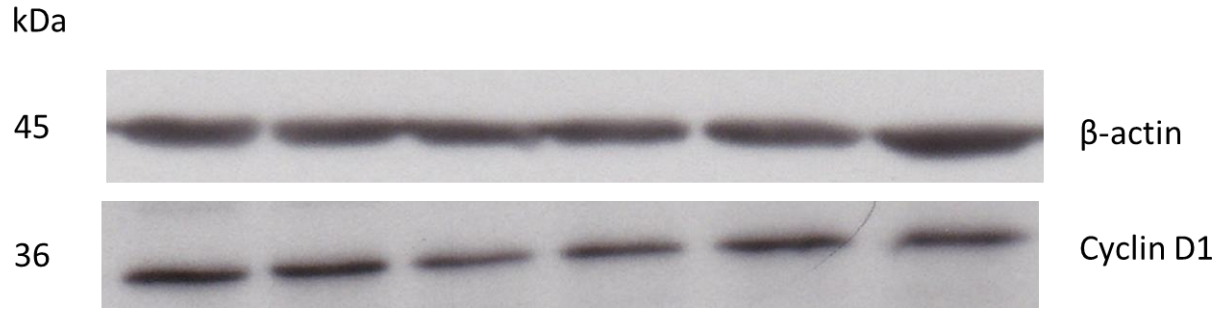


Figure 2.11. Inhibitive Effects of Ursolic Acid on Cyclin D1 expression in MDA-MB-231 Human Breast Cancer Cells. Data were expressed as mean ± SD, n=3. Bar with no letters in common are significantly different ($p<0.05$).



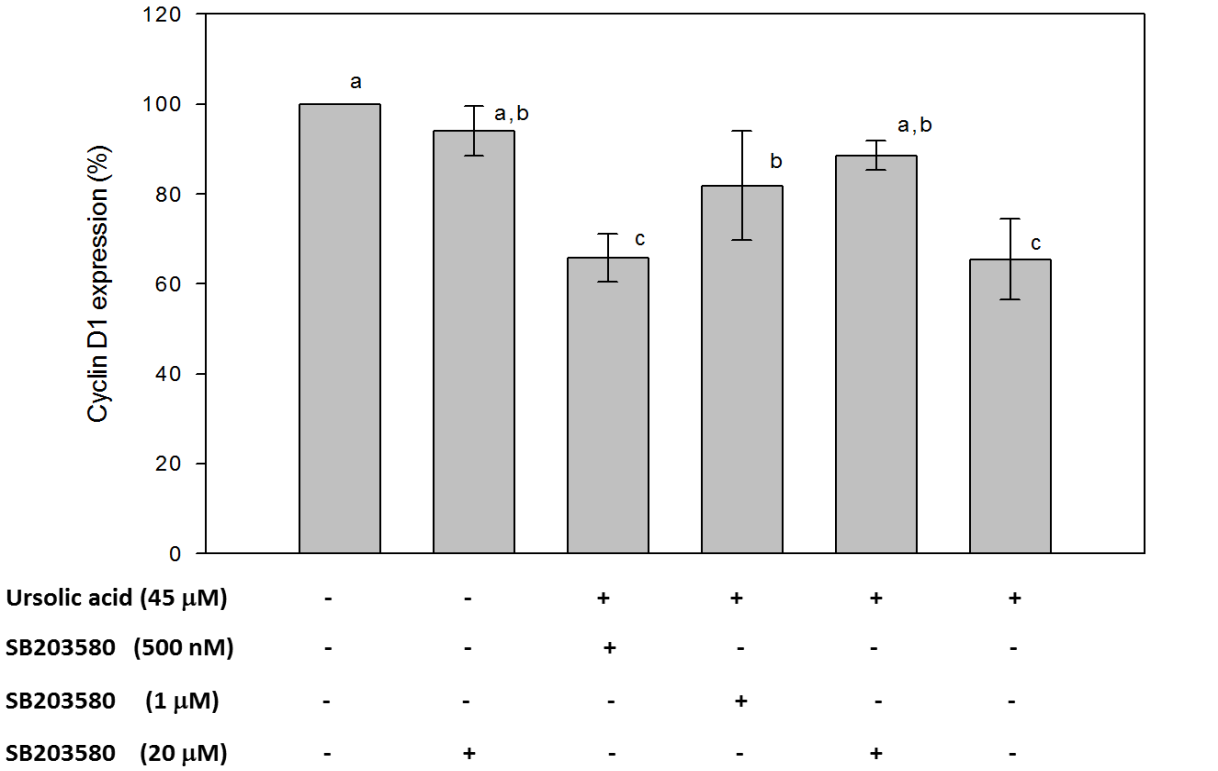
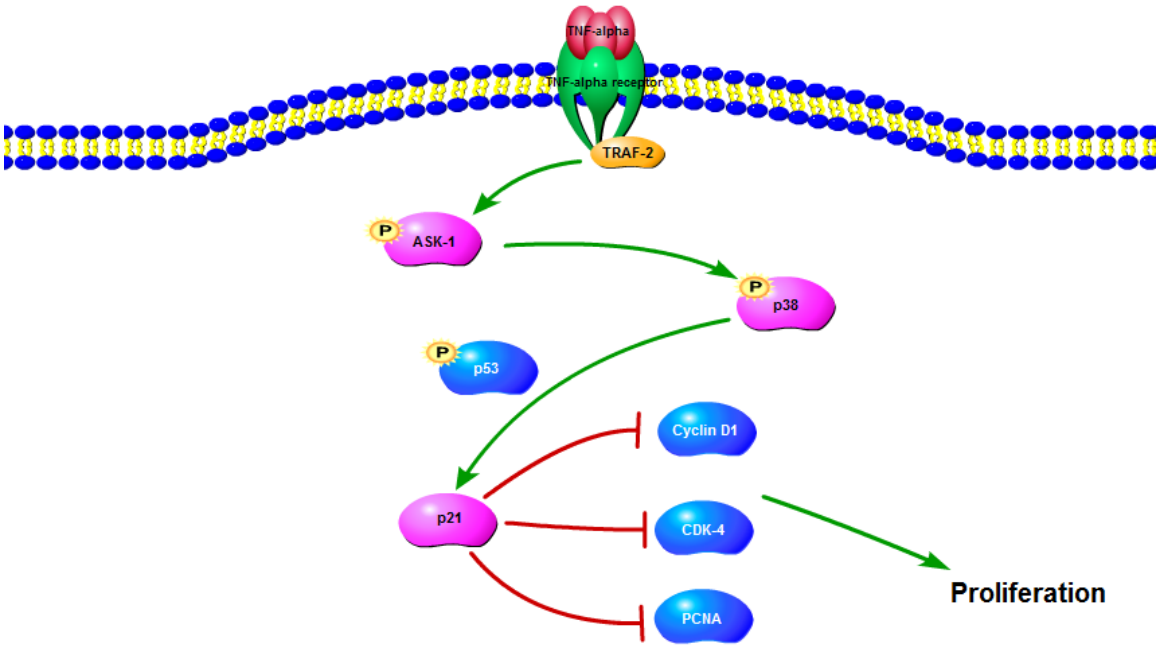


Figure 2.12. Proposed mechanism of actions of ursolic acid in regulation of cell proliferation through p38/MAP kinase pathway



CHAPTER THREE

URSOLIC ACID INDUCES CELL APOPTOSIS BY REGULATING THE INTRINSIC MITOCHONDRIA PATHWAY IN MDA-MB-231 HUMAN BREAST CANCER CELLS

3.1. Introduction

Breast cancer is the most frequently diagnosed cancer and the second most common cause of cancer death among women in United States(Siegel et al., 2015). It is estimated that 1 in 8 of women will develop breast cancer in her lifetime and 17.4% of breast cancer patients will die due to the disease in 2015. In particular, 231,840 new cases of invasive breast cancer are expected to be diagnosed in 2015 with 40,730 new cases of deaths. The survival rate is highly associated with the stage of breast cancer. Rebecca *et.al* reported that at the localized stage, the 5-year relative survival rate is 99%, however, if the cancer spreads to lymph nodes or organs, the survival rate will drop to 25%. Modern therapy for breast cancer often involves surgery, which may be combined with one or more rounds of radiation therapy, chemotherapy to ensure to remove all cancer tissues and cells from breast and reduce the risk of recurrence. Recently, an improved understanding of hormone receptor status in breast cancer led to the introduction of hormone therapy. Tamoxifen, a hormone therapy drug, has been determined to exhibit anticancer effect on estrogen receptor (ER) positive breast cancers. Epidemiological studies suggested that 5-year adjuvant treatment of tamoxifen reduced the annual death rate by 31% on ER positive breast cancers(Early Breast Cancer Trialists' Collaborative, 2005). Others suggested that treatment of the

disease with tamoxifen for 5 years would eliminate the rate of ER^+ breast cancer recurrence by 39% throughout the first decade and reduced the mortality by 30% throughout the first 15 years(Early Breast Cancer Trialists' Collaborative et al., 2011). However, severe side effects, post-surgical recovery, as well as financial cost are still unresolved. Thus, there is a high demand for the development of a practical and effective breast cancer therapy.

Epidemiological studies indicated that frequent intake of fruits and vegetables is highly associated with reduction of chronic diseases such as cardiovascular disease and cancer(McPherson et al., 2000),(Block et al., 1992). Phytochemicals, the bioactive compounds widely distributed in most of fruits and vegetables, are primary contributors for these health benefits. Adams et al. determined that blueberry extracts significantly reduced the metastatic potential and cell proliferation of MDA-MB-231 human breast cancer cells(Adams et al., 2010). Quercetin, a flavonoid found in many fruits, vegetables and grains, induced both cell arrest G2 phase and apoptosis in estrogen positive MCF-7 human breast cancer cells(Choi et al., 2001). In addition, combined phytochemicals, such as apple extracts with quercetin 3-beta-d-glucoside has been examined to possess synergistic effect against MCF-7 cell proliferation(Yang and Liu, 2009). All these finding leads to the exploration on the mechanism of health benefits from fruits and vegetables intake, yet still not well understood.

Ursolic acid is a pentacyclic triterpenoid ubiquitously distributed in fruits and vegetables. Effects of ursolic acid against various human cancer cells have gained much attention recently for its anti-proliferation, apoptosis and anti-metastasis properties determined both *in vitro* and in *vivo*. Sheng et al. reported that ursolic acid elevated the activity of caspase-3 and caspase-8 in different human liver cancer cells

HepG2, Hep3B and HA227 cells, indicating that ursolic acid induced apoptosis were accomplished from both extrinsic and intrinsic signaling pathways(Yan et al., 2010). Moreover, ursolic acid showed significant inhibitory effects on skin tumor promoted by 12-O-tetradecanoylphorbol-13-acetate (TPA)(Tokuda et al., 1986). In this study, ursolic acid was introduced by single time and multiple times at the dosage of 41 nM on the back skin of mouse 1 hour before TPA treatment. After 20 weeks, both applications were determined to delay the formation of papilloma in mouse skin. The rate of papilloma-bearing mouse and the numbers of papilloma per mouse were also shown significantly decreased. Previously, we determined that ursolic acid inhibited the cell proliferation in MDA-MB-231 human breast cancer cells through p-38MAP Kinase dependent pathway. However, whether ursolic acid apoptotic activity was dependent on mitochondria death pathway is still unclear.

