NEAR EAST UNIVERSITY

HEALTH SCIENCES INSTITUTE

THE EFFECT OF CHENOPODIUM QUINOA SAPONINS ON THE PROLIFERATION OF MCF-7 AND MDA-MB-231 BREAST CANCER

IBTEHAL A ALBADRI
MASTER THESIS

HISTOLOGY AND EMBRYOLOGY DEPARTMENT

THESIS SUPERVISORS

Prof. Dr. AYSEL KÜKNER
Assoc.Prof. Pınar TÜLAY

NICOSIA- 2020

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DIRECTORATE OF HEALTH SCIENCES INSTITUTE

This work has been adopted as a master thesis in the program of Histology and Embryology by the jury.

Examining Committee in Charge:

Jury Member (Supervisor):	Prof.Aysel KÜKNER
Jury Member (Second Supervisor):	Assoc.Prof. Dr. Pınar TÜLAY
Jury Member	Prof.Gamze MOCAN
Jury Member	Prof.Candan ÖZOĞUL
Jury Member	Prof.Bekir ERGÜR

APPROVAL:

This thesis has been approved by the above jury members in accordance with the relevant articles of the NEU postgraduate education, training and examination regulations and has been accepted by the decision of the board of the Institute.

Prof. Dr. Hüsnü Can BAŞER

Director of Health Sciences Institute

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Student Name: Ibtehal A Albadri

Signature: _____

Date: _29th/05/2020_____

COMPLIANCE AND APPROVAL

Her master thesis “Ibtehal Albadri” was written in accordance with the NEU Postgraduate thesis proposal and thesis writing directive.

Prepared by Thesis	Supervisor
Ibtehal Albadri	Prof. Aysel KÜKNER Assoc.Prof. Pınar TÜLAY

Prof. Aysel KÜKNER
Head of Histology and Embryology

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The effect of Chenopodium Quinoa Saponins on the proliferation of mcf-7 and mda-mb-231 breast cancer

Ibtehal Albadri

Supervisors: Aysel KÜKNER, Pınar Tülay

Department of Histology and Embryology, Department of Medical Genetic

ABSTRACT

AIM: The aim of this study was to evaluate the effect of Chenopodium Quinoa Saponins on the proliferation of MCF-7 and MDA-MB-231 breast cancers. The study's main objectives were to evaluate the cytotoxic effects of bioactive compounds, present in Chenopodium quinoa seed.

Methods: The potential anticancer effects of Chenopodium Quinoa Saponins seeds on breast (MCF-7 and MDA-MB231) cancer cell lines using TEBU-BIO cell counting kit 8 and IC50 values were analyzed by using GraphPad® Prism software version 8. Cell apoptosis was evaluated by using Apoptosis detection kit: (ApopTag® Plus Peroxidase In Situ, USA). P-values were analyzed by using PAWS STATISTIC 18.

Results: MCF-7 cells were treated with different concentrations of chenopodium quinoa saponins (200µM, 100µM, 50µM, 25µM, 10µM) and absorbency percentages were measured after 24h, 48h and 72 hours of exposure. There were significant differences between means when 24h-48h, 24h-72h, 48h-72h values were compared in control group, 100µM group, and 25µM group. MDA-MB231 cells were treated with different concentrations of chenopodium quinoa saponins (200µM, 100µM, 50µM, 25µM, 10µM) and absorbency percentages were measured after 24h, 48h and 72 hours of exposure. There were significant differences between means when 24h-48h, 24h-72h,48h-72h values were compared in control group and all concentrations of saponins.

Detection of apoptosis via tunnel stain in MCF-7 cells and in MDA-MB231 cells the results showed that there was no significant difference compared with control group according to the p-values.

Conclusion: The current study demonstrates that the chenopodium quinoa saponins have potential cytotoxic effects on breast cancer cell lines MCF-7 and MDA-MB231 by mechanism involving apoptosis were examined by using TUNNEL-ASSAY and result showed that there was no statistically differences between control group and saponins groups.

KEYWORDS: Apoptosis, Breast Cancer, Cytotoxicity, MCF-7, MDA-MB231, Chenopodium Quinoa Saponins.

ÖZET

Chenopodium Quinoa Saponins'in mcf-7 ve mda-mb-231 meme kanseri proliferasyonu üzerindeki etkisi

İbtehal Albadri

Danışman: Aysel KÜKNER, Pınar Tülay

Histoloji ve Embriyoloji Anabilim Dalı, Tıbbi Genetik Anabilim Dalı

AMAÇ: Bu çalışmanın amacı Chenopodium Quinoa Saponins'in MCF-7 ve MDA-MB-231 meme kanserlerinin proliferasyonu üzerindeki etkisini değerlendirmektir. Çalışmanın ana hedefleri, Chenopodium Quinoa tohumunda bulunan biyoaktif bileşiklerin sitotoksik etkilerini değerlendirmekti.

Çalışma planı: Chenopodium Quinoa Saponins tohumlarının meme üzerindeki potansiyel antikanser etkileri (MCF-7 ve MDA-MB231) TEBU-BIO hücre sayma kiti 8 ve IC50 değerleri kullanılarak kanser hücre hatları GraphPad® Prizma yazılım sürüm 8 kullanılarak analiz edildi. hücre apoptozisi Apoptoz ispat kiti kullanılarak değerlendirildi: (ApopTag® Artı Peroksidaz in Situ, ABD). P-değerleri PAWS STATISTIC 18 kullanılarak analiz edildi.

Bulgular: MCF-7 hücreleri farklı kenopodium Kinoa saponin (200µM,100µM,50µM,25 µM,10µM) ve absorbency yüzdeleri 24h,48h ve 72 saatlik maruziyet ten sonra ölçüldü. Kontrol grubunda 24h-48h, 24h-72h, 48h-72h değerleri ve 100 µM grubu, 25 µM grubu ile karşılaştırıldığında ortalamalar arasında anlamlı farklar vardı. MDA-MB231 hücreleri farklı kenopodium Quinoa saponins (200 µM,100 µM,50 µM,25 µM,10 µM) ile tedavi edildi ve absorbency yüzdeleri 24h,48h ve 72 saatlik maruziyet ten sonra ölçüldü. 24h-48h, 24h-72h,48h-72h değerleri kontrol grubunda ve saponin tüm konsantrasyonlarında karşılaştırıldığında ortalamalar arasında anlamlı farklar vardı. MCF-7 hücrelerinde ve MDA-MB231 hücrelerinde tünel lekesi ile apopoz saptanması sonuçları p-değerlerine göre kontrol grubuna göre anlamlı bir fark olmadığını göstermiştir.

Sonuç: Mevcut çalışma, kenopodium Kinoa saponinlerinin meme kanseri hücre hatları nda MCF-7 ve MDA-MB231 üzerinde potansiyel sitotoksik etkilere sahip olduğunu göstermektedir.

ANAHTAR KELIMELER: Apoptoz, Meme Kanseri, Sitotoksisite, MCF-7, MDA-MB231, chenopodium Quinoa saponinler.

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LIST OF ABBREVIATIONS

ASC	American Society of Cancer
CC	Column Chromatography
CD8	Cluster of differentiation 8
CHO	Carbohydrate
C-MYC	Cellular-myelocytomatosis
C-T	Computer tomography
CYP	Cytochrome p450
Cytochrome p450	(CYP) which result in DNA single strand break (SSDNA)
DAPI stain	2,2-diphenyl-1-picrylhydrazyl fluorescent stain
Db	Dry weight basis
DNA	Deoxyribonucleic Acid
DSB	Double Strand Break
DSDNA	Double Strand DNA
ER	Estrogen Receptor
ESI	Electrospray Ionization Mass
ESI-Ms	Electrospray Ionization-Mass spectrometry
FAO	Food and Agriculture Organization
Gene BCR	Gene breakpoint cluster region protein
HeLa	Henrietta Lacks cervical cell lines
HER2	Human epidermal growth factor receptor 2
IC50	The half maximal inhibitory concentration
IHC	Immunohistochemistry
LA	Lung Adenocarcinoma
MCF-7	Michigan Cancer Foundation-7
MDA-MB-231	Breast cancer cell line isolated by M. D. Anderson
MS	Mass Spectroscopy
NMR	Nuclear Magnetic Resonance
N-MYC	human gene n-myelocytomatosis
PAM50	Prediction Analysis of Microarray 50
SMMC	Statewide Medicaid managed care
SSDNA	Single Strand DNA
TLC	Thin Layer Chromatography
WHO	World Health Organization

1.0 INTRODUCTION AND AIM

The word cancer is a general name for disease that occur as a result of uncontrolled division and growth of cells (i.e. abnormal growth of cells). According to World Health Organization (WHO), cancer is described as set of diseases that can occur at almost every tissue or organ due to uncontrolled or abnormal growth of cells which progress beyond their boundaries and spreading or invading other parts of the body. Cancer is one of the leading causes of death in the world with an estimate of close to 10 million deaths every year, which accounts for 1 in 6 deaths (WHO 2018).

Cancer affects both men and women with some mostly specific to a certain gender. In 2018, the most common types of cancer in men was shown to be stomach, lung, colorectal, liver and prostate cancer, while breast, thyroid, cervical and lung cancer are the common types of cancer in women. In children, the most common types of cancer are blood cancer, which is the major cancer type with the highest percentage, followed by lymph nodes and brain cancer (Schoenfeld & Fraumeni 2006; WHO 2018; Hassanpour & Dehghani 2017).

Chenopodium quinoa seeds poses diverse bioactive components, ranging from saponins, phenolic compounds, flavonoids, triterpenoids, tannins etc. Saponins derived from other plants have shown potential when used as anticancer treatments.

The aim of this study is to evaluate the effect of Chenopodium quinoa saponins on the proliferation of MCF-7 and MDA-MB-231 breast cancers. The study's main objectives are to evaluate the cytotoxic effects of bioactive compounds, present in Chenopodium quinoa seed. This research will add to the state of art in this field, and will demonstrate clear results regarding the effects of using saponins on breast cancer cell lines.

2.0 GENERAL INFORMATION

2.1 TYPES OF CANCER

Currently, according to WHO there are more than 200 different types of cancer. The common ones are breast cancer, prostate cancer, lymphoma, colon cancer, skin cancer, lung cancer etc. (Siegel & Jemal 2019).

Table 1 Common types of cancer

Cancer Type	**Cases**	Deaths
Lung	**2.09 million**	1.76 million
Breast	**2.09 million**	627000
Colorectal	**1.80 million**	862000
Prostate	**1.28 million**	33000
Non-Melanoma Skin Cancer	**1.04 million**	Less than 10000
Stomach	**1.03 million**	783000
Liver	**-**	782000

There is a great variation between cancer and other diseases due to the fact that cancer can develop in almost all body parts and every stage of life from infancy, adolescent, adulthood and old age. Different types of cancer behave differently from one another, some may be very aggressive and proliferate rapidly, while others maybe dormant and proliferate slowly for years (Kaplan et al 2013).

2.2 CAUSES OF CANCER

The scientists continued to discover new types of cancer and search for their causes. The general causes of different types of cancer are either occupational, as a result of exposure to toxic or carcinogenic chemicals in the environment, or to less extent, due to genetic factors which are inheritable (Blackadar 2016). The two common causes of cancer consist of both genetic and non-genetic factors. Non-genetic factors contribute highly to development of cancer due to their role in triggering or activation of genes that are mutated, and thus lead to uncontrollable cell proliferation and neoplasmic transformations. Even though there are few studies that describe processes that occur at the early stages of cancer growth, and the overall environmental or occupational factors that induce tumor development (Kaplan et al 2013).

Several studies have revealed that 80% of cancer types are formed due to environmental factors and lifestyle, and thus, they are preventable (Blackadar, 2016). Most of the current types of cancers and the risk factors associated with their formation have been identified, only few remain problematic and thus adequate research is needed. It is also estimated that 30% of future cancer development can be prevented by taking different measures (Dumalaon-Canaria et al 2014; Blackadar, 2016).

Toxic chemical compounds induce gene mutations in cells and thus leading to cancer cells. Occupational and habitual lifestyle, such as smoking tobacco, which contain carcinogenic compounds can lead to lung cancer (Aizawa et al 2016). Moreover, any compound that possesses carcinogenic properties for use in food industries, fertilizers, drinks and environment can directly or indirectly affect both nucleus and cytoplasm of cells, and thus result in genetic disorders or gene mutation, that can lead to formation of cancer at tissue site or cell, Other causes of cancer include radiation rays, bacteria and viruses account for 7% (Poon et al 2014).

2.2.1 Genetic Factors

activation of oncogenes and development of genetic disorders occurs due to genetic changes which comprise point mutation (i.e. Ras gene) in colon cancer, amplification (i.e. N-MYC) in neuroblastoma, insertion activation (i.e. C-MYC) in acute blood cancer, translocation of the chromosome (i.e. oncogene ABI and gene BCR) in chronic blood cancer. In elderly people, the exchange of genetic constituent between two chromosomes (i.e. chromosome 22 and 9) lead to chronic blood cancer (Hassanpour & Dehghani 2017). The most common genetic factors are due to p53 gene mutation which results in development of odd protein that plays a vital role in disruption of molecular activities of p53. Under favorable conditions, p53 plays a vital role in cell differentiation, cell division, DNA metabolism, angiogenesis, cell death and senescence (Blackadar, 2016). However, most of the mutations associated with p53 gene arise in DNA binding position, as well as, the inability of p53 to regulate genes during replication. The eccentricity of these biological and molecular processes results in development of cancer cells and tissue.

Moreover, there is a complex connection between cancer cells and p53 gene, and thus the abnormality of p53 gene occurs in more than 50% of all cancer cases (Chae et al 2011).

2.2.2 Environmental and Occupational Factors

For over half century, scientist have been studying the impact of cigarette smoking on the health of individuals and its carcinogenic property. Several studies have demonstrated that cigarette induces cell damage and mutation *in vivo* due to its carcinogenic nature. The profiling of cigarette has been shown to contain more than 73 carcinogenic elements specific to tobacco nitrosamines. These chemicals constitute NNK (4-(N-methylN-nitrosamino)-1–(3-pyridyl)-1-butanone) and its derivatives, NNK constitutes the most active and major carcinogen in different tobacco products (Maser 2004).

The mechanism behind carcinogenic effect of NNK rely on activation by an enzyme known as cytochrome p450 (CYP), which results in DNA single strand break (SSDNA), which in turn can induce gene mutation resulting in cancer formation in liver, lung and other tissues or organs. The risk of developing different types of cancer increases in direct relation to the number of cigarettes an individual smoke and the duration of smoking. A study has revealed that a person who smokes have 95% higher risk of developing cancer diseases than a person who had never smoked (Zheng, & Takano 2011).

2.2.3 Ultraviolet Radiation (UV)

Ultraviolet Radiation (UV radiation), and other forms of radiation induce gene mutations and cellular damage (Clapp et al 2006). When a UV radiation is exerted on a cell, it leads to conformational changes, and double strand break in cellular genetic constituents. Those mutations include Cytosine-Thymine (C-T) and double mutations (CC-TT). These mutations occur due to cytosine dimers formed by UV light which result in adjacent cytosine mutation to thymine (Alexendrov et al 2013). The common type of cancer caused by UV light is skin cancer. A study has shown that more than 90% of melanoma and non-melanoma skin cancers are caused by high exposure to UV radiation from sources such as the sun, sun lamps, solariums and sunbeds (Narayanan et al 2010). Skin cancer occurs when UV light penetrates into epidermis which form the top outer layer of the skin. UV light thus causes genetic damage as a result of double strand break of DNA which leads

to mutations and formation of cancer cells, as well as, immune suppression, ageing, blotchiness, wrinkles and sunburn which contribute to the risk of formation of skin cancer (e.g. melanoma skin cancer) (Diepgen et al 2012).

2.3 Diagnosis and Treatment

Advances in molecular biology, epidemiology, oncology, genetic engineering and imaging allowed scientists to diagnose cancer diseases by focusing on various changes that affect genes, genomic surroundings of both cancer cells, normal cells and precursors, and their link to environmental factors. To design an effective cancer therapies and prevention approaches, identifying several changes that lead to tumor formation and surrounding tissues link with both validated and suspected environmental and occupational risk factors are very critical.

In the past, some cancer types experienced high cure rates, but other types had low cure rates due to their complexity, lack of proper and accurate diagnosis procedures and therapy. Cancers can be treated at different stages, starting from diagnosis at early stages to progression stages, with therapies that vary extensively according to cancer type, stage and patient's genetics make-ups, metabolism and history of other diseases mostly associated with the immune system (Kaplan et al 2013).

The type of cancer treatment given to a patient is based on diagnosis procedure, the location of the cancer in the body, the type or grade of the cancer, the standard approach, medical therapies and treatment procedures in patient's country, and the patient's ability to finance and cover the cost of treatment using either national insurance scheme or private funds. For solid types of cancer, at an early stage of diagnosis, the use of surgery as a treatment approach is very effective and hence recommended. Other approaches combine surgery with radiotherapy to destroy cancer tumors. Changing the treatment approach is related to the cancer progress, from the early stage, most of the treatment procedures include chemotherapy, radiotherapy, hormone ablation treatment and hormone regulated treatment. These are the types of treatment that focus on activating the immune system to induce immune response against tumor types, or the process of stabilizing and suppressing tumors. The most common target cancer types for cancer immunotherapy include

melanoma and kidney cancer as a result of spontaneous regression process and CD8+ lymphocyte frequent infiltration (Drake et al 2013).

2.4 BREAST CANCER

Breast cancer is among the most common cancer types in women, along with skin cancer. In 2012, it was estimated that there are over 1.6 million diagnosed new cases of breast cancer in women globally, and most of the cases are seen in women above the age of 55 years (WHO 2012; Mitsuk 2016). Formation of breast cancer begins when cells in the breast start to grow uncontrollably. The resulting growth of these cells results in tumors which can be diagnosed using X-ray, or seen as a lump growing in the breast. Breast cancer can become malignant as a result of proliferation of the cells and spreading or invading nearby tissues and other parts of the body (American society of Cancer 2016).

2.4.1 Classification of Breast Cancer

Breast cancers can be classified based on their tumor size, grade, molecular classification, histological type, lymph node and location of the tumor, response to treatment, and estrogen receptor α (ER) and HER-2 receptor status.

2.4.1.1 Classification based on ER Status

Evaluation of ER status is very critical concerning the treatment approach for breast cancer. This classification method is employed to determine if the patient should undergo endocrine therapy or another therapy, as patients who are ER negative do not need endocrine interventions. To evaluate ER status in patients, IHC is employed which is the gold standard approach that is carried out on formalin fixation and paraffin-embedded cancer tissues (Pusztai et al 2006).

2.4.1.2 Classification based on Aggressiveness (Invasive and Non-Invasive)

Invasive breast cancer is the type of cancer that spreads beyond the breast. The most common example of invasive breast cancers are invasive ductal breast cancers (i.e. ductal carcinoma), which are found in cells or tissues of the ducts that are responsible for transporting milk to the nipples. Other examples are inflammatory breast cancers and invasive lobular breast cancers. A non-invasive breast tumor is regarded as in situ

carcinoma, which is located in the breast. This type of cancer can only be detected in a mammogram, but difficult to detect using breast palpation. Moreover, other types of breast cancer include metastatic breast cancer, triple negative breast cancer and other rare types (Al-Ejeh et al 2014; Lehmann-Che et al 2013).

2.4.1.3 Classification based on Grade

This type of classification is one of the common classification approaches for breast cancer, in which tissues that undergo biopsy are classified based on the appearance of the tumor compared to healthy or normal tissues, and the speed in which cells divide. When cells grow or divide slowly it is termed "lower grade", but when cells grow or divide very fast, and have tendency to spread to other neighboring tissues or cells, it is called "high grade". Other grade classifications include Nottingham grade, Elston-Ellis grade, Bloom Richardson grade, Scarf Bloom Richardson grade (Malhotra et al 2010). This classification can also be represented as: Grade 1 cancer is categorized as grade 1 when it looks mostly like normal cells but can be well differentiated. Grade 2 cancer is categorized as grade 2 when it can be moderately differentiated. Grade 3 cancer is categorized as grade 3 when it is highly unrecognizable, and is spreading or growing at very high rate or speed.

2.4.1.4 Classification based on Gene Expression

Categorizing breast cancers based on gene expression is the recent classification approach (ASC 2014; Lehmann-Che et al 2013; Viale 2012; Gusterson et al 2005). According to ASC, breast cancer is classified base on molecular structures evaluated by PAM50 test. This approach classifies breast cancers into 4 types:

Luminal breast cancer: This type consists of luminal A and B breast cancer types, which have similar genes expression patterns with healthy cells. Like the classification based on grade, luminal A grow slowly and are termed as low grade, while luminal B grow faster and behave aggressively. HER2: Is a different classification approach based on the presence of more copies of HER2 gene after diagnosis. Basal type classification: Is another classification approach based on basal type (i.e. triple negative breast cancer cells). Other approaches: Breast cancer cells can be classified due to the lack of progesterone and estrogen receptors etc.

2.4.2 Causes and Risk Factors of Breast Cancer

Even with the advances in technology and computer aided detection techniques, the cause of breast cancers and other forms of cancers are completely uncertain or unknown. Some of the factors that play a vital role in breast cancer formation include genetics, age, female sex hormones and predisposition. The mechanisms on how these factors induce or enhance the development of tumors in the breast remain unknown (Hashemi et al 2014). Many studies have shown that undergoing abortion, size of breast, use of antiperspirants and deodorants, artificial breast, breast implants and wearing brazier does not cause or increase the risk of breast cancer (Bernstein, 2002).

However, few studies have shown that women who undergo surgery related to breast shape correction, breast augmentation such as breast enlargement, have higher risk of developing breast cancer compared to other women who do not undergo such type of surgery, due to formation of scars during operations which may lead to change in cell growth (McLaughlin at al., 2006). It is also revealed that injuries to the breast, and the taking of oral contraceptives, may slightly increase the chance of developing breast cancers. Heavy smoking of cigarettes and tobacco has been shown to induce cell disruption and significantly increase the chance of developing breast cancers (Hashemi et al 2014).

Risk factors

Currently, oncologists have difficulties on ascertaining why some women cannot develop breast cancer and others can (Nelson et al 2012; Dumitrescu et al 2005). Some of the factors that lead to breast cancer can be prevented, while some can only be suppressed. The most common risk factors include:

Family history this risk factor is based on genetics, where cancer genes are transferred from parents to offspring. Women who inherited these genes have higher chance of developing breast cancer. Age research has shown correlation between older age and breast tumor development. According to ASC, eight out of ten women can develop breast cancer after 50 years of age. Breast density the risk factor of breast density is far below that of genetics. Few researches have shown the relationship between breast density and the risk of developing breast cancer. Previously diagnosed breast cancers due to the ability

of breast cancer cells to spread to other tissue or organs (i.e. invasive breast cancer). Patients who have been diagnosed, and treated, with breast cancer have higher chances of developing the same, or other type of, cancer.

Overweight, obesity and diet: According to several studies, diet play major role in preventing cancer. Many diets rich in vitamins, minerals and bioactive compounds have shown to reduce risk of different cancer types (Nelson et al 2012). Radiotherapy for chest and breast: Previous contact with radiation for treatment purposes, or as hazard, improve the chances of developing breast cancer.

Other risk factors: These include Estrogen treatments of breast cancer, giving birth of a first child at an older age (i.e. above 50), first menstruation at an early adolescent stage (below 12 years and women who drink too much alcohol) (Bernstein, 2002).

2.4.3 Diagnosis of Breast Cancer

One of the first diagnosis methods of breast cancer is biopsy, which is carried out when a physician suspects the presence of the tumor. Biopsy method is based on collecting breast sample tissues which will be analyzed under the microscope. Diagnosis or screening of breast cancer is achieved using mammography, which is a simple, safe and affordable technique. This approach has been shown to be able to detect cancer cells at early stages (i.e. zero stage, and 1A-1B stage), when symptoms are unnoticeable. Undergoing regular mammography has also, been shown to prevent, or reduce the risk, of developing malignant or chronic cancer that is difficult to cure (i.e. stage 3-A, 3-B, 3-C and 4) (Løberg et al, 2015). This technique uses X-ray to take images of breast mammary glands, which is carried out in a room with installed mammography machines.

2.4.4 Treatments of Breast Cancer

The treatment of breast cancers is carried out after diagnosis, and oncologists usually propose a suitable treatment approach and prognosis. This treatment approach mostly depends on patient's breast cancer classification, according to a standard protocol based on grade, type, gene expression and stage of the breast cancer. There are few numbers of approaches for the treatment and prevention of breast cancer. Some of these approaches include chemotherapy, or the use of drugs, surgery, radiotherapy, hormone therapy etc. (NHS 2020; Maughan et al 2010).