In this paper, our objective is to elucidate ursolic acid apoptotic activity in MDA-MB-231 human breast cancer cells. Kim et al. proposed that ursolic acid triggered both intrinsic and extrinsic apoptosis pathway in MDA-MB-231 cells (Kim et al., 2011b). This is very crucial in understanding which signaling pathway ursolic acid is involved in initiation of cell apoptosis

3.2. Material and methods

3.2.1 Chemicals

Ursolic acid was isolated and purified in our laboratory as reported previously(He and Liu, 2007a). Dimethyl sulfoxide (DMSO) was purchased from VWR (Radnor, PA). DPBS was obtained from Thermo Scientific (Waltham, MA), and polyoxyethylene (tween 20) sorbitan monolaurate was obtained from Calbiochem

(Billerica, MA). Trypsin-EDTA (0.05%, 1X), Trypan Blue Stain (0.4%) were obtained from Life Technologies (Grand Island, NY). Protein standard (100 mg/ml), Biuret reagent (150-1,000 μM/mL protein detection level), Sample buffer (Laemmli concentrate), Glycine, Carestream® Kodak® autoradiography GBX developer and fixer (replenisher), and Carestream® Kodak® BioMax® MR film were obtained from Sigma Aldrich (St. Louis, MO). Precision Plus Protein Standards were obtained from Bio-rad biotechnology (Hercules, CA). Tris-Base and Sodium dodecyl sulfate (SDS) were obtained from Amresco (Solon, OH). ApopTag®Plus Peroxidase in situ Apoptosis detection kit were obtained from CHEMICON (Temecula CA). 200 Proof Pure ethanol was obtained from KOPTEC (Baltimore, MD). LumiGLO chemoluminescent substrate was obtained from Cell Signaling Technology (Beverly, MA).

3.2.2 Cell culture

Minimum Essential Media α (α-MEM) (Invitrogen, Carlsbad, CA) were used to support the growth of MDA-MB-231 human breast cancer cells obtained from Animal Tissue Culture Collection (ATCC, Rockville, MD). Working medium contained 10% heat-inactivated fetal fobine serum (FBS) (Atlanta Biologicals, Inc, Lawrenceville, GA), 10 mM Hepes (Sigma Aldrich, St. Louis, MO), 50 unites /ml pencilin and 50 μg/ml Streptomycin(Life Technologies, Grand Island, NY). All cell culture were performanced at 37°C and 5% CO_2 within incubakers.

3.2.3 Antibodies and inhibitor

Primary antibody against cleaved caspase-3, cleaved caspase-9 and SB203580 (p38 MAPK inhibitor) were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies against β-actin and α-tublin, and HRP-conjugated secondary

antibodies (anti-mouse IgG and anti-rabbit IgG) were obtained from Sigma-Aldrich Inc. (St. Louis, MO).

3.2.4 Determination of cell apoptosis

The cell apoptosis was determined by ApopTag®Plus Peroxidase in situ Apoptosis detection kit based on the terminal deoxynucleotidyl-transferase mediated dUTP nick end labeling (TUNEL) assay. MDA-MB-231 human breast cancer cells were plated into a Falcon 8-chamber culture slide at the concentration of 3×10^4 cells/chamber (Becton Dickinson Labware, Franklin Lakes, NJ). Medium with 10% FBS were changed after the first 24 hours then every 48 hours until cell reached 60-70% confluence. Then cell were starved with serum-free medium for overnight and treated with different ursolic acid at the dosage of 20, 35 and 45 μM for 12 hours. Cells were washed with PBS, fixed with 1 % paraformaldehyde for 20 min, then post-fixed into pre-cooled ethanol:acetic acid 2:1 (v:v) for 5 mins at -20°C. Cells were quenched with 3.0% hydrogen peroxide for 15 mins at room temperature. DNA fragment from apoptotic cells labeled with peroxidase, stained brown color by peroxidase subtract, diaminobenzidine (DAB). At last, the cells were counter-stain with 0.5% (w:v) methyl green. All results were expressed as the percentage of apoptotic cells for triplicate.

3.2.5 Determination of specific proteins expression by Western blot

All protocols of protein analysis and Western blotting were followed by our previous work(Liu et al., 1997) with modification(Yoon and Liu, 2008). Briefly, MDA-MB-231 human breast cancer cells were plated at the concentration of 3.0×10^5 cells/mL in 6 –well plates and cultured at 37 ^oC and 5% CO_2. Then, different dosages of ursolic acid (20, 35 and 45 μM) were applied in MDA-MB-231 cells with control

group containing 1% DMSO only for 24 to 48 hours. Cells were collected in PBS buffer solution, lysed in RIPA buffer (50 mM Tris Base, 1% Igepal, 150 mM sodium chloride, 1 mM EDTA, pH=7.4) with protease inhibitors (1 mg/mL aprotinine, 1 mg/mL leupeptin, 1 mg/mL pepstain, 200 mM sodium orthovanadate). Proteins were subjected to 10% (w/v) SDS-polyacrylamide gel for electrophoresis, transferred to polyvinylidene fluoride (PVDF) membrane and incubated in 5% non-fat milk for 2 hours at room temperature. Western Blot results were revealed by LumiGLO chemoluminescent substrate followed by Phototope-HRP detection assay. Protein expression was quantified on Kodak Biomax MR Film by developing and fixing process. The band analysis was produced by ImageJ software 1.43u.

3.2.6 Statistical analysis

All data were collected as mean ± SD for triplicate. Statistical results was performed by SigmaPlot software 11.0 (Systat Software, Inc. Chicago, IL) and all dose-effect analysis was performed by Calcusyn Software version 2.0 (Biosoft, Cambridge, UK). The significant difference in western blot result was performed by T-test with a p-value of 0.05. Results were analysis by JMP software 10.0.0 for ANOVA method (SAS institute, Inc.)

3.3. Results

3.3.1 Cell apoptosis induced by ursolic acid in MDA-MB-231 cells

To detect the apoptotic effect of ursolic acid in MDA-MB-231 human breast cancer cells, TUNEL assay were performed at the ursolic acid concentration of 0, 20, 35 and 45 μM (**Figure 3.1**) to measure stained nuclei mediated by DNA-fragmentation. Compared to the control group, treated cells exhibited significant

increased brown color (apoptotic cells) in a dose-dependent manner, indicating that cell apoptosis were induced by ursolic acid.

3.3.2 Regulation of Bax expression by ursolic acid in MDA-MB-231 human breast cancer cells

Ursolic acid up-regulated the expression of Bax in MDA-MB-231 human breast cancer cells in a dose-dependent manner (**Figure 3.2**). At the dosage of 45 μM, ursolic acid significantly increased the expression of Bax by 187.1 ± 21.9%. At and above 35 μM, ursolic acid inhibition effect was significantly different when compared to the control.

3.3.3 Regulation of Bcl-2 expression by ursolic acid in MDA-MB-231 human breast cancer cells

Ursolic acid down-regulated the expression of Bcl-2 in MDA-MB-231 human breast cancer cells in a dose-dependent manner (**Figure 3.3**). At the dosage of 45 μM, ursolic acid significantly decreased the expression of Bcl-2 by 24.96 ± 6.60%. At and above 35 μM, ursolic acid inhibition effect was significantly different when compared with control.

3.3.4 Regulation of Cleaved-caspase 9 expression by ursolic acid in MDA-MB-231 human breast cancer cells

Ursolic acid up-regulated the expression of cleaved-caspase 9 in MDA-MB-231 human breast cancer cells in a dose-dependent manner (**Figure 3.4**). At the dosage of 45 μM, ursolic acid significantly increased the expression of cleaved-caspase 9 by 133.27 ± 8.23%. At and above 45 μM, ursolic acid inhibition effect was significantly different when compared with control.

3.3.5 Regulation of cleaved-caspase 3 expression by ursolic acid in MDA-MB-231 human breast cancer cells

Ursolic acid up regulated the expression of cleaved-caspase 3 in MDA-MB-231 human breast cancer cells in a dose-dependent manner (**Figure 3.5**). At the dosage of 45 μM, ursolic acid significantly decreased the expression of cleaved-caspase 3 by 265.6 ± 49.9%. At and above 20 μM, ursolic acid inhibition effect was significantly different when compared with control.

3.4. Discussion

Apoptosis, or programed cell death, is a fundamental process in hemostasis regulation and Carcinogenesis prevention in most multicellular animals(Kerr et al., 1972),(Hengartner, 2000). Being an opposite but complementary role of cell proliferation and migration, apoptosis is programed to eliminate the cell numbers and tissue sizes by certain signaling transdcution pathways. The regulation of apoptosis in cancer cells has been recognized as one of the most advanced approach in recent cancer therapetic development. So far, the induction of the apoptotic effect is proposed to be activated in two main pathways: the extrinsic pathway which involves the death receptor on cell surface (initiator) and the intrinsic pathway which involves penetration of mitochondria outer membrance, cytochormone *c*, caspase family proteins (signaling ampilifer)(Fulda and Debatin, 2006). Contradictorily, caspase-independent necrotic pathway(Holler et al., 2000) and a few cell death models(Kitanaka and Kuchino, 1999) were reported that cell death was achieved by Fas and TNF-related apoptosis-inducing ligand (TRAIL), without relying on the caspase-associated pathways, emphasizing how diverse signals were involved in the control of cell death. Therefore, a better

understanding of these regulatory proteins and their functions in cell apoptosis would provide novel opportunity to improve the current therapies against cancer.

Caspase (cysteinyl ASPartate-specific proteASE) is a family of cysteine proteases involved in cell apoptosis and necrosis. Caspase families are considered as the central excutioners in apoptotic pathways and any elimination of caspase activity, either genetic knockout or chemical inhibitors would weaken and prevent the apoptotic effect(Matikainen et al., 2001),(Deveraux et al., 1998). All caspases are synthesized as a inactive pro-caspase consisting three structural domains: a prodomain, a large subunit and small subunit. Activation of caspases involved the proteolytical cleavage responded to the apoptotic stimulis either from CD95 (Fas ligand), TNF-related apoptosis-inducing ligand and its receptor (TRAIL receptor) of extrinsic pathway or cytochrome *c* of intrinsic pathway(Kallenberger et al., 2014),(Suliman et al., 2001),(Liu et al., 1996). Kim et al reported that ursolic acid, abundently distributed in fruits vegetables, especially in apples and burries, induced apoptosis toward MDA-MB-231 human breast cancer cells through both the extrinsinc and intrinsic pathway(Kim et al., 2011b). In this paper, ursolic acid was determined induce MDA-MB-231 human breast cancer cell apoptosis by different methods including mitochondrial membrane potential, flow cytometry and immunoblotting. In the immunoblot analysis, ursolic acid significantly increased the expression of Fas receptor, cleaved caspase-8 and 3 at the dosage of 40 μM in 36-hour treatment, suggesting that ursolic acid induced cell apoptosis through death receptor pathway(Debatin and Krammer, 2004). In addition, ursolic acid also induced apoptosis through the intrinsic mitochondria pathway by releasing the cytochrome *c* to cytoplasma and activated the pro-caspase 9. The level of cytochrome *c* in

mitochondria dramatically decreased, wherase the amount of cytochrome *c* in cytoplasm increased. Then, pro-caspase-9 and cleaved caspase-9 also showed in a dose-dependent manner by ursolic acid treatment. Other phytochemicals, for example EGCG [(−)epigallocatechin-3-gallate)](Hwang et al., 2007), quercetin(Granado-Serrano et al., 2006), or whole food extracts,such as berry extracts(Seeram et al., 2006a) were also observed to induce cell apoptosis through multiple caspase-dependent signaling pathways. Though knowledge about the mechainsm of actions of phytochemicals initiating the apoptotic signals are still poorly suitable for clinical application, researches provide tremendous amount of insights and show great potency for advanced cancer therapeutic development.

The Bcl-2 family proteins are members of pro-apoptotic(Yin et al., 1997) and anti-apoptotic(Hockenbery et al., 1993) proteins which are proposed to controll the mitochondria outer membrane permeabilization. Evidence suggested that apoptotic stimulis were regulated by Bcl-2 family proteins, and then released cytochrome *c* from the intermembrane space of mitochondria, promoted the formation of apoptosome which consisted the adapter protein apoptotic protease activating factor 1 (Apaf1)(Li et al., 1997), (Gross et al., 1999),(Adams and Cory, 1998). Once formed, these apoptosome initiated the activation of pro-caspase 9, further activated the caspase 3 to complete apoptosis(Zou et al., 1999),(Brentnall et al., 2013), Nishikawa et al.(Nishikawa et al., 2006) reported that epigalocatechin-3-gallate (EGCG), one of major polyphenols in teas, significantly induced apoptosis through Bcl-2 family proteins in human liver cancer HLE cells. It was proposed that EGCG decreased the level of Bcl-2α and bcl-xl in a dose-dependent manner by inhibiting the activation of NF-κB. Nguyen et al.(Nguyen et al., 2004) reported the similar results on quercetin

induced apoptosis on human lung cancer A549 cells. The protein expression Bcl-2 familys bax, bad, cleaved-caspase 3, 7 and poly ADP-ribose polymerase (PARP) showed increased in a dose-depdent manner by quercetin treatment. The MEK-ERK signaling pathways were suggested to play an dominate role in the regulation of quercetin induced apoptosis. In our study, ursolic acid significantly altered the ratio of Bax/Bcl-2 and increased the expression of cleaved-caspase 9 and 3. However, the mechanism of ursolic acid induced intrinsic apoptosis pathway, such as the initiation signals received by mitochondria still remain unclear. Thus, it is essential to fully understand the function of ursolic acid in apoptosis induction so it can be used either as an individual compound or in combinaion with other chemotherapeutic agents in treatment or prevention of breast cancer.

Epidermological studies suggested that high consumption of fruits and vegetables is inversely associated with risk of the chonric diseases such as cancer, cardiovescular diseases and obesity(Block et al., 1992),(Bazzano et al., 2002). Phytochemicals derived from fruits and vegetables, such as resveratrol, quercetin and others, have been introduced as chemoprotective agents to improve the current cancer therapy. For example, tamoxifen is currently used in estrogen receptor positive (ER^+) breast cancer treatment among pre- or post-menopausal women. Shin *et al*. reported that coadministration of quercetin at the dosage of 7.5 mg /kg would significantly improved the bioavailability of tamoxifen metabolites, 4-hydrotamoxifen in *vivo*(Shin et al., 2006). In this study, both the absolute and relative bioavaiability of tamoxifen was increased by orally administrating quercetin. In addition, quercetin effectivly inhibited the efflux of multi-drug resistance (MDR) and reduced the first-pass effect of tamoxifen to enhance the bioavailbility. Celecoxib is a nonsteroidal anti-inflammatory

drug specifically targeting cyclooxygenase 2 (COX2). Kiskova et al. determined that resveratrol, combined with celecoxib significantly reduced the tumor volume (60% compared with celecoxib alone), tumor frequency (29%) in methyl-N-nitrosourea (NMU) induced mammary cancer in rats(Kiskova et al., 2014). Furthemore, it was observed that reactive oxygen species generated in blood lymphocytes and the expression of COX-2 mRNA was significantly reduced and the expression of growth differentiation factor 15 (GDF15), a regulatory proteins involved in inflammatory and apoptotic pathways, was increased by combined treatment. All these data suggested that the combination of resveratrol and celecoxib was more effective than the use of any of them individually isolated compound.

Ursolic acid induced the apoptosis in MDA-MB-231 human breast cancer cells through intrinic mitochondira pathway. In detail, ursolic acid significantly increased the expression of Bax, cleaved caspase-9 and cleaved caspase-3. Ursolic acid significantly decreased the expression of Bcl-2. Finally, the TUNEL results morphologically confirmed that ursolic acid could significantly induced cell apoptosis in MDA-MB-231 human breast cancer cells.

Figure 3.1. TNUEL asssay of ursolic acid in induction of apoptosis in MDA-MB-231 human breast cancer cells.

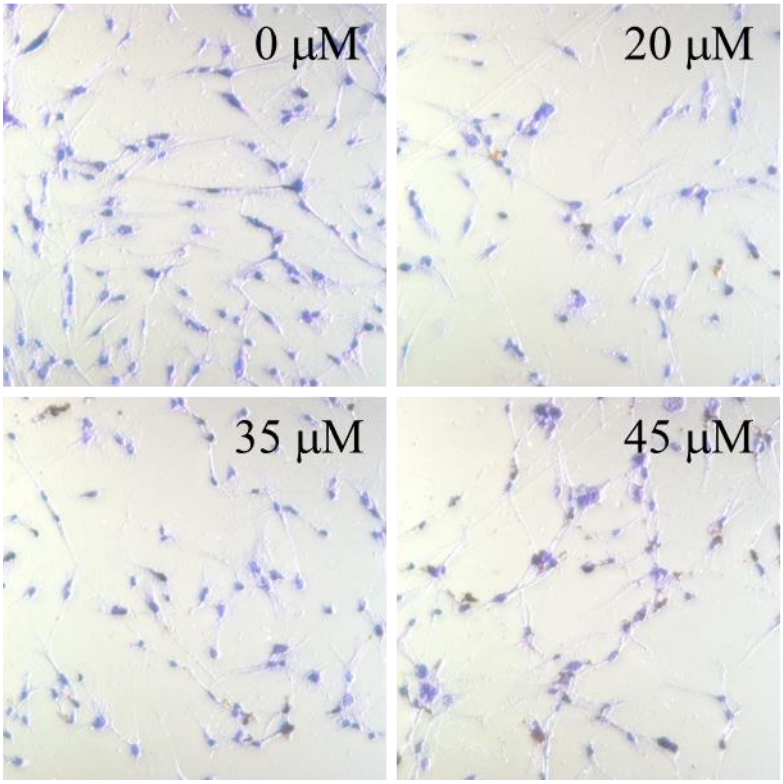


Figure 3.2. Effects of ursolic acid on expressions of Bax in MDA-MB-231 human breast cancer cells. Data were expressed as mean ± SD, n=3. Bar with no letters in common are significantly different ($p<0.05$).

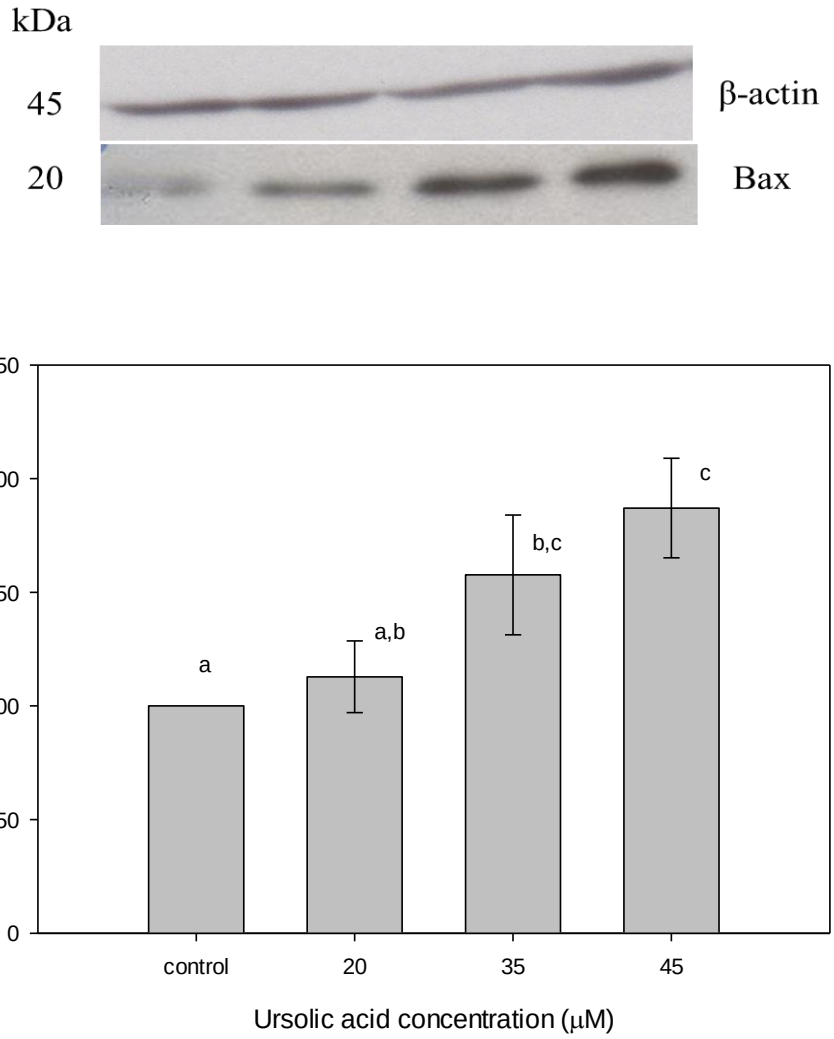
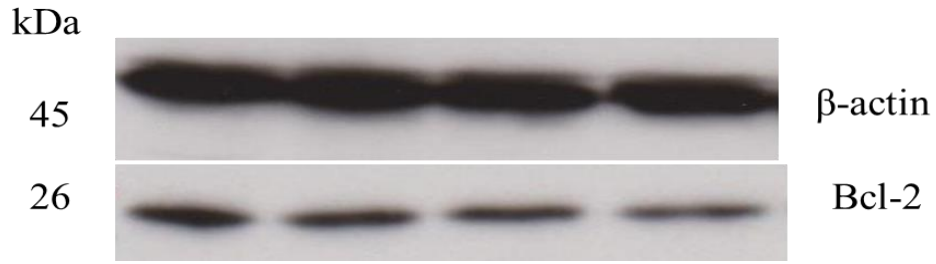


Figure 3.3. Effects of ursolic acid on expressions of Bcl-2 in MDA-MB-231 human breast cancer cells. Data were expressed as mean ± SD, n=3. Bar with no letters in common are significantly different ($p<0.05$).