2.4.4.1 Chemotherapy

Chemotherapy is the use of drugs that kill cancer cells, and stop cancer from spreading to other cells or neighboring tissues. Cancer drugs are termed as anticancer agents, and they are cytotoxic in nature. The use of anticancer drugs varies from one patient to another, and according to the availability of anticancer drugs present in the clinic, or patient's country.

Administering drugs to cancer patients before surgery is termed as neo-adjuvant chemotherapy. This type of chemotherapy is employed in order to shrink or reduce larger tumor sizes, and hence reduce the risk of complications during surgery. Adjuvant chemotherapy, is another type of chemotherapy which is administered after surgery. In this therapy, patients are prescribed with cytotoxic drugs to eliminate the remaining cancer cells that escaped surgery (NHS 2020; Cancer Net 2019).

The mechanism behind the application of anticancer drugs is mostly to induce cytotoxic effects as a result of inhibition of cancer gene, inhibition of enzymes responsible for different pathways, such as: krebs cycle, glycolysis, and phosphorylation. Other approaches use chemical compounds which are toxic to eliminate cancer cells. Most common anticancer drugs include Pertuzumab, Tamoxifen, Ribociclib, Abemaciclib, Everolimus, Eribulin, Aromatase inhibitors, and ovarian ablation, or suppression. Oncologists administer cancer drugs based on the type, stage and grade of the cancer cells, and its aggressiveness (whether it spread to other tissues). Chemotherapy drugs are mostly administered intravenously or using drips, few drugs are in tablet form (NHS 2020; Cancer Net 2019).

Side effects of chemotherapy

Several studies have reported adverse side effects of synthetic (chemical) anticancer drugs on healthy or normal cells (e.g. immune cells). The most common side effects of chemotherapy include: Cytotoxicity to healthy cells, which leads to several infections and Cough and Shivering, Loss of hair, Diarrhea, Loss of appetite, Headache, Skin rash, Sore mouth, Fatigue and Stoppage of period in women experiencing menopause.

2.4.4.2 Surgery

The treatment of breast cancer using surgery include either cutting out the entire breast, or only the part that contains the tumor. This treatment approach includes extirpation of the breast (i.e. mastectomy), and breast preservation approach along with removal of mammary nodules (Nazarali & Narod 2014). Treatment of breast cancer depends on the stage (0, 1-A, 1-B, 2-A, 2-B, 3-A, 3-B, 3-C and 4), the age of the patient, cost and the available treatment. However, complete removal of pre-cancerous tissues is a preventive measure, recommended only for women who have potential or higher risk of developing breast cancer. The side effects of surgery are related to chemotherapy and radiotherapy which are employed after surgery (NHS 2020; Cancer Net 2019; Maughan et al 2010).

2.4.4.3Radiotherapy

Radiotherapy is a treatment technique which utilized regulated doses of radiation to kill breast cancer cells. Radiotherapy is mostly employed, after chemotherapy or surgery, to kill cancer cells remnant. There are different types of radiotherapy, and each type depends on the type or grade of breast cancer and the surgical approach utilized. Patients with stage 1 or grade 1 breast cancer usually do not need surgery. The types of radiotherapy include: Breast radiotherapy, Chest wall radiotherapy, Breast boost and Lymph nodes radiotherapy. (Cancer Net 2019; Maughan et al 2010).

Side effects of Radiotherapy

Some of the side effects of radiotherapy include: Lymphedema: Blockage of the lymph nodes under patients arm due to excess build of fluid, Fatigue (extreme feeling of tiredness), Darkening and extreme irritation on the breast skin, Redness and weepy skin, Sore and rashness in the breast.

2.4.5 BREAST CANCER CELL LINES

2.4.5.1 MCF-7 Breast Cancer Cell Line

MCF-7 was isolated through pleural effusion in 1973 (pirsko et al 2018). The name is an acronym of Michigan Cancer Foundation. MCF-7 cell lines are regarded as the most

utilized breast cancer cell line for research purposes worldwide. The characteristics and profiling of this cancer has shown to be noninvasive, estrogen dependence and positive for functional estrogen receptor for proliferation *in vivo* and *in vitro*. Other characteristics delineate MCF-7 as xenografts which express p53 (wild type). MCF-7 has been utilized for identifying the mechanism of cancer cell resistance to drugs and preclinical analysis and testing of therapies such as antiestrogen using aromatase inhibitors and tamoxifen (Welsh et al 2013). Figure 1 shows the Morphology of MCF-7 cells grown in 2D and 3D cultures.

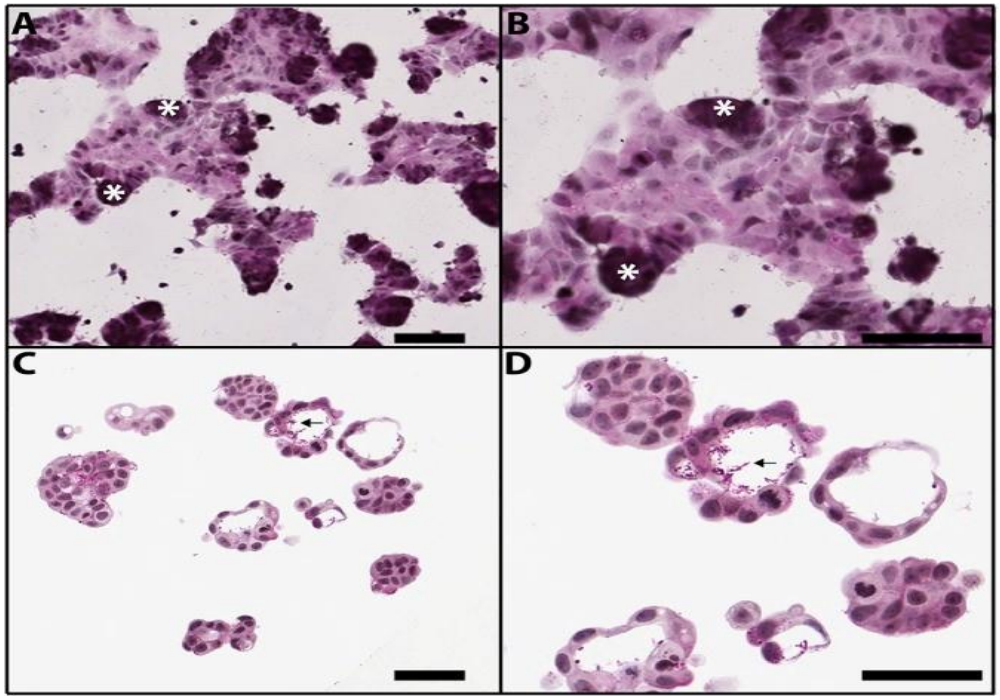


Figure 1: Morphology of MCF-7 cells grown in 2D and 3D cultures (Vantangoli et al 2015).

2.4.5.2 MDA-MB-231 Breast Cancer Cell Line

Originally, MDA-MB-231 cells were isolated from a Caucasian female name MD Anderson at the age of 51years old from Houston Texan USA and has been used worldwide in laboratories as a model for breast cancer research. MDA-MB-231 is an invasive form of cancer and very aggressive. Triple negative breast cancer. This type of breast cancer is epithelial and metastatic in nature. It lacks expression of HER, progesterone receptor and estrogen receptor which are differentiated as MDA-MB-231

cells were initially classified as basal B breast cancer cells; however, it is now categorized as Claudine low molecular subtype (journal of cancer et al 2017). This recent classification is due to exhibition of downregulation of claudin-4 and claudin-3 and also low expression of Ki-67 proliferation marker (Welsh et al 2013). Figure 2 shows the bright field and field and fluorescence images of MDA-MB-231 cells in 2D and 3D with and without Cytochalasin D treatment (Mak et al 2015).

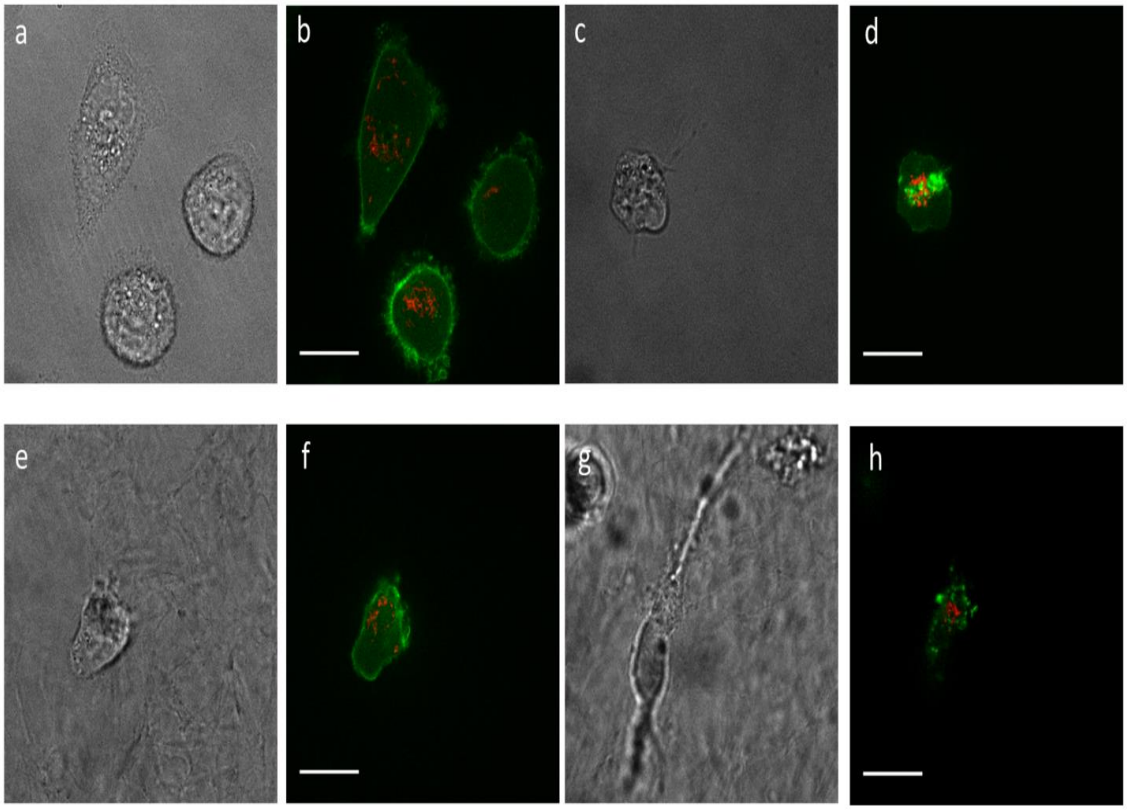


Figure 2: Bright field and fluorescence images of MDA-MB-231 cells in 2D and 3D with and without Cytochalasin D treatment (Mak et al 2015).

2.5 ADVANTAGES OF NATURAL TREATMENT

Disease is one of the horrific conditions that affect human beings, and most of acute or chronic mild diseases are associated with parasites, viruses, fungi and bacteria such as gastroenteritis, headache, fever and cough. On the other hand, chronic diseases are mostly associated with genetic disorders, as a result of gene malfunction or mutation such as: Sickle cell anemia, Cystic fibrosis, Some types of cancer, Huntington's diseases,

Hemophilia, Duchene muscular dystrophy, Immunodeficiency, as well as, Uncontrolled spread of pathogens such as HIV, Zika virus, Dengue virus and Coronavirus (Namita & Mukesh 2012).

There is wide range of bioactive metabolites that form building blocks of therapeutic drugs synthesis, nutraceuticals and pharmaceuticals. From ancient times, where folk medicine is practiced, plants have been the major source of drugs against diseases worldwide, due to their low side effects (Newman & Cragg 2007). Bioactive compounds from plants have been isolated and used as antimicrobial drugs to treat ailments related to infectious diseases caused by fungi and bacteria. Moreover, these metabolites derived from different medicinal plants have been used as anticancer, antioxidant, antidiabetic, antihypertensive, anticoagulants and other cardiovascular disorders (Mukheriee & Wahile2006).

3. LITERATURE REVIEW

This chapter overviews the taxonomy of Chenopodium quinoa wild, nutritional, bioactive and chemical compositions. It discusses saponins, chemical structure and health benefit of saponins, and presents empirical review on state of art of using plant extracts for anticancer activity.

3.1 CHENOPODIUM QUINOA WILD

Chenopodium quinoa wild, is a member of the Chenopodiaceae family, also commonly known as "Quinoa", and is a popular staple food native to South American Andean communities. It serves as food alternative, and utilized as porridge soup in some parts of the region (Figure **3** below)

Figure 3: A photograph of Chenopodium quinoa wild (Lim 2012).

Profiling of the plant seeds, has shown them to possess so many key elements as nutritional diet and medical properties (Kuljanabhagavad et al 2008). Quinoa seeds are

found to possess high amount of carbohydrate (energy), compared to other carbohydrate-rich foods such as rice, corn, wheat, cereals and sorghum.

Table **2** Biological activity of Chenopodium quinoa (Yadav et al 2007)

Specie	Part/Extract	Biological Activity
Chenopodium quinoa	Seed (extraction of saponins using chemical solvent)	Anticancer, antifungal and immunomodulatory
Chenopodium anthelmenticum	Essential oil	Cytotoxic
Chenopodium multifidum	Aerial part (aqueous extract)	Cytogenic
Chenopodium murale	Leaf using ethanol for extraction and flavonoid from aerial part	Cytotoxic and Hypotensive
Chenopodium album	Fruit using ethanol for extraction	Antipruritic, Antinociceptive
Chenopodium chilense	Aerial part using methanol for extraction	spasmolytic
Chenopodium abrosiodes	Leaf using methanol for extraction	Antipruritic, Antinociceptive
Chenopodium botrys	Aerial part (essential oil)	Antibacterial
Chenopodium amaranticolar	Leaf	Hemagglutination and antiviral

The seeds are also rich in high quality proteins, which contain diverse class of amino acids (Dini et al 2005). Moreover, the plant is rich in minerals such as cupper (Cu), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), and manganese (Mn).

The seeds were also utilized and processed for use in animal diet formulations, and for consumptions by humans (Lindeboon et al 2005).

The common preparation of Quinoa is similar to wheat and rice preparations, which are grinded into a flour for use in drinks fermentation, cakes and bread preparations (Bhargava et al 2006). With all the benefits that come along using this seed, it has a limitation as a result of bitterness in taste, due to the presence of chemical constituents known as saponins (Brady et al 2007). *Chenopodium quinoa* has also been shown to contain Folic Acid (Vitamin B9), Tocopherols (Vitamin E), Riboflavin (Vitamin B2) and Carotene, more than those contained in oats, rice, maize and wheats. According to Food and Agriculture

Organization (FAO), the nutritional components such as carbohydrates, vitamins, proteins and minerals content, provide daily nutritional requirements for children less than 3 years old.

3.2 SAPONINS

Saponins are a class of triterpene glucosides. They consist of a linear arrangement between 1 to 6 pentose-glycoside, or hexose-glycoside units, linked to sapogenin aglycone which exists in two forms (triterpenoid aglycone or steroidal). In either triterpenoid aglycone or steroid, the CHO (Carbohydrate) side chain is mostly attached to the 3-C (carbon) of the sapogenin (Savage, 2003). They are found in the outer layer of *Chenopodium quinoa* seeds, and can be easily removed by washing with water or polishing. Saponins are widely found in different plants, but mostly present in legumes such as beans derived from Phaseolus vulgaris, Soya beans and Chickpeas.

Several studies have reported the presence of saponins in more than 100 different families of plants, and 150 plants were found to poses anticancer properties (Man et al 2010). Saponins are classified into 11 different classes, which include steroids, hopanes, cucurbitanes, lupanes, lanostanes, tricullanes, cycloartanes, dammaranes, taraxasteranes, ursanes, and oleananes. Due to their diverse structure, saponins poses antitumor effects against different types of cancer cells through various anticancer pathways (Man et al 2010).

3.2.1 Chemical Structure and Properties of Saponins

There is a wide variety of plants that contain saponins. A study has shown the complexity of saponins isolated from *Chenopodium quinoa*, which were found to contain a mixture of derivatives of triterpene glycosides, including Phytolaccagenic acid, 3b,23,30-Trihydroxy Olean-12-en-28-oic acid, that bear Carboxylate group and Hydroxyl group at C-28 and C-3 respectively, Oleanolic acid, Serjanic acid, Hederagenin, which bear Hydroxyl and Carboxylate groups (Mad et al 2006).

The common carbohydrates present include Galactose, Arabinose and Glucose, while the less common are Carbohydrates including Xylose and Glucuronic acid (Mad et al 2006;

Kuljanabhagavad et al 2008). Saponins extracted from *Chenopodium quinoa* are highly soluble in water and methanol, but saponins combine with iron to form chemical complexes to reduce absorption. In aqueous solutions, saponins produce stable foams; i.e. they form soapy lather when agitated in water, (Savage et al 2003). The chemical structure of saponins is shown in Figure **4**

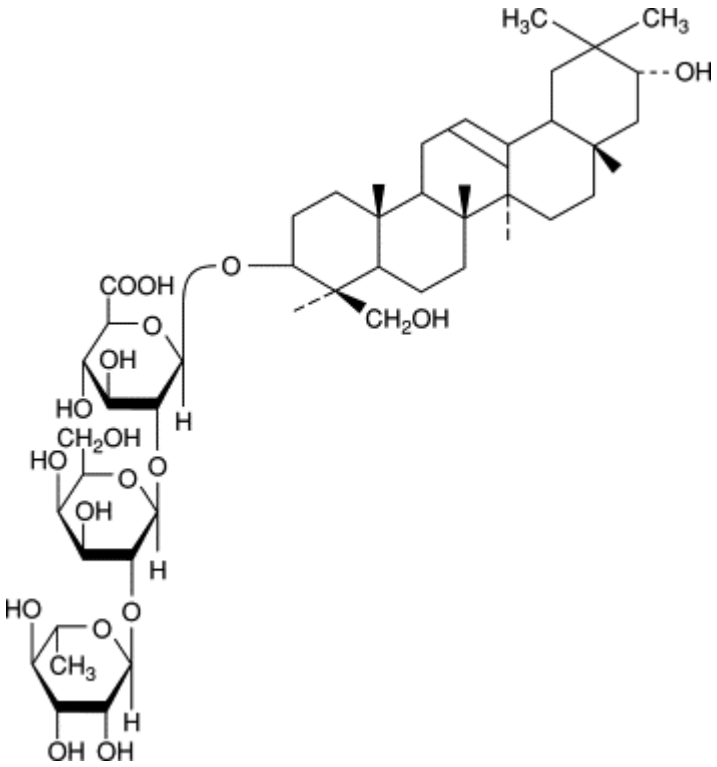


Figure 4 Chemical Structure of Saponins (Savage, 2003).

3.2.2 Hederagenin, Hydroxy-Hederagenin and Phytolaccagenic Acid

Hederagenin also called hederagenic acid. Hederagenol is a chemical compound which belongs to the Triterpenoid family, and is one of the chemical components of many plants' biome. The most common plants that poses high amounts of hederagenin is common ivy (Hedera helix) and Chenopodium quinoa. These plants possess diverse forms of hederagenin with alpha hederin, and hederacoside C is the most common type. Other types include delta-hederin, beta hederin and their derivatives, such as Hederagenin 3-O-Alpha-L-rhamnosyl (1-->2)-Alpha-L- arabinoside and Hederagenin Alpha-L-arabinoside. Hederagenin saponins possess diverse biological activities such as anticancer, or cytotoxic properties, as a result of their interaction with cell membrane, antifungal, antiviral, hemolytic and molluscidal activities (shi et al 2004).

Hederagenin has a molecular weight of 472.7 g/mol. Hydroxy hederagenin is a saponin in the form of olean-12-en-28-oic acid, which is substituted by hydroxy groups at position 2 and 23. (figure **5**)

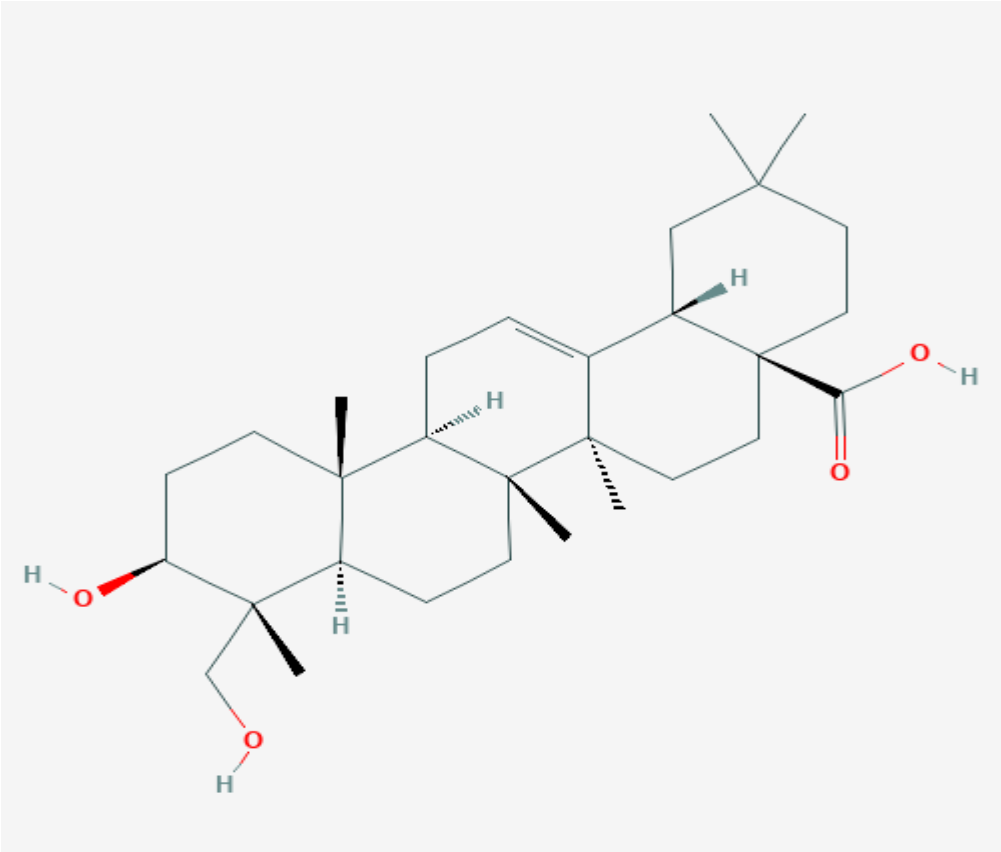


Figure 5 Chemical structure of Hederagenin (PumChem.com)

Phytolaccagenic acid, also called 10-Hydroxy-9-(hydroxymethyl)-2-(methoxycarbonyl), exists in other forms such as phytolaccinic acid and phytolaccinate. It has an average molecular weight of 516.7092 g/mol, and a molecular formula of $C_{31}H_{48}O_6$. It is a very strong acid, with water solubility of 0.0024 g/L. Phytolaccagenic acid belongs to the Triterpenoids class (organic compounds known as "terpene"), which contain 6 different isoprene units.

Dini et al (2001) carried out an analysis on Chenopodium quinoa. The team isolated and characterized Triterpene saponins from Chenopodium quinoa on the basis of spectral evidence using NMR and ESI-Ms. Among many chemical compounds, different forms of

phytolaccagenic acid were isolated including oleanolic acid and its derivatives and Oleanane saponins. Figure **6** shows the chemical structure of Phytolaccagenic acid.

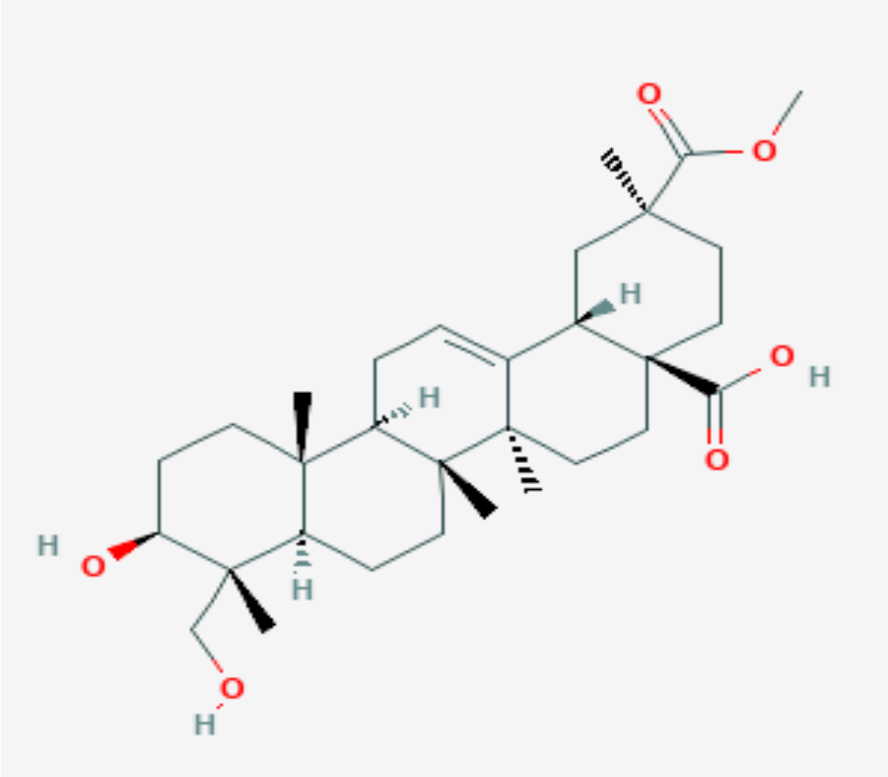


Figure 6: Chemical Structure of Phytolaccagenic acid (PumChem.com)

Several researchers have isolated saponins from Chenopodium quinoa, and their analysis revealed that saponins possesses antimicrobial properties against a broad range of gram positive and gram-negative bacteria and antiviral properties (PumChem.com). A popular study has shown saponins to be very toxic to brine shrimps (Kuljanabhagavad et al 2008). Other health benefits of saponins include the ability to lower cholesterol level, intensify or boost drug absorption *via* mucosal membranes. They also were found to enhance immunity through absorption adjuvants for antibody antigen reaction (Kuljanabhagavad et al 2008).