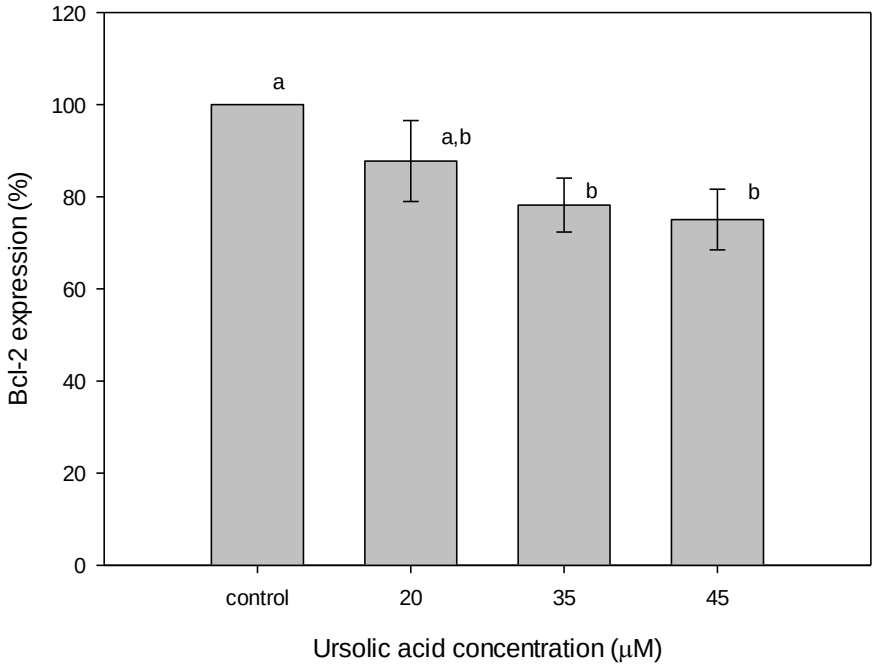


Figure 3.4. Effects of ursolic acid on expressions of cleaved-caspase 9 in MDA-MB-231 human breast cancer cells. Data were expressed as mean ± SD, n=3. Bar with no letters in common are significantly different ($p<0.05$).

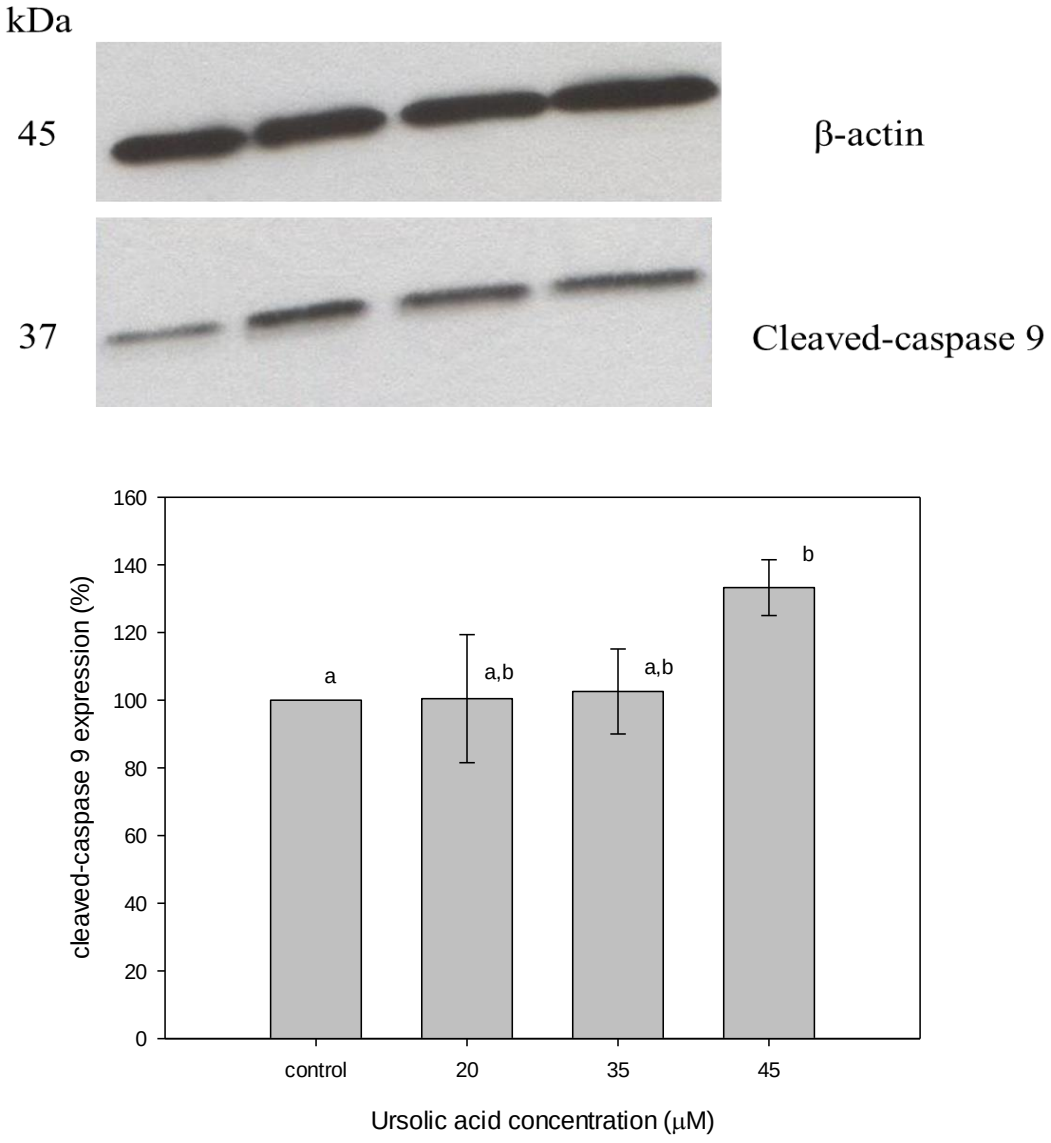
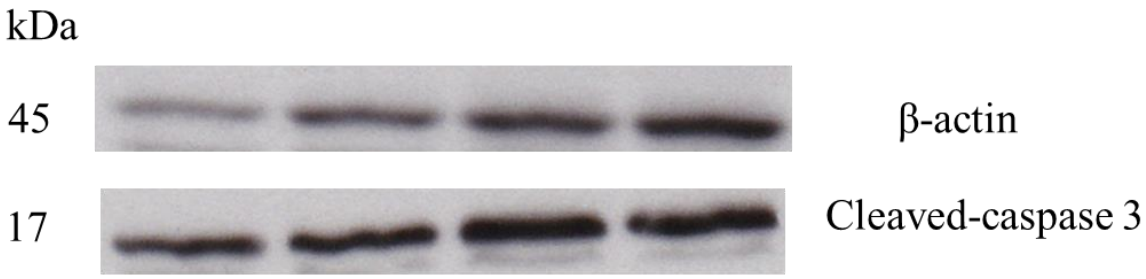
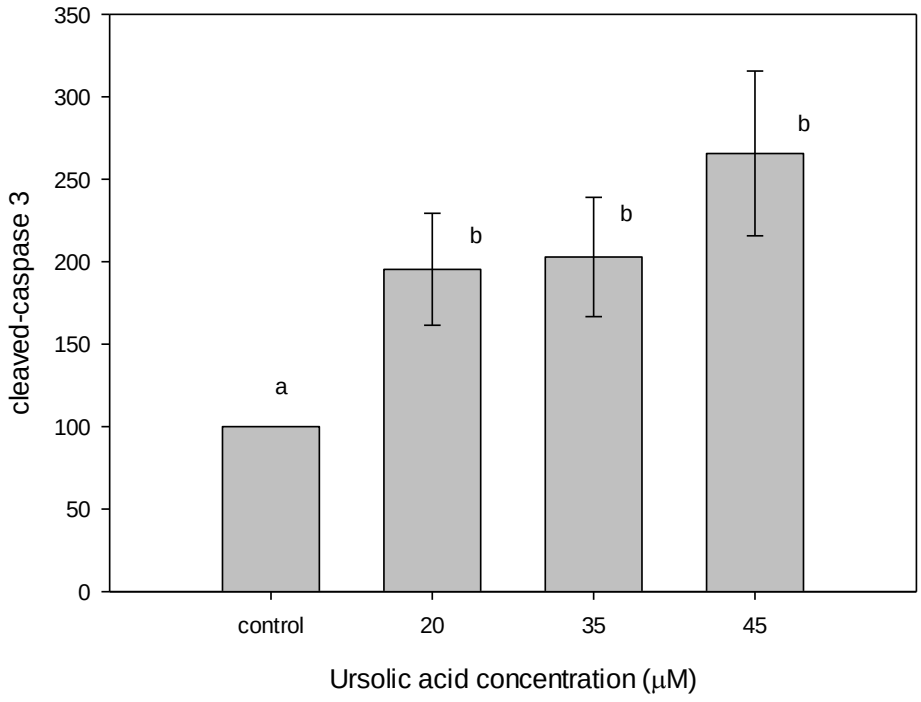


Figure 3.5. Effects of ursolic acid on expressions of cleave-caspase 3 in MDA-MB-231 human breast cancer cells. Data were expressed as mean ± SD, n=3. Bar with no letters in common are significantly different ($p<0.05$).





CHAPTER FOUR

POTENTIAL ADDITIVE AND SYNERGISTIC EFFECTS OF URSOLIC ACID COMBINATION WITH OTHER PHYTOCHEMICALS IN MDA-MB-231 HUMAN BREAST CANCER CELLS

4.1. Introduction

American Cancer Society has estimated a total number of 1,658,370 new breast cancer cases diagnosed and 589,430 new cases of death of breast cancer would occur in the United States in 2015(Siegel et al., 2015). Prostate and breast cancer are leading types of cancers in men and women with numbers of 220,800 and 231,840, respectively. Current cancer therapies, such as radiation, chemotherapy, hormone therapy and other targeted therapies were reported to have multi-drug resistance (MDR)(Gottesman et al., 2002), headache, nausea, fatigue and severe unexpected tissue damage(Shapiro and Recht, 2001),(Dearnaley et al., 1999), as well as mental stress(Holland, 2003),(Sellick and Crooks, 1999),(Spiegel and Giese-Davis, 2003). Moreover, cancer therapy is time consuming and expensive. Therefore, it is necessary to develop an alternative therapy with less side-effective beside conventional cancer therapy.

Epidemiological studies suggested that high consumption of fresh fruits and vegetables reduced the risk of chronic disease, such as cancer and CVD. Several case-control studies supported that the reduction of risks of esophagus, lung, stomach and colorectal cancers were strongly associated with fruits and vegetables intake(Riboli and Norat, 2003). Some case-control and cohort studies indicated that

high fruits and vegetables intake significantly reduced the risk of prostate cancer and invasive breast cancer (Smith-Warner et al., 2001). Berry fruits, such as blackberries, blueberries, cranberries, black raspberries and strawberries are widely consumed in our regular diet. The whole extracts of these berries has been reported to exhibit strong antiproliferative activities against human colon, prostate and breast cancer cells (Seeram et al., 2006b). The pro-apoptotic activities of these extracts were evaluated on human HT-29 colon cancer cells. The expression of COX-2 enzymes was significantly increased 3-fold by black raspberry, and increased 1-fold by other berry extracts. Appel peel extracts was shown to inhibit cell proliferation by inducing G2/M phase arrest in human liver cancer HepG2 cells (Sudan and Rupasinghe, 2014). The apoptotic activity of peel extracts was also observed by regulating the activation of caspase-3, one of regulatory proteins that control the cancer cell death. For cruciferous vegetables, 5 serving per week had been proved to significantly reduce the risk of prostate cancer by 40% (Cohen et al., 2000). High intake of carrots containing β-carotene are reported to significantly reduce the risk of prostate cancer(Giovannucci et al., 1995), lung cancer(Michaud et al., 2000) and breast cancer(Longnecker et al., 1997). All these results indicated that fruits and vegetable are able to prevent carcinogenesis and control cell fate through certain process. In these processes, phytochemicals have been determined to act as a primary contributor. Thus, it is necessary to under how these phytochemicals works in order to provide insights in developing brand-new cancer therapy.

Phytochemicals are bioactive, non-nutrient compounds abundantly distributed in fruits and vegetables. Research has focused on as the antioxidant, anticancer and anti-inflammation activities. For example, epigallocatechin gallate (EGCG), a

polyphenol abundantly distributed in green tea inhibited human colorectal cancer cells growth by inhibiting the activation of vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) axis. In this study, EGCG decreased the level of VEGFR-2 and phosphorylated form of VEGFR-2 in Caco2, HCT116, HT29, SW480, and SW837 human colorectal cancer cell lines. Furthermore, EGCG at the concentration of 25 μg/ml significantly reduced the level of phosphorylated ERK and Akt proteins in SW837 cells, suggesting that EGCG might partially exert the cancer preventive activity through regulating these major growth factors. Quercetin is a common flavonoid found in many fruits and vegetables and recently was reported to exhibit high antioxidant activity by scavenging the free oxidative species(Rice-Evans et al., 1996). For its anticancer activity, some studies determined that quercetin induced G1 arrest in HGC-27 human gastric cancer cells(Yoshida et al., 1990). Based on the flow cytometry data, quercetin reduced the numbers of cancer cells in S phase and G2/M phase during the first 24 hours at the concentration of 70 μM. In addition, the progression of cell cycle on starving HGC-27 cells were significantly delayed (12-24 hours) by quercetin compared with control group, indicating that quercetin also induced G1 arrest on HGC-27 cells in a starvation condition. At last, quercetin showed no effect on inhibition of G2/M phase shifting. It was also reported that quercetin induced cell apoptosis through intrinsic mitochondria death pathway by regulating the expression of caspase family proteins on human breast cancer MDA-MB-231 cells (Chien et al., 2009). The mitochondria membrane potentials, key proteins, such as caspase-3,9 and PRAP were all regulated by quercetin. Our previous studies have determined that ursolic acid, a pentacycli triterpenoid, inhibited cell proliferation through p38/MAP kinase pathway. Protein expression of cyclinD1, CDK4 and PCNA

were shown dose dependent inhibition by ursolic acid treatment. Moreover, it was also determined that ursolic acid induced apoptosis through mitochondria intrinsic pathway. The expression of Bax, Bcl-2, as well as the cleaved- caspase 3 and 9 were all regulated in a dose-dependent manner. Conclusively, ursolic acid would have great potent to be used in new cancer therapy. However, due to the lack of evidence from animal studies and possible low bioavailability(Chen et al., 2011b),(Yin et al., 2012a), ursolic acid alone might not function well as anticancer drug, but work as single compound in a food complex which have bioactivity in cancer prevention and treatment. Thus, it is necessary to evaluate the health benefits of ursolic acid in food/dietary complex, especially its additive and synergistic effects with other bioactive compounds.

Synergy is defined as a greater effect of two or more compounds combined in the system than the sum of each compound's effect individually. The combination of drugs is used for their synergistic advantages in enhancing drug sensitivity, reducing the risk of drug toxicity and multi-drug resistance in clinical therapies. Tamoxifen is commonly used in hormone therapy, especially targeting on estrogen receptor positive breast cancer. The combination of tamoxifen with sodium phenylacetate significantly suppressed the tumor growth in MCF-7 human breast cancer cells, compared with tamoxifen and sodium phenylacetate alone(Adam et al., 1997), Among many phytochemicals, the anticancer synergism has also been observed with difference combinations. Two-way combination study of quercetin-3-glucoside and apple extracts showed synergistic effect on antiproliferation in MCF-7 human breast cancer cells(Yang and Liu, 2009). Vitamin E and selenium were also reported to have synergy in apoptosis induction in human prostate cancer PC-3 cells(Zu and Ip, 2003).

Resveratrol and catechin combination protected rat pheochromocytoma PC12 cells from β-amyloid induced toxicity (Conte et al., 2003). These synergistic phenomena indicated that phytochemicals in fruits and vegetables do function independently but act synergistically to achieve anticancer and other health benefits.

The objective of this study was to determine whether ursolic acid, when combined with quercetin, EGCG and resveratrol, has synergistic or additive effects against MDA-MB-231 human breast cancer cells.

4.2. Material and methods

4.2.1 Chemicals

Ursolic acid was isolated and purified previously in our lab(He and Liu, 2007a). Quercetin, (−)-Epigallocatechin gallate (EGCG), and resveratrol were obtained from Sigma Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from VWR (Radnor, PA). Trypsin-EDTA (0.05%, 1X), Trypan Blue Stain (0.4%) were obtained from Life Technologies (Grand Island, NY). Methylene blue was obtained from BBL (Division of BioQuest, cockeyville, MD).

4.2.2 Cell culture

Human breast cancer MDA-MB-231 cells obtained from Animal Tissue Culture Collection (ATCC, Rockville, MD). Cell culture were performeanced at 37°C and 5% CO_2 with minimum essential media α (α-MEM) (Invitrogen, Carlsbad, CA). Working medium contained 10% heat-inactivated fetal fobine serum (FBS) (Atlanta Biologicals, Inc, Lawrenceville, GA), 10 mM Hepes (Sigma Aldrich, St. Louis, MO), 50 unites /ml pencilin and 50 μg/ml Streptomycin(Life Technologies, Grand Island, NY).

4.2.3 Measurement of cytotoxicity activity of ursolic acid, EGCG, resveratrol and quercetin

The cytotoxicity activity of ursolic acid was measured by methylene blue assaydeveloped by our laboratory(Felice et al., 2009). In brief, MDA-MB-231 human breast cancer cells were plated at the concentration of $4.0x10^4$ per well into a 96-well plate and incubated for 24 hours. Then cells were treated with different concentrations of ursolic acid (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μM), quercetin (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μM), EGCG (20, 40, 60, 80, 100, 120, 140, 160, 180, 200 μM), and resveratrol (20, 40, 60, 80, 100, 120, 140, 160, 180, 200 μM) for another 24 hours. All phytochemicals were pre-disssolvoed in DMSO and minimized at less than 1% (v/v) in growth medium. Cells were then rinsed with phosphate buffered saline (PBS) twice and stained with methylene blue solution containing 1.25% glutaldehyde and 0.6% methylene blue at 37 ^oC and 5% CO_2 for 1 hour. Methylene blue stain was eluted by 49% (v/v) PBS, 50% (v/v) ethanol and 1% (v/v) acetic acid elution buffer at room temperature for 30 mins. The absorbance was measured at 570 nm by using MRX II Dynex plate reader (Dynex Technologies, Inc., Chantilly, VA). Data were collected in triplicate for statistical analysis.

4.2.4 Measurement of antiproliferation activity of ursolic acid, EGCG, resveratrol and quercetin

The antiproliferation activity of ursolic acid, EGCG, resveratrol, and quercetinwere measued by methylene blue assay previously described(Felice et al., 2009; Oliver et al., 1989). Briefly, MDA-MB-231 cells were plated at the concentration of $2.5x10^4$ cells/well and were incubated for 6 hours at 37 ^oC and 5%

CO_2. Then cells were treated at designed concentrations of ursolic acid, quercetin, EGCG and resveratrol for another 72 hours with control containing less than 1% (v/v) DMSO. Then cells were rinsed with phosphate buffered saline (PBS) twice and stained with methylene blue solution containing 1.25% glutaldehyde and 0.6% methylene blue at 37 ^{o}C and 5% CO_2 for 1 hour. Methylene blue stain was eluted by 49% (v/v) PBS, 50% (v/v) ethanol and 1% (v/v) acetic acid elution buffer at room temperature for 30 mins. The absorbance was measured at 570 nm by using MRX II Dynex plate reader (Dynex Technologies, Inc., Chantilly, VA). Data were collected in triplicate for statistical analysis. The effective mediam dose (EC_{50}) were determined and expressed as micromolarity ± SD.