3.2.3 Health Benefits of Saponins

Some of the biological activities of saponins include hemolysis of red blood cells when administered intravenously, but the degree to which saponins effect red blood cells varies

among different mammalian species. The mechanism behind the hemolysis activity of saponins is due to its interaction with membrane bound sterols, leading to high permeability of the cellular plasma membrane and resulting in cell destruction. Furthermore, saponins hemolytic activity has been utilized for assays regarding detection of saponins in plant components, used as fish poison due to their toxicity to cold blooded animals, and they are also shown to poses pharmacological activity, fungicidal, insecticidal, and antibiotic activity (Shi et al 2004). The cholesterol-binding activity of saponins has been utilized for feed formulations of chicks, due to its ability to reduce plasma cholesterol level. A series of experiments of feeding saponins to monkeys, rats and rabbits have demonstrated the liver-cholesterol lowering effects of saponins (i.e. Lucerne-saponins) derived from Chenopodium quinoa, but exert no effect on plasma-cholesterol compared to controlled test sample (Shi et al 2004). Similar experiments carried out on normal human subjects lead to variable results. Another study utilized patients with hypercholesterolemia with a variety, and different concentrations of saponin supplement, in a dietary food such as soy flour, and no evidence of cholesterol lowering effects were noticed.

3.3 EMPIRICAL REVIEW

Effects of Saponins (Hederagenin, Hydroxy-Hederagenin and Phytolaccagenic acid) Isolated from C. Quinoa

3.3.1 Anti-cancer effects of saponins

Kuljanabhagavad et al (2008) carried out an analysis on the isolation of triterpene saponins, its structure elucidation and biological properties such as cytotoxicity of the chemicals isolated from Chenopodium quinoa's seed, seed coats, fruit and flowers. The resulting chemical components were extracted *via* exhaustive maceration by using methanol and petroleum ether. The chemical constituents were analyzed using Thin Layer Chromatography (TLC) and the result yields were as follows 8% yield from seed, 10.7% from seed coats, 4% from fruit and 4% from flowers. These chemicals contained Hederagenin, Phytolaccagenic acid, Oxo-oleanolic acid and its derivatives, and Serjanic acid. HeLa cells are used to test the cytotoxic activity of saponins using MTT assays, with the result demonstrating high toxicity effects (i.e. potent cytotoxic activity).

Hu et al (2017) evaluated the chemical characterization, antioxidant anticancer activity, and immune regulating activities, of bioactive polysaccharide components isolated from Chenopodium quinoa seeds. The experiment utilized ultrasound-assisted extraction process to extract polysaccharide components from the plants, and purification using column chromatography. Evaluation of anticancer activity was carried out using C. Quinoa Polysaccharide (CQP) on MCF-7 breast cancer cell lines and human liver cancer SMMC 7721. The result has shown that CQP possesses cytotoxic activity against breast cancer cells and liver cancer cells, with no effects on normal cells. Moreover, the result indicated that the bioactive components present in Chenopodium quinoa has a potential for use as anticancer, antioxidant and immune regulating agents.

A study conducted by Chwalek et al (2006) focused on the cytotoxicity activity of Hederagenin (i.e. α-hederin) on Keratin forming tumor cell lines (KB cells). The research explored the sugar moieties of the compound hederagenin diglycosides (α-L-rhamnose, β-D-xylose or β-D-glucose). Confocal microscopy was utilized to evaluate the effect of Hederagenin on KB cells. The result has shown some saponins induced apoptosis to cells and anticancer activities due to the sugar moiety present in the compounds.

Another study by Wang et al (2009) investigated the *in-vivo* and *in-vitro* antitumor activities of Hederagenin saponin isolated from Lonicera macranthoides. The research adopted different assays such as DAPI staining, cell proliferation assays, flow cytometry analysis and xenograft tumor growth assays, on different types of cancer cells. The result has shown Hederagenin to inhibit the proliferation of cancer cells in a range of 10 to 20 micro molar with IC50.

3.3.2 Anti-inflammatory effects of saponins

One of the causes of inflammation is the body's reaction due to immune response, injury and infections which result in reddening and swollen of either external or internal body parts. Health expert classified inflammatory into acute and chronic, acute inflammation are less severe while chronic inflammation contributes to formation of severe disorders, diseases related to inflammation include chronic peptic ulcer, rheumatoid arthritis, acute

appendicitis, acute bronchitis, infective meningitis, tuberculosis, asthma, sore throat and dermatitis. There are wide range of drugs available that relieve inflammation, majority of these drugs are synthesized in the laboratories and thus have side effects and are expensive. In order to optimize the side effects, scientist extracted chemical compounds such polyphenols, saponins, tannins and flavonoid from plants to analyze their anti-inflammatory activities (Khansari et al 2009).

To evaluate the anti-inflammatory activity of saponins from quinoa (Chenopodium quinoa Wild.) seeds, Yao et al (2014) extracted saponins using exhaustive maceration with MeOH as solvent. The extract is analyzed using liquid chromatography–mass spectrometry (LC–MS). Mouse macrophage cell line RAW 264.7 was employed for evaluation of the anti-inflammatory activity of the extracted saponins. The analysis indicated that saponins can be used for treatment of inflammation related diseases and as functional food components for prevention.

Ibironke & Ajiboye (2007) investigated the anti-inflammatory activity of saponins extracted from chenopodium ambrosioides. The study utilized extracted saponins from the dried leaves of the plant using methanol. Evaluation of anti-inflammatory activity is carried out on formalin and carrageenan induced rat models (male albino Wister rats). The result has indicated saponins extracted from Chenopodium ambrosioides has potential in alleviating pain and reducing swelling as a result of inflammation.

3.3.3 Antibacterial effects of saponins

Bacteria are one of the most abundant organisms in nature. Some strains are very beneficial to humans as the aid in digestion and metabolomics, they also play important role in the environment such as decomposition of waste. However, some strains of these microorganisms are pathogenic in nature and thus they can infect human beings and animals. Antibacterial agents are classified as natural-derived or synthetic. Several studies have shown the presence of antibacterial agents extracted from wide range of food crops and medicinal plants. Some of the food crops that are susceptible against bacterial species include lemon, garlic and ginger. (Klančnik et al 2010).

Korcan et al (2013) analyzed the antibacterial effects of saponins derived from Chenopodium albums. Saponins are extracted from the leaf using solvent extraction method with ethanol as the main solvent. Determination of the antibacterial effect is carried out on both the Gram (+) and Gram (-) microorganisms (*Salmonella typhimurium, Proteus vulgaris, Bacillus cereus, Bacillus subtilis, Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa, Listeria monocytogenes and Micrococcus luteus*) based on inhibition zone. The result has shown susceptibility of saponins against antibacterial activity was recorded *Bacillus subtilis* with 13 mm of inhibition zone.

A similar approach was employed by Amjad & Alizad (2012), the study examined the antimicrobial activity of chemicals extracted from *Chenopodium album L*. collected from East Esfahan, Iran. The extraction method is based on solvent extraction using ethanol and methanol. The crude extracts were evaluated using disc diffusion method against four bacterial strains (*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli and Bacillus cereus*). The result has shown minimal inhibitory effect of extract against the bacteria used in this study.

3.3.4 Antioxidants effects of saponins

Antioxidants are very important compound required for proper metabolism due to their ability to repair cells from damage and prevent cellular oxidation as a result of free radicals. The main function of antioxidants is to neutralize the damage cause by free radicals which contribute to the body becoming prone to diseases and to prevent apoptosis. The mechanism of antioxidants function by scavenging for free radicals in cells and terminating chain reactions (Poprac et al 2017). The body produced antioxidants, but more are needed from external sources such as food and supplements. Studies has shown many food crops and plants contain antioxidants in variable amounts, some of the dietary foods includes banana, lemon, citric fruits, oranges, strawberries, gooseberries, apples, spinach, cabbage and lettuce etc. as source of ascorbic acid (Vitamin C), green tea, nuts, vegetable oils, as source of tocopherols (Vitamin E) (Hoyos et al 2017).

Korcan et al (2013) investigated the antioxidant effects of Saponins extracted from *Chenopodium albums*. Ethanol is used as the main solvent for extraction of chemicals from plant's leaves. Rel Assay Diagnostic kit is utilized to evaluate the total antioxidant status (TAS) levels and Total oxidant status (TOS). The result has shown a good correlation between chemicals derived from Chenopodium and total antioxidants status levels. Escribano et al 2017 characterized betalains and saponins and their antioxidant power in differently colored quinoa (*Chenopodium quinoa*) varieties. The chemicals were analyzed using different techniques (HPLC, DAD, MS/MS, Q-TOF. Different assays such as ORAC (oxygen radical absorbance capacity), ABTS ([2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and ferric reducing antioxidant power (FRAP) are used to determine the antioxidant activity of the extracted chemicals. The result has shown these chemicals poses high antioxidant activities.

3.4 Aim and hypothesis

The cost of cancer treatment is very high and unaffordable by patients in underdeveloped countries. The use of chemotherapy approaches for treatment of viable cancer cells has contributed to a lot of side effects. Cutting out the entire breast, using surgery, has its own disadvantages. The use of natural bioactive components derived from plants has shown a great promise for treatments of cancer, with low side effects and reasonable affordability. Chenopodium quinoa seeds poses diverse bioactive components, ranging from saponins, phenolic compounds, flavonoids, triterpenoids, tannins etc. Saponins derived from other plants have shown potential when used as anticancer treatments.

This research will add to the state of art in this field, and will demonstrate clear results regarding the effects of using saponins on breast cancer cell lines

4.0 MATERIALS AND METHODS

4.1 Chemicals

- Penicillin-Streptomycin (+10000µg/mL streptomycin, (+10000 units'/mL penicillin) thermo fisher Germany
- Insulin, Human (at 4mg/ml) thermo fisher, Germany

- Fetal Bovine Serum and DMEM /F-12(1:1) (1x) (Dulbecco's modified Eagle medium F-12, +L-glutamate, +15mM HEPES, Nutrient Mixture (ham), 500mL.sigma-aldrich, Germany
- Trypsin/EDTA Solution, 0.25% (Stored at (-200C) were purchased from Gibco, USA.
- DMSO-Dimethyl Sulfoxide, 100ml, was obtained from Che Cruz, USA.
- PBS (phosphate buffered saline) thermo fisher, Germany
- Formaldehyde 400µl.

4.2 Kits

Cell viability/cytotoxicity: TEBU-BIO cell counting kit 8 (CCK8) (Stored at 0-50C) was purchased from Tebu, France.Non-radioactive CCK-8 kit is used for sensitive colorimetric assays to calculate number of viable cells in the cytotoxicity assays and cell proliferation. This one-bottle solution does not require premixing of components. It uses tetrazolium salt WST-8 [2-(2-methoxy4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]. WST-8 is reduced by dehydrogenases to give water-soluble formazan dye (orange colored) in cells. The amount of formazan is directly proportional to the number of viable cells.

Apoptosis detection kit: (ApopTag® Plus Peroxidase in Situ, USA), characterize the apoptotic cells by modifying DNA fragments using terminal deoxynucleotidyl transferase (TdT) and detection of apoptotic cells by TUNNEL assay in the samples.