4.2.5 Experimental design of combination study

Two way combination of ursolic acid with quercetin, ursolic acid with EGCG and ursolic acid with resveratrol antiproliferation activity in MDA-MB-231 human breast cancer cells were designed as the method previously published from our lab (Yang and Liu, 2009). Based on the EC_{50} value of ursolic acid, quercetin, EGCG and resveratrol determind on the basis of the dose-response curve, the combined concentrations of each phytochemical were 0.25xEC_{50}, 0.5x EC_{50}, 0.75x EC_{50}, 1.0x EC_{50}, and 1.25x EC_{50}. The inhibition of cell proliferation of two-way combinations of ursolic acid and other phytochemicals in MDA-MB-231 human breast cancer cells was determined by the method described previously (Yang and Liu, 2009). The combination effects were analyzed by calculating the combination index based on the isobologram equation(Chou et al., 1994).

2.6 Statistical analysis

Data were collected as mean ± SD for triplicate. Statistical results was performed

by SigmaPlot software 11.0 (Systat Software, Inc. Chicago, IL) and all dose-effect analysis was performed by Calcusyn Software version 2.0 (Biosoft, Cambridge, UK). The significant difference was analysis by JMP software 10.0.0 for student T-test(SAS institute, Inc.).

4.3. Results

4.3.1 Antiproliferative activity and cytotoxicity of ursolic acid in MDA-MB-231 human breast cancer cells

The antiproliferative activity and cytotoxicity of ursolic acid in human breast cancer MDA-MB-231 cells were evaluated and shown in **Figure 4.1**. EC_{50} (half-maximal effective concentration) of ursolic acid inhibition of MDA-MB-231 human breast cancer cell proliferation was observed at 26 μM. At the concentration of 50 μM, ursolic acid inhibited cell proliferation in MDA-MB-231 human breast cancer cells by 90%. There was no cytotoxicity (<10%) was observed at the concentration below 60 μM in MDA-MB-231 human breast cancer cells..

4.3.2 Antiproliferative activity and cytotoxicity of quercetin in MDA-MB-231 human breast cancer cells

The anti-proliferative activity and cytotoxicity of quercetin in MDA-MB-231 human breast cancer cells were evaluated and shown in **Figure 4.2**. EC_{50} (half-maximal effective concentration) of quercetin inhibition of MDA-MB-231 cell proliferation was observed at 31 μM. At the concentration of 40 μM, quercetin acid inhibited cell proliferation on MDA-MB-231 by 50%. There was no cytotoxicity (<10%) was observed at the concentration below 40 μM in MDA-MB-231 human

breast cancer cells.

4.3.3 Antiproliferative activity and cytotoxicity of EGCG in MDA-MB-231 human breast cancer cells

The anti-proliferative activity and cytotoxicity of EGCG in MDA-MB-231 human breast cancer cells were evaluated and shown in **Figure 4.3**. EC_{50} (half-maximal effective concentration) of EGCG inhibition of MDA-MB-231 cell proliferation was observed at 156 μM. At the concentration of 200 μM, EGCG inhibited cell proliferation on MDA-MB-231 by 58%. There was no cytotoxicity (<10%) was observed at the concentration below 200 μM.

4.3.4 Antiproliferative activity and cytotoxicity of resveratrol in MDA-MB-231 human breast cancer cells

The anti-proliferative activity and cytotoxicity of resveratrol in MDA-MB-231 human breast cancer cells were evaluated and shown in **Figure 4.4**. EC_{50} (half-maximal effective concentration) of resveratrol inhibition of MDA-MB-231 cell proliferation was observed at 71 μM. At the concentration of 100 μM, resveratrol inhibited cell proliferation on MDA-MB-231 by 52%. There was no cytotoxicity (<10%) was observed at the concentration below 100 μM,

4.3.5 Combination effect of ursolic acid plus quercetin, EGCG and resveratrol

The combination effects of ursolic acid plus quercetin, ursolic acid plus EGCG and ursolic acid plus resveratrol were determined by combination index (CI) described previously(Chou et al., 1994). The CI values of each combination were calculated and presented in **Table 4.1, 4.2, 4.3**. The CI value of the two-way combination of ursolic acid plus quercetin at 50% inhibition of MDA-MB-231 cell proliferation was 1.09,

indicating that there was an additive effect. The CI value of the two-way combination of ursolic acid plus EGCG at 50% inhibition of MDA-MB-231 cell proliferation was 1.38, suggesting that there was a relative additive effect. The CI value of the two-way combination of ursolic acid plus resveratrol at 50% inhibition of MDA-MB-231 cell proliferation was 2.17, indicating that there was no effect.

4.4. Discussion

Breast cancer is the leading type of cancer diagnosed among women in United States(Siegel et al., 2015). Clinical surgeries and treatments including breast-conserving surgery (BCS), hormone therapy and targeted therapy aimed at specific proteins, such as HER2, are common cancer therapies applied on patients recently. The very first radical mastectomy (Halsted mastectomy) was performed in early 1880s(Halsted, 1898). The procedure was to remove entire breast tissues along with nearby muscles on the chest, yet is rarely done now unless for patients with large and invasive tumors. Breast conserving surgery is considered as partial mastectomy and small parts of breast tissues with limited surrounding normal tissues(Winchester and Cox, 1998),(Veronesi et al., 1995),(Fisher et al., 1995). Veronesi. et al. conducted 20 years follow-up of a randomized study for early breast cancer patients(Veronesi et al., 2002). Compared with radical mastectomy, breast-conserving surgery showed a very similarity in long-term survival rate and the probability of tumor recurrence, however with less pain in post-surgical recovery and lower dosage of radiotherapy. BCS was considered as replacement for mastectomy, yet was found to be suitable for relatively small size of in situ breast carcinoma(Fisher et al., 1993),(Solin et al., 1996).

Systemic therapies, such as chemotherapies, hormone therapies and targeted

therapies are treatments administered through the bloodstream and affect not only cancer tissues but also the entire body. Usually such treatments are given to patients before or after surgery to effectively control cancer metastasis and kill undetected or undead cancer tissues(Mauri et al., 2005),(Kelly and Goldberg, 2005).

Epidemiological studies and clinical trials have determined that combination of drugs may function synergistically and more effective than the use of individual drug alone for cancer treatment. Two-way combination studies of trastuzumab with carboplatin, trastuzumab with 4-hydroxycyclophopsphamide, trastuzumab with docetaxel and vinorelbine with trastuzumab were all shown to have synergistic effect on four HER-2 overexpressed breast cancer cell lines SK-BR-3, BT-474, MDA-MB-361, and MDA-MB-453(Pegram et al., 2004). Moreover, the triple combination of docetaxel, carboplatin and trastuzumab was also observed to have a synergistic interaction on these breast cancer cells. For phytochemicals and clinical drugs combination, green tea has been proved to exhibit synergistic effect with tamoxifen in the inhibition of cell proliferation on estrogen receptor positive breast cancer MCF-7, ZR75 and T47D cells(Sartippour et al., 2006). Pancratistatin, a bioactive compound distributed in Hawaiian Spider Lily was determined to induce cell apoptosis synergistically with tamoxifen in a mitochondria ROS generation related pathway on human breast cancer MCF-7 cells (Siedlakowski et al., 2008). Synergy effect was also observed on basal-like breast cancer MDA-MB-231 cells by the combination of EGCG and tamoxifen (Chisholm et al., 2004). All these evidence suggests that utilization of combined phytochemicals in cancer treatment is more effective than individual. Therefore, fully understanding the synergy between phytochemicals are crucial to explore the health benefits from fruits and vegetables.

Ursolic acid is a pentacycli triterpenoid abundantly distributed in fruits and vegetables. Evidences suggest that ursolic acid exhibited high anticancer, antioxidant and other health benefits. Hsu et al. reported that ursolic acid inhibited the cell proliferation by inducing G1 arrest in human non-small lung cancer A549 cells (Hsu et al., 2004b). Further, DNA fragmentation and Western blotting data indicated that ursolic acid induced cell apoptosis through both Fas-receptor (Fas ligand, Fas/APO-1) and mitochondria (Bcl-2, Bcl-x_L) pathways. Li et al. determined that ursolic acid, and its isomer oleanolic acid regulated the cell proliferation through G0/G1 arrest in human colon carcinoma cell line HT15(Li et al., 2002). Our previous studies have demonstrated that ursolic acid inhibited the cell proliferation through p38/MAP kinase pathway on human breast cancer MDA-MB-231 cells. PCNA, cyclinD1 and CDK4 protein expressions were regulated by ursolic acid in a dose-dependent manner. In addition, ursolic acid induced apoptosis through intrinsic mitochondria pathways on MDA-MB-231 cells by regulating the expression of Bcl-2, Bax and cleaved-caspase 3 and 9. Even though the molecular mechanisms of actions of ursolic acid in cancer cell lines are investigated, there is still few studies focused on the combination effects. In our study, it is first time that the additive effect and potential synergistic effect of ursolic acid with quercetin and EGCG on antiproliferative activity has been determined in MDA-MB-231 human breast cancer cells. Further research is needed to determine three or multiple ways of combination in order to elucidate the mechanism of synergy of bioactive compounds in the prevention of breast cancer.

Figure 4.1. Effects of ursolic acid on cell proliferation and cytotoxicity in MDA-MB-231 human breast cancer cells. Each value represents the mean ± SD with triplicates.

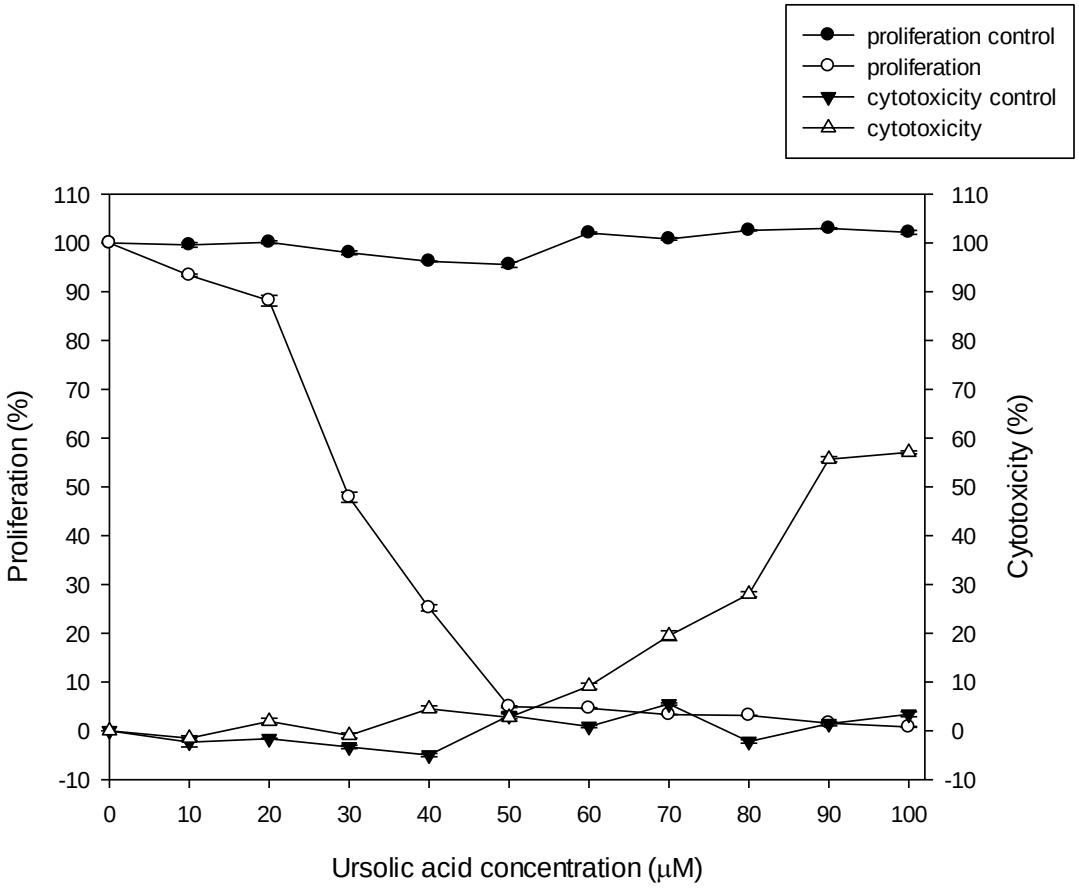


Figure 4.2. Effects of quercetin on cell proliferation and cytotoxicity in MDA-MB-231 human breast cancer cells. Each value represents the mean ± SD with triplicates.

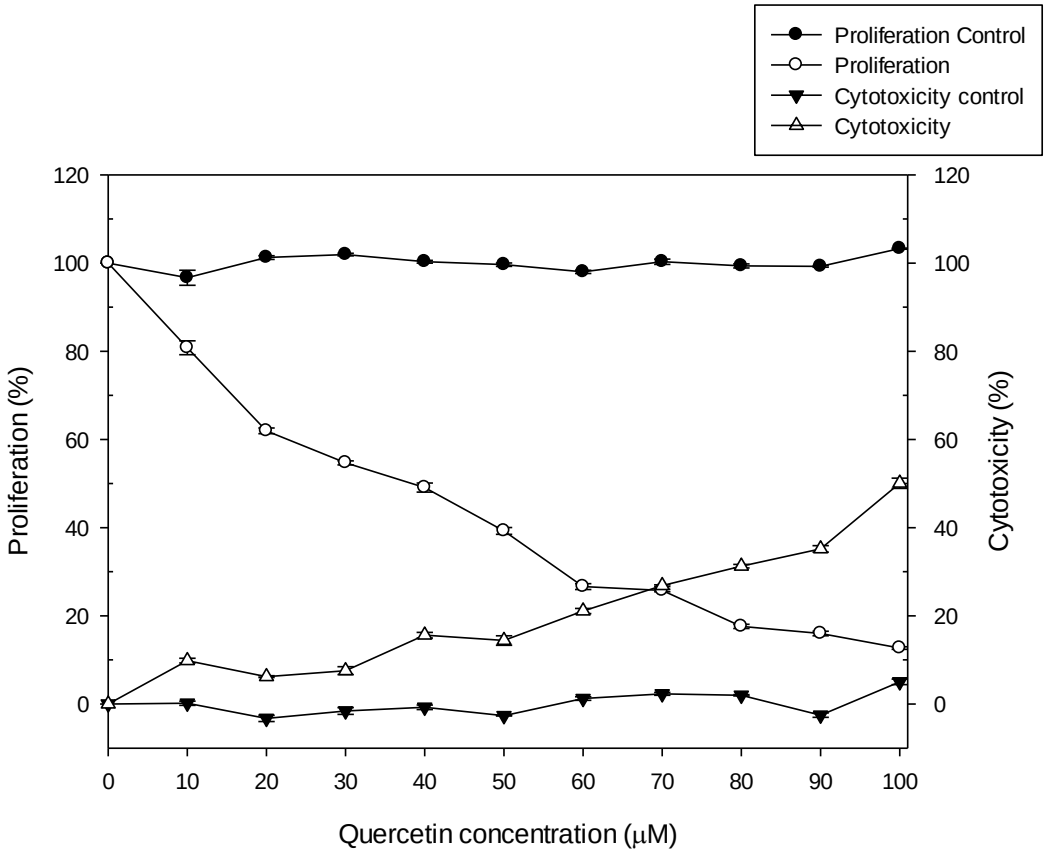


Figure 4.3. Effects of EGCG on cell proliferation and cytotoxicity MDA-MB-231 in human breast cancer cells. Each value represents the mean ± SD with triplicates.

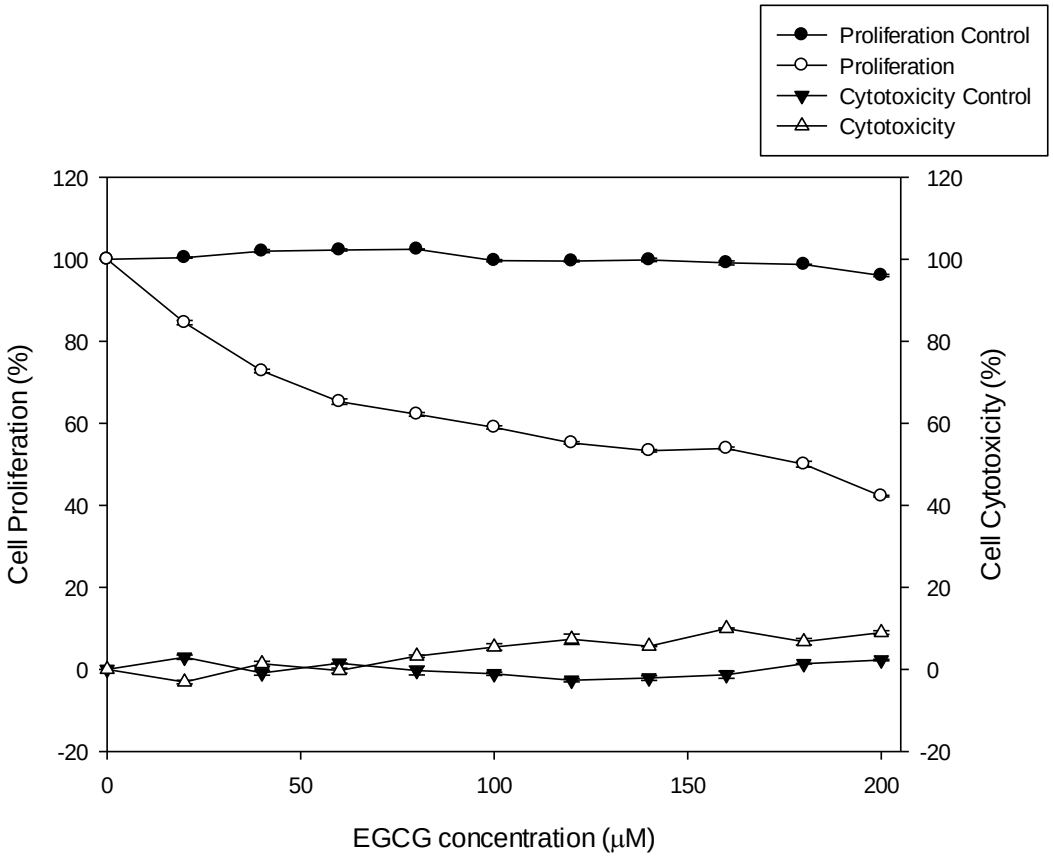


Figure 4.4. Effects of resverstrol on cell proliferation and cytotoxicity in MDA-MB-231 human breast cancer cells. Each value represents the mean ± SD with triplicates.

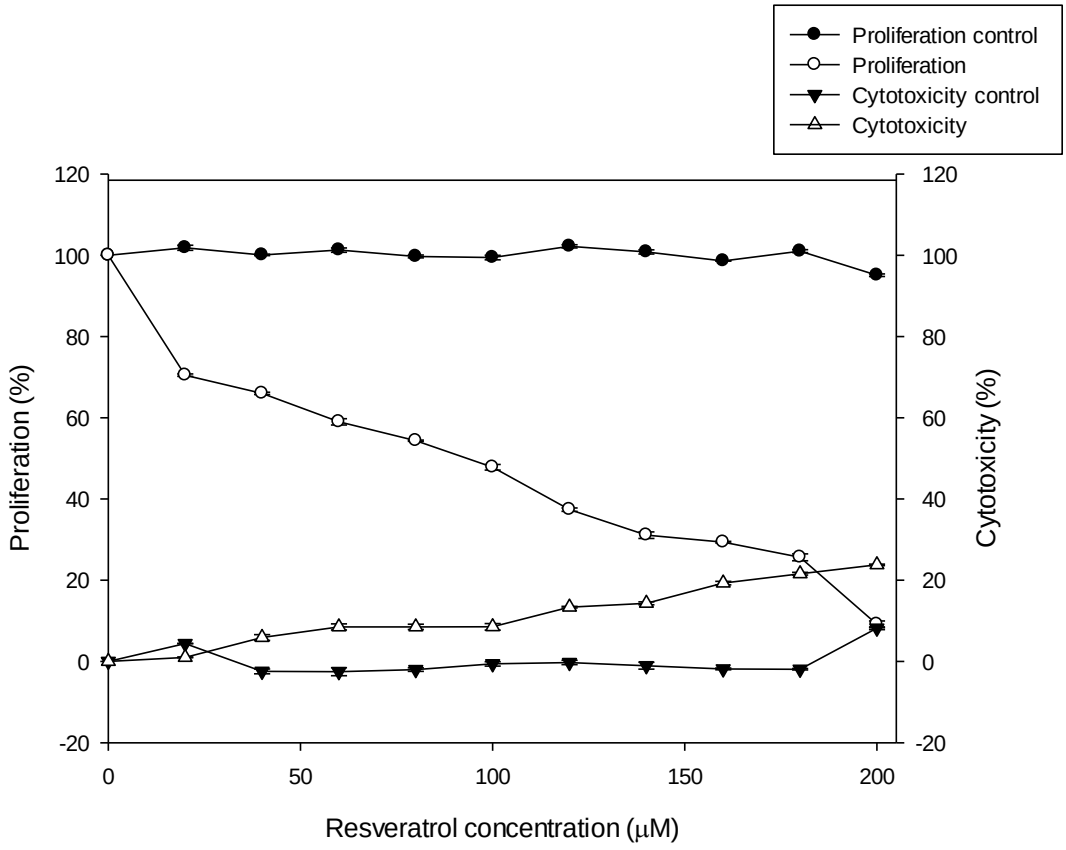


Figure 4.5. Synergistic interactions between ursolic acid and quercetin in inhibition of MDA-MB-231 human breast cancer cell proliferation. Each value represents the mean ± SD with triplicates.

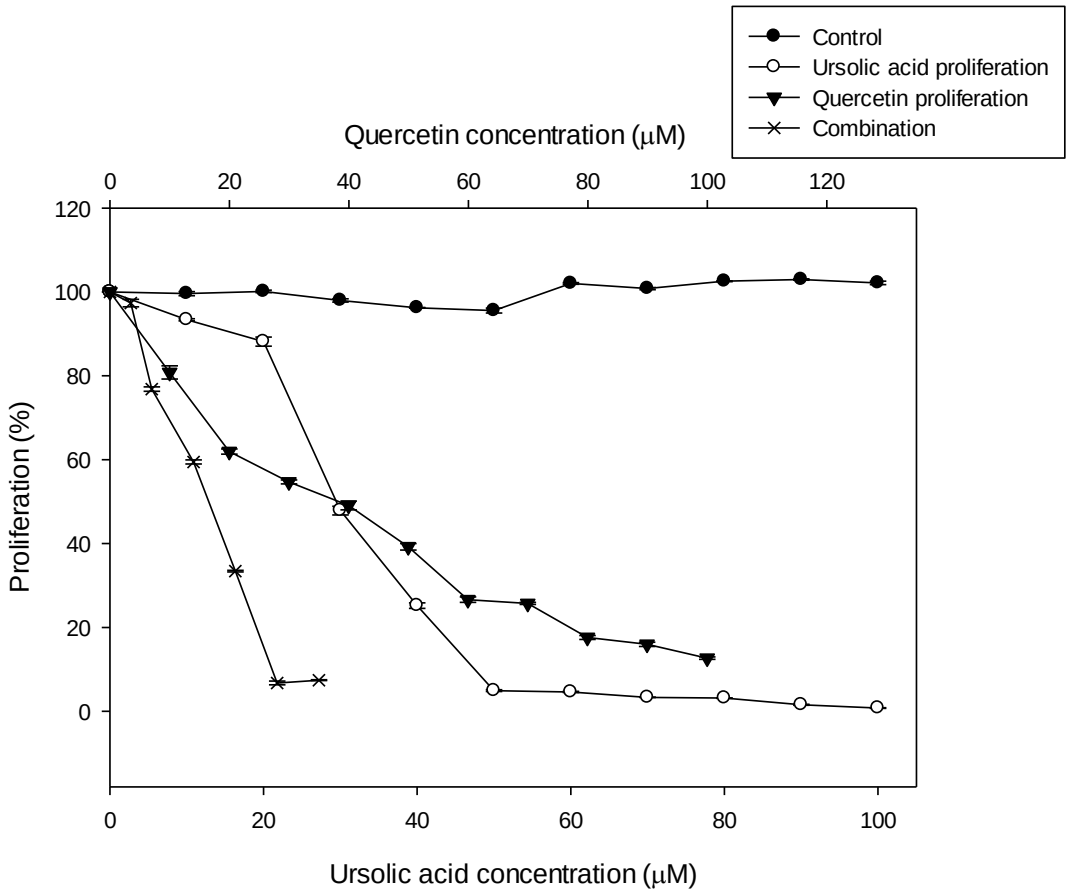


Figure 4.6. Synergistic interactions between ursolic acid and EGCG in inhibition of MDA-MB-231 human breast cancer cell proliferation. Each value represents the mean ± SD with triplicates.

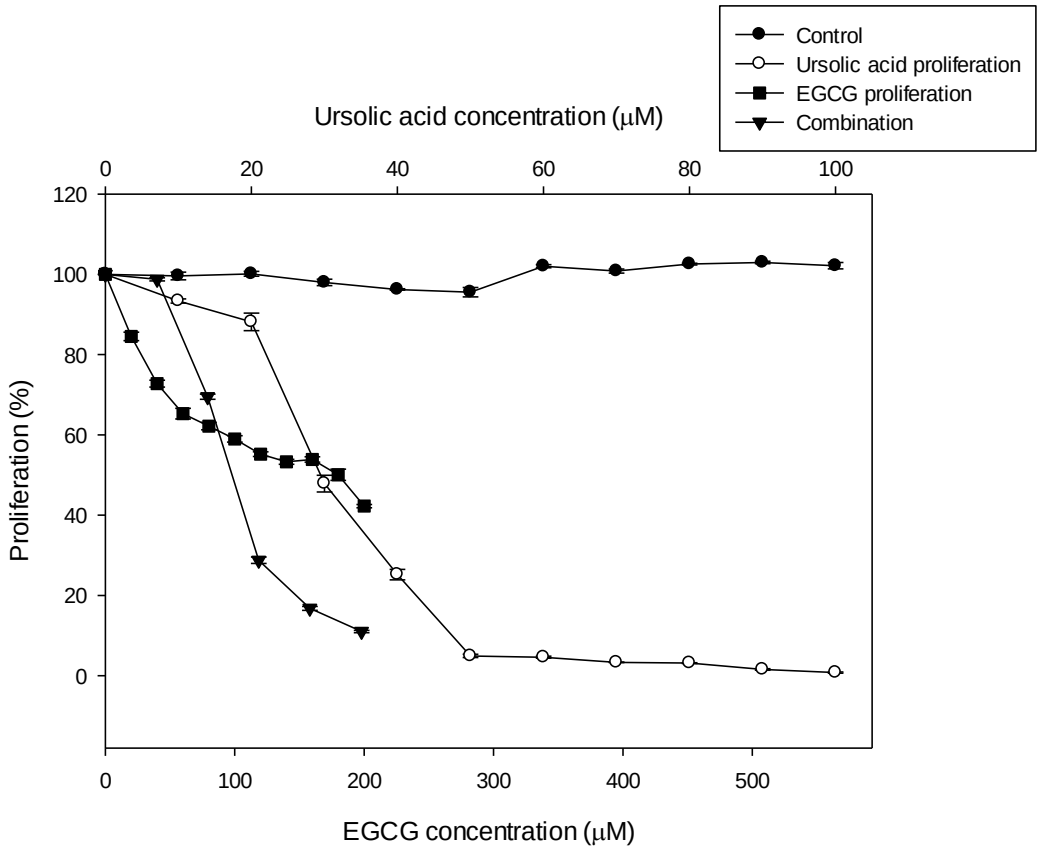


Figure 4.7. Synergistic interactions between ursolic acid and resveratrol in inhibition of MDA-MB-231 breast cancer cell proliferation. Each value represents the mean ± SD with triplicates.

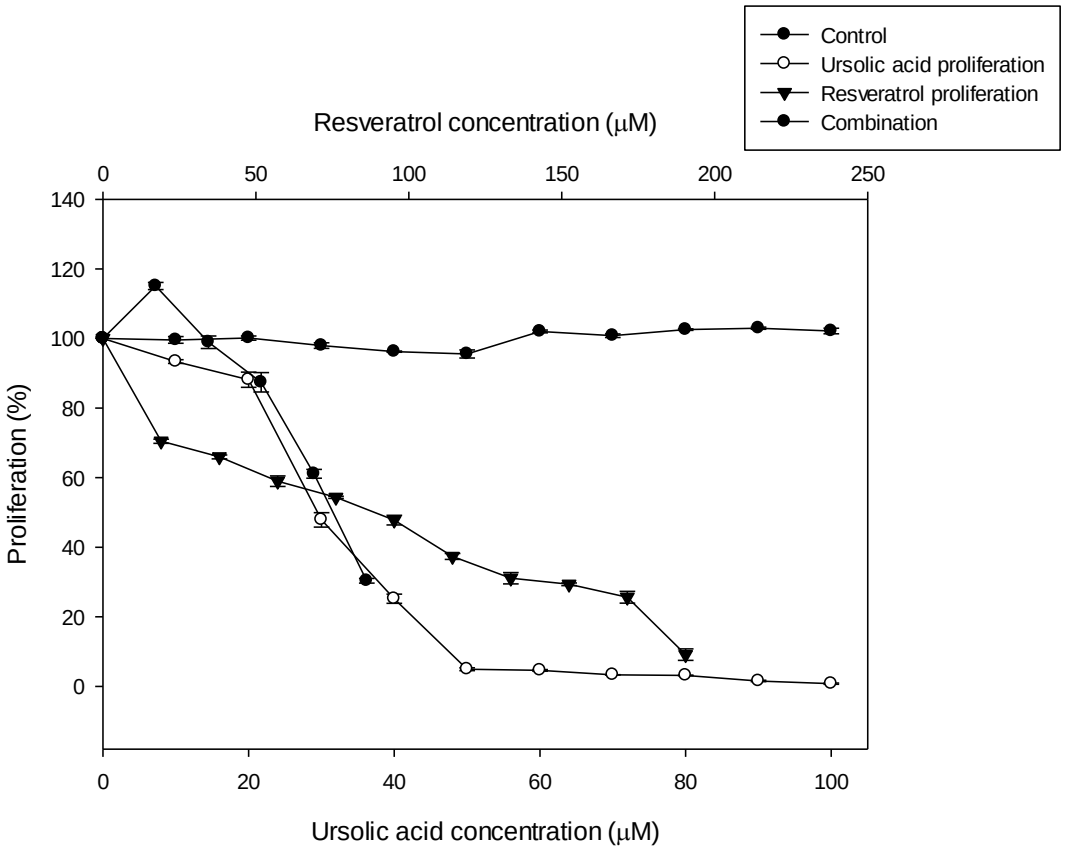


Table 4.1. EC_{50} value of ursolic acid, quercetin and ursolic acid in combination with quercetin in inhibiting MDA-MB-231 cell growth

	EC_{50} value	
component	single	combined
Ursolic acid (μM)	26.68	13.29
Quercetin (μM)	30.79	18.37
Combination index	1.09	

Table 4.2. EC_{50} value of ursolic acid, EGCG and ursolic acid in combination with EGCG in inhibiting MDA-MB-231 cell growth

	EC_{50} value	
component	single	combined
Ursolic acid (μM)	26.68	18.78
EGCG (μM)	155.77	106.29
Combination index	1.38	

Table 4.3. EC_{50} value of ursolic acid, resveratrol and ursolic acid in combination with resveratrol in inhibiting MDA-MB-231 cell growth

	EC_{50} value	
component	single	combined
Ursolic acid (μM)	26.68	30.11
Resverstrol (μM)	72.33	74.19
Combination index	2.17	

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