4.3 Instrumentation

- Centrifuge (Sigma 3-18K), Germany
- Cell culture T-75 (Thermo fisher), Germany
- Cryotubes (Thermo fisher), Germany
- Safety Cabinet ESCO Class II Type A2 Biological, Germany
- External computer
- Flasks
- Freezer (Panasonic, Tweenguard), UK
- Falcon tubes (14 mL* 17x100 mm), USA

- Hemocytometer (counting chamber) (Sigma-Aldrich), USA
- Humidified incubator (Binder) (at 37 °C, 5% CO2), Germany
- Inverted microscope (Olympus IX53), Germany
- Pipette controller, pipettes, micropipettes (Eppendorf research), Germany
- SOFTmaxÆ PRO software (absorbance at 450 nm)
- microplate reader (Versa max tunable), USA
- Water bath (Huber, Combatible control, CC1), Germany
- 24-well plates (Sigma-Aldrich), USA
- 96-well plates (Sigma-Aldrich), USA

4.4 Plant Extract

The saponins (CQ1, CQ3, CQ5 and MIX) was extracted from the *Chenopodium Quinoa* by Prof. Dr. İhsan Çalış (Faculty of Pharmacy, Near East University).

4.5 Cell Lines

Two different breast cancer cell lines were used for this project. These cell lines vary according to stage at which they are extracted, grade, type and immunoprofiles such as ER+, ER-, PR+/-, HER2+, HER2- (Holliday & Speirs 2011).

Breast cancer luminal a molecular subtype MCF-7 and MDA-MB-231 cell lines were obtained from the American Type Culture Collection (ATCC), Rockvilles, Maryland, USA.

4.6 METHODOLOGY

4.6.1 Freezing and Thawing Cell Lines

Cell lines were stored in a freezer at - 80 ℃ in Cryotubes contained 5% DMSO (dimethyl sulfoxide) as a cryoprotectant. The frozen cells were thawed in an incubator at 37℃ then transferred into a centrifuge tube containing 15ml culture medium. The cells were pelleted by centrifugation at 1000rpm for 8 minutes at room temperature. The supernatant was discarded and the cells were re-suspended in 1ml of fresh medium containing DMEM with F-12 Nutrient Mixture (Ham), L-Glutamate and 15mM HEPES that is supplemented with 10% Fetal Bovine Serum and 1% penicillin-streptomycin mixture.

4.6.2 Cell Culture

MCF-7 and MDA-MB-231 cell lines were cultured in T75 flasks with the culture medium at 37 ˚C and in a 5% CO2 containing humidified chamber. The medium was refreshed every other day.

4.6.3 Sub Culturing

T-75 flasks were taken out from the incubator and examined under inverted microscope at low power. Cells were sub cultured if there were no floating cells and they were 80-100% confluent. Culture medium was removed from the flasks. Then cell layer was rinsed briefly with 2ml, 0.25% (w/v) trypsin to remove traces of serum having trypsin inhibitor. Seven ml trypsin was added to flask and placed in incubator and left for 10 minutes at 37°C. Then cells were examined under inverted microscope to be sure that all the cells are detached and floating. Up to eight mL of growth medium was added into flask and cells were aspirated by pipetting gently. After transfer of cell suspension into the centrifuge tube containing medium and cells, this tube was centrifuged for 5 minutes (RCF:192, temperature: 24°C, speed: 1000). Then supernatant was discarded. One ml of medium was added into the centrifuge tube containing only cells. One hundred microliter of suspension is placed on hemocytometer (counting chamber) and number of cells were counted 3 x 10^6 cells were seeded into a new sterile flask containing 15ml medium. Then flask was placed in incubator and incubated at 37°C, 5% CO_2 containing humidified chamber.

4.6.4 Preparation of stock solution and various concentrations of *chenopodium quinoa*

Five mg of four types of saponins was dissolved in 1000µl of distilled water. Then stock solution containing 100µl saponins and 900µl of medium was prepared. This stock solution was used to prepare various concentrations of saponins as 200µM, 100µM, 50µM, 25µM and 10µM.

4.6.5 Measuring cell viability/Cytotoxicity

Cell viability/cytotoxicity activity of saponins was evaluated by using TEBUBIO cell counting kit 8 on breast cancer cell lines MCF-7 and MDA-MB231. following manufacturer's protocol. One hundred µl of cell suspension was applied (3000 cells/ well) in each of 3 x 96-well plate. Plates were pre-incubated for 24 hours' in a humidified incubator (at 37°C, 5% CO_2). Plates were examined under inverted microscope to be sure that they were attached. Then medium in each well was removed. Thereafter were utilized five different concentrations for each type of chenopodium saponins (10 µM ,25 µM, 50 µM, 100 µM, 200 µM, respectively), then five different amounts (2µl, 5µl, 10µl, 20µl, 40µl) were added to be tested to the plates. Each of the three plates was labeled as duration of exposure, name of cells and date. The effect of different concentration of saponins were investigated in three time periods (24, 48, 72 hours, respectively). Ten µl of CCK-8 was added into each well of the plates. The plates were incubated for four hours in the incubator. The absorbance was measured at 450nm by using Versa max tunable microplate reader connected to a computer.

4.6.6 Statistical Analysis

GraphPad® Prism software version 8 was used to calculate the statistical significance of different concentrations of saponins at different time intervals and IC50 values were calculated by the application of non-linear regression curve fit analysis.

PASW STATISTICS 18® was used to calculate the statistical significance of different concentrations of saponins at different time interval were calculated by KRUSKAL-WALLIS TEST.

4.6.7 Tunnel Assay Apoptosis

The concentration of CQ1, CQ3, CQ5 and MIX of saponins with the least cell proliferation was chosen for the apoptosis analysis. The CQ5 and MIX of saponins were used at a concentration of 25µM, for both breast cancer cell lines, and were incubated for 24 hours. As for the CQ1 and CQ3 they were incubated for 48 hours, but with different concentrations (200µM, 25µM respectively, for both breast cancer cell lines). Thereafter,

400µl of cell suspension was applied (7500/well) in each of 2 x 24-well plates. Then were added 160µl stock volume from CQ1 and 20µl from other saponins to be tested to the plates, each of the two plates was labeled with the duration of exposure, name of cells and date. The plates were incubated for (24 hrs and 48 hrs, respectively) in a humidified incubator (at 37°C, 5% CO_2).

Fixation and staining processes:

The medium was removed from the plates, then the plates were washed by PBS (phosphate buffered saline). The formaldehyde of 400µl was added in each well, and kept for 30 minutes, then the Plates were washed again using PBS. Next 15µl PBS +15µl TRITON-X 100 were added to each well and washed with PBS three times. After that we added hydrogen peroxide for 5 minutes, and washed again three times using PBS. Equilibration buffer was then added for 5 minutes, then appended by 55µl TDT (terminal deoxynucleotidyl transferase enzyme) without washing by PBS, and kept for 1 hour.

In the next step, a stop/wash buffer was applied for 10 minutes, subsequently the plates were washed three times by PBS, and then Anti-Digoxigenin conjugate 65µl/5cm2 was added, before they were incubated in humidified chamber for 30 minutes at room temperature. Then, the plates were washed again three times by PBS, followed by applying 50µl of DAB (3,3' diaminobenzidine) substrate on each well for 5 minutes. Then they were washed with distillated water three times, before adding hematoxylin stain five minutes, and then washed again. The final step was applying a mounting medium over the slide to place a coverslip.

5.0 RESULTS

5.1 CYTOTOXICITY RESULTS FOR MCF-7 CELL LINE

MCF-7 breast cancer cells were exposed to a different concentration of saponins 10, 25, 50, 100, and 200µM, and the absorbance values were measured at 450nm using Versa max tunable microplate reader. Then all absorbance values were normalized (max.100-min.0). The normalized values were analyzed using IC50 (%95 CI) values and R^2 values were calculated. IC50 values for MCF-7 cell line are shown in the table **3** below:

Exposure time to saponins	24 hours	48 hours	72 hours
R^2	0.3655	0.9193	0.5499
IC50	0.006223 to 14.05	0.006223 to 14.05	0.006223 to 14.05

Table 3: IC50 values for MCF-7 cell line, R^2 values

Further statistical investigations were performed using Two-way Anova Multiple Comparisons Test to determine the significance difference between control and other concentrations of saponins on MCF-7 cell line for 24h, 48h and 72h respectively. There were significant changes between control group and all of the 25µM, 50µM and 200µM groups at 24 hours. After 48 hours of exposure, there was a significant change between control group and 25µM sample group only. However, there was a significant change between control group and 10µM, 25µM, 50µM and 100µM groups at 72 hours.

Tukey's multiple comparison test was performed for MCF-7 cell line to determine the significance of mean difference within each group of concentration of saponins and means obtained after 24h, 48h and 72h were compared. There were significant differences in the following cases:
Between means when 24h-48h, 24h-72h, 48h-72h values were compared in control group, between means when 24h-48h and 24h-72h values were compared in 200µM group, between means when 48h-72h values were compared in 100µM group and between means when 24h-72h values were compared in 25µM group.

Two-way Anova multiple comparisons test results for MCF-7 cell line for 24h, 48h and 72 hours' exposure of saponins are shown in the following figures:

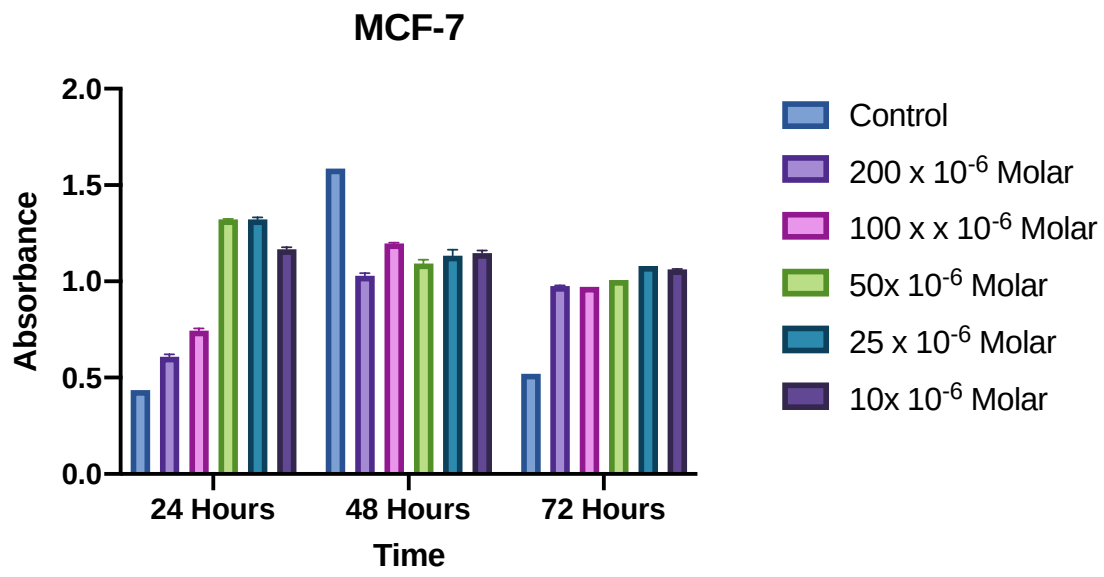


Figure 7.1 Two-way Anova results for CQ1 saponin on MCF-7

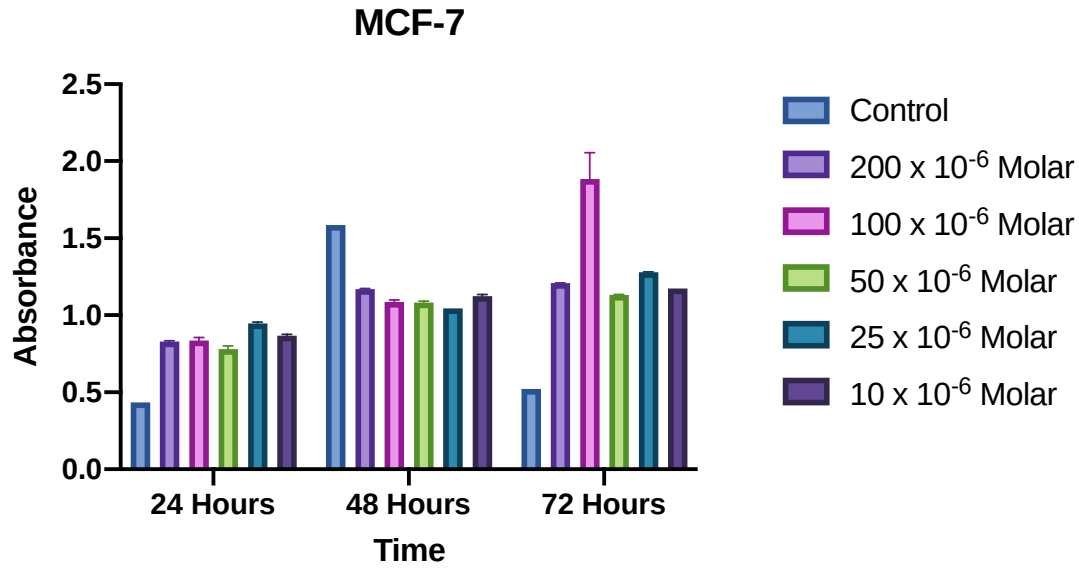


Figure 7.2 Two-way anova results for CQ3 saponin on MCF-7

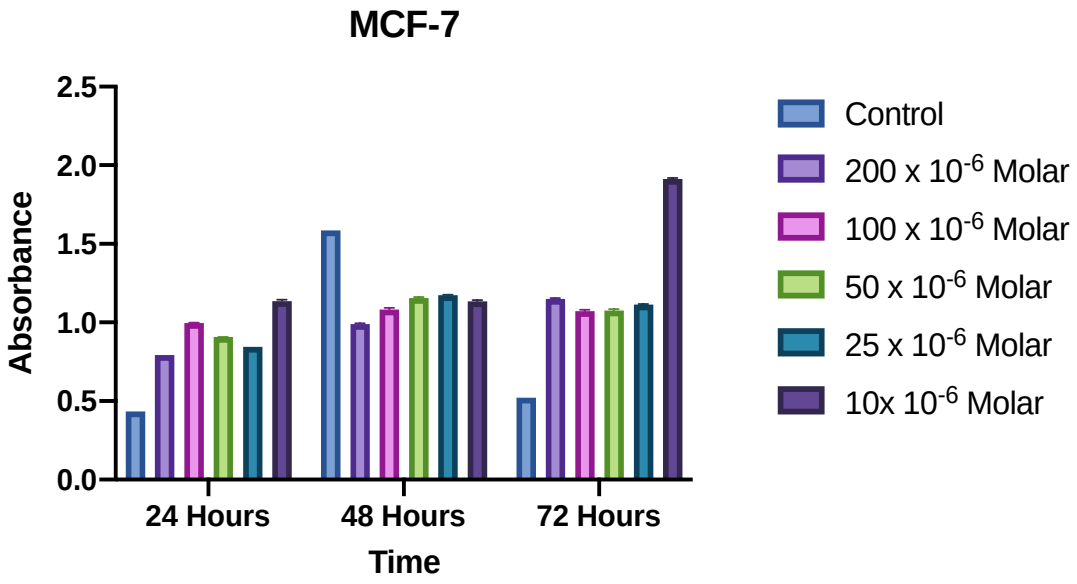


Figure 7.3 Two-way Anova results for CQ5 saponin on MCF-7

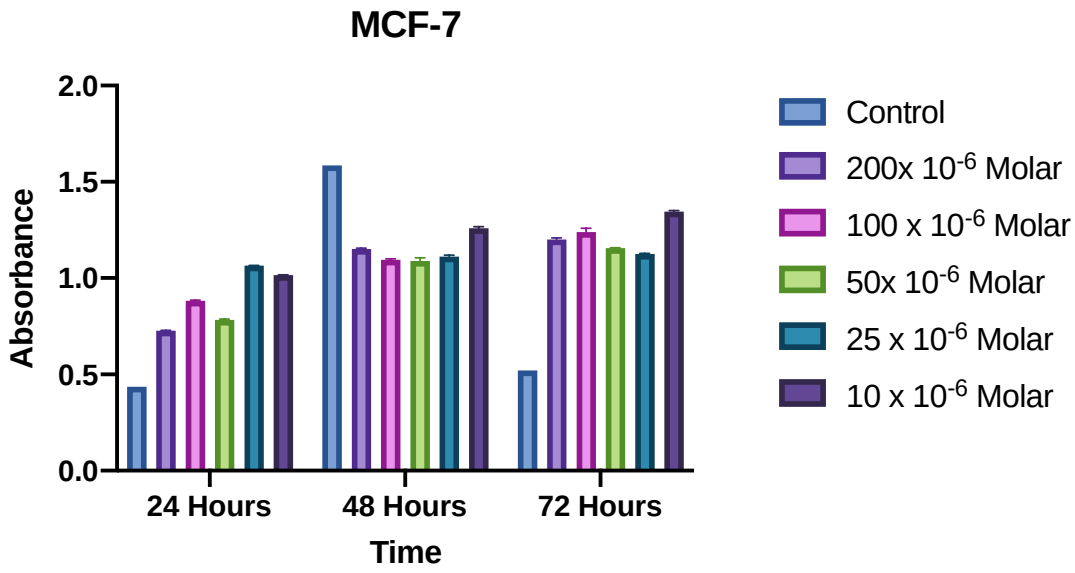
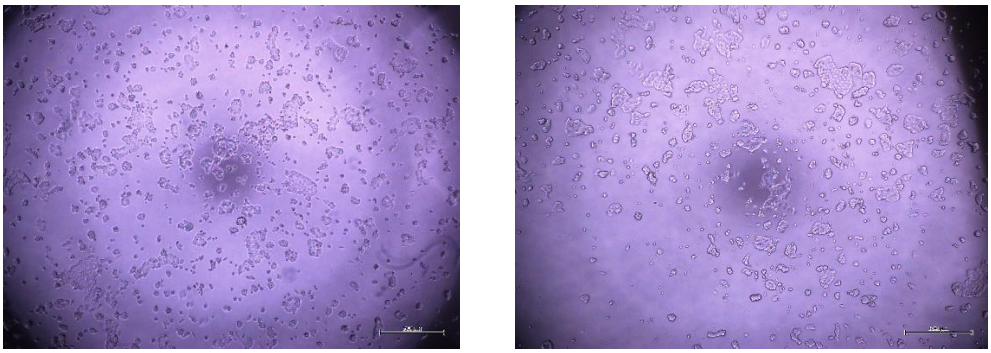


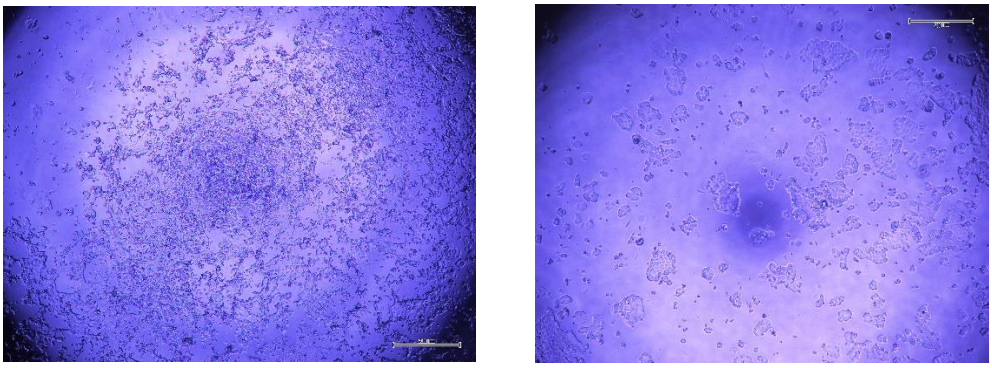
Figure 7.4 Two-way Anova results for MIX of saponins on MCF-7

MCF-7 cell lines morphology under microscope after exposure of saponins at 24hrs, 48hrs and 72hrs respectively are shown in the following figures:



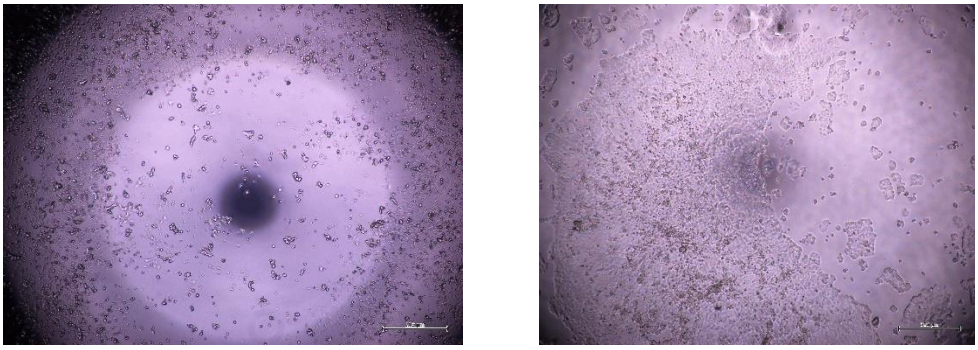
Control group 200 μM

Figure 8.1 MCF-7 cells after 24 hours of CQ5 saponin exposure



Control group 10 μM

Figure 8.2 MCF-7 cells after 48 hours of MIX saponins exposure



Control group 50 μM

Figure 8.3 MCF-7 cells after 72 hours of CQ3 saponin exposure

5.2 CYTOTOXICITY RESULTS FOR MDA-MB 231 CELL LINE

MDA-MB231 breast cancer cells were exposed to a different concentration of saponins 10, 25, 50, 100, and 200µM, and the absorbance values were measured at 450nm using Versa max tunable microplate reader. Then all absorbance values were normalized (max.100-min.0). Normalized values were analyzed using IC50 (%95 CI) values were calculated and R^2. IC50 values for MDA-MB231 cell line are shown in table **4:**

Exposure time to saponins	24 hours	48 hours	72 hours
R^2	0.1997	-0.002739	0.3835
IC50	2.511 to 70.37	2.511 to 70.37	2.511 to 70.37

Table 4: IC50 values for MDA-MB231 cell line, R^2 values

Similar to MCF-7 cell investigations, two-way anova Multiple Comparisons Test was also performed to determine the significance of mean difference between control and other concentrations of saponins on MDA-MB 231 cell line for 24h, 48h and 72h respectively. For 24 hours of exposure there were significant changes between control group and both 25µM and 50µM groups. After 48 hours there were significant changes between control group and 10µM and 200µM groups. While after 72 hours of exposure there were significant changes between control group and 10µM and 200µM groups.

Tukey's multiple comparison test was performed for MDA-MB231 cell line to determine the significance of mean difference within each group of concentration of saponins and means obtained after 24h, 48h and 72h were compared. The significant differences were as follows:

Between means when 24h-48h, 24h-72h, 48h-72h values were compared in control group, between means when 24h-72h values were compared in 200µM group, between means when 24h-48h and 24h-72h values were compared in 100µM group, between means when 24h-48h and 24h-72h values were compared in 50µM group, between means when 24h-72h values were compared in 25µM group and between means when 24h-72h values were compared in 10µM group.

Two way Anova multiple comparisons test results for MDA-MB 231 cell line for 24h, 48h and 72 hours' exposure of saponins are shown in the figures **9** below:

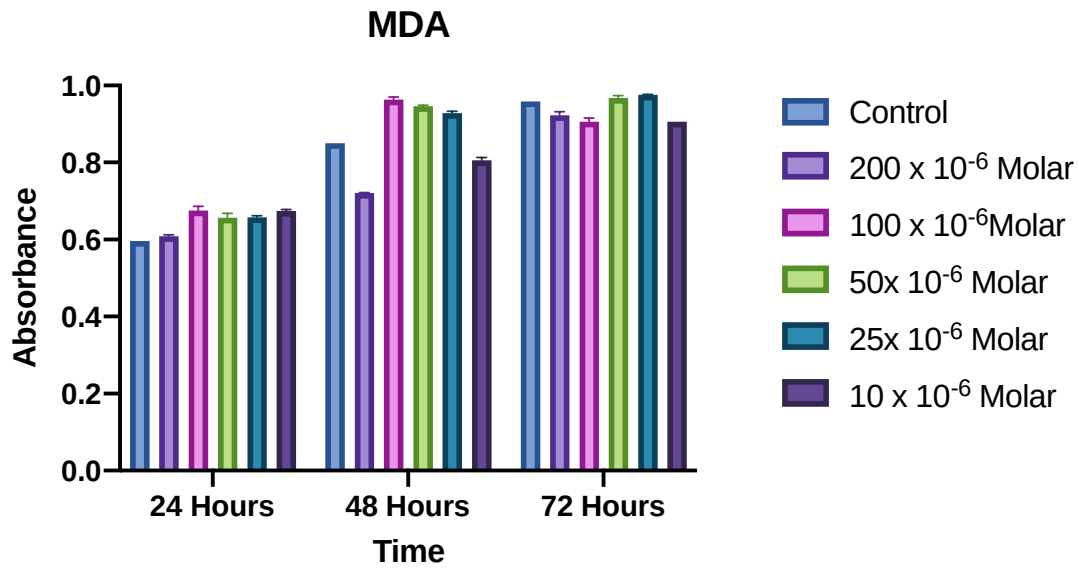


Figure 9.1 Two-way Anova results for CQ1 on MDA-MB231 cells

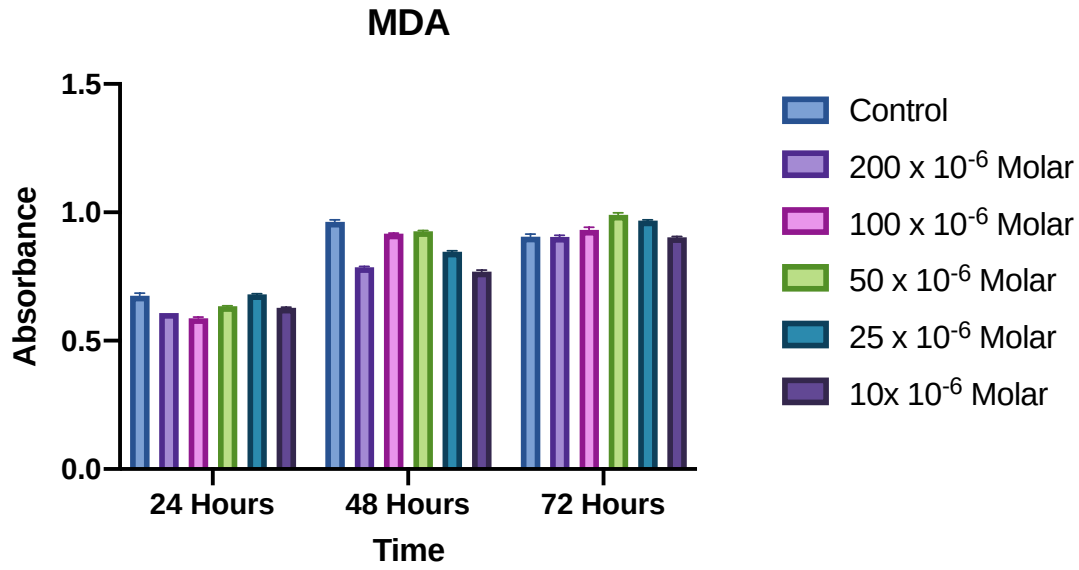


Figure 9.2 Two-way Anova results for CQ3 on MDA-MB231 cells

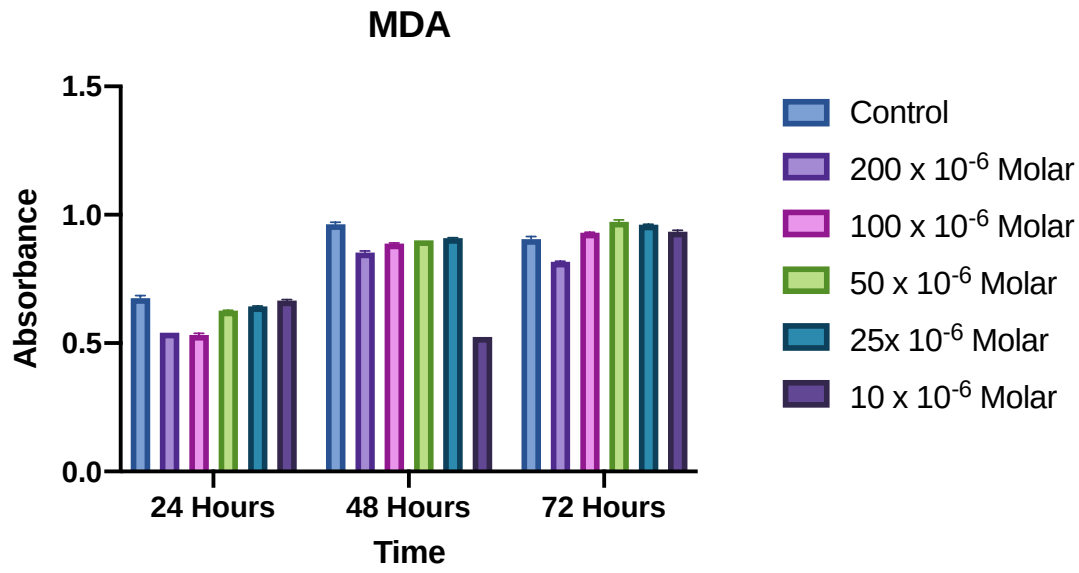


Figure 9.3 Two-way Anova results for CQ5 on MDA-MB231 cells

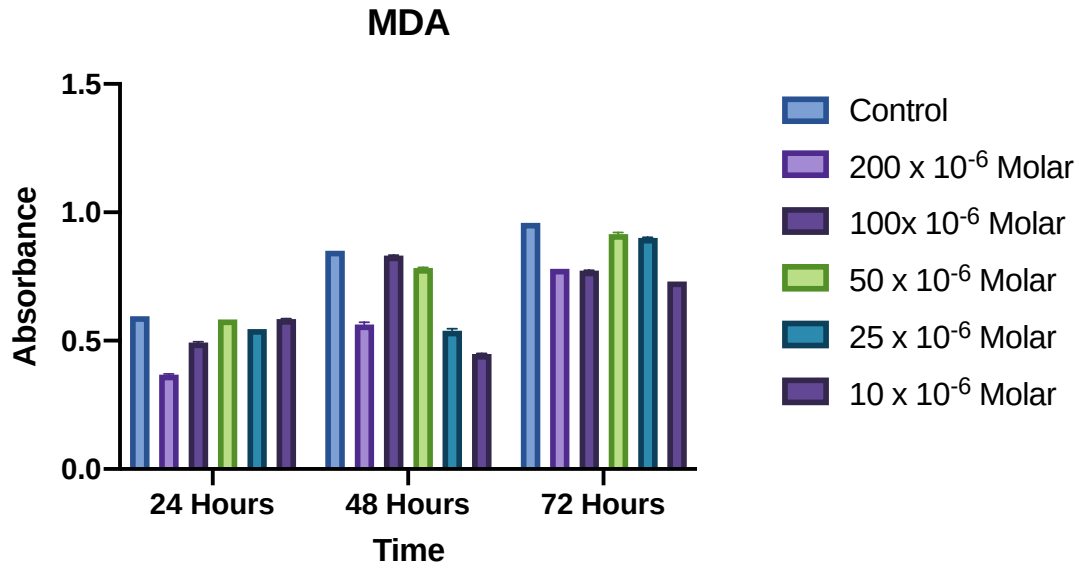
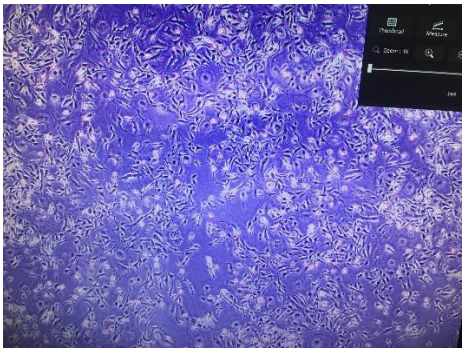
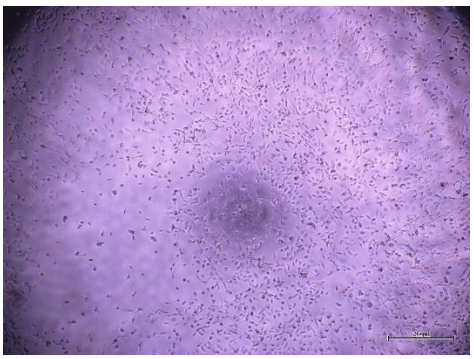


Figure 9.4 Two-way Anova results for mix of saponins on MDA-MB231 cells

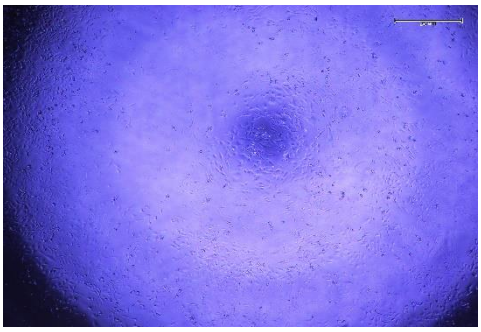
MDA-MB231 cell lines morphology under microscope after exposure of saponins at 24hrs,48 hrs and 72 hrs respectively are shown in the following the figures below:

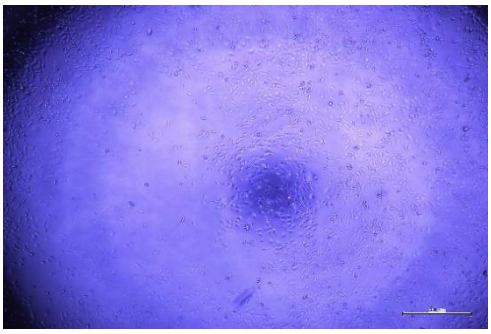




Control group 200 μM

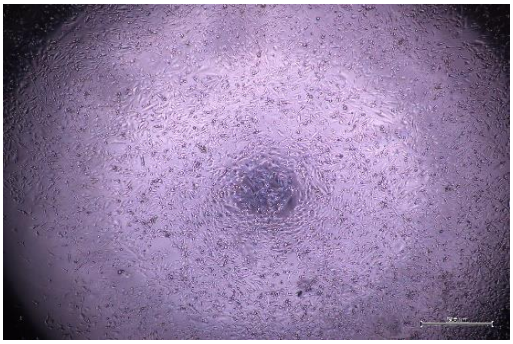
Figure 10.1 MDA-MB231 cells after 24 hours of CQ3 exposure

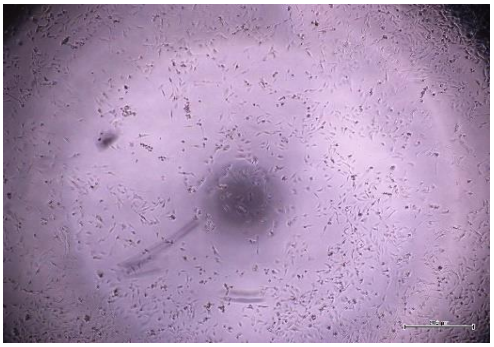




Control group 10 **μ**M

Figure 10.2 MDA-MB231 cells after 48 hours of CQ5 exposure





Control group 50 **μ**M

Figure 10.3 MDA-MB231 cells after 72 hours of mix saponins exposure

5.3 DETECTION OF APOPTOSIS VIA TUNNEL ASSAY IN MCF-7 CELLS AND IN MDA-MB231 CELLS

After 24, 48 hours of treatment with 25μM and 200μM of saponins on MCF-7 and MDA-MB231 breast cancer cell lines. The slides were examined under the light microscope, were the stained cells with Tunnel counted, and the results were evaluated using KRUSKAL-WALLIS TEST by SPSS program. The results showed that there was no significant difference compared with control group, the p-values readings were as follows: For MCF-7 cells the p-values at 24 hours were **(0.362, 0.362 respectively),** while the p-values at 48 hours were **(0.344, 0.202 respectively).** For MDA-MB231 the p-values at 24 hours were **(0.429, 0.617 respectively),** while the p-values at 48 hours were **(1.000, 0.147 respectively).**

The following Figures show Tunnel Assay Apoptosis of MCF-7 and MDA-MB231 cells under light microscope power of **(×40)**:

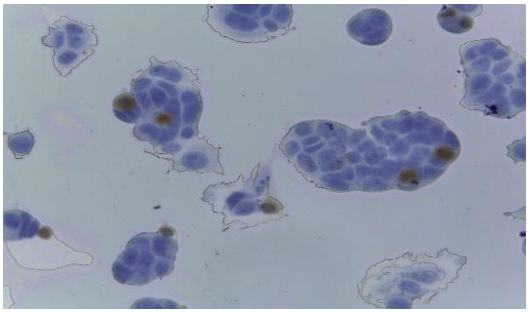


Figure A : control

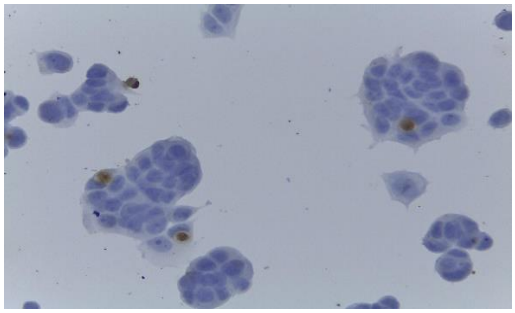


figure B : CQ5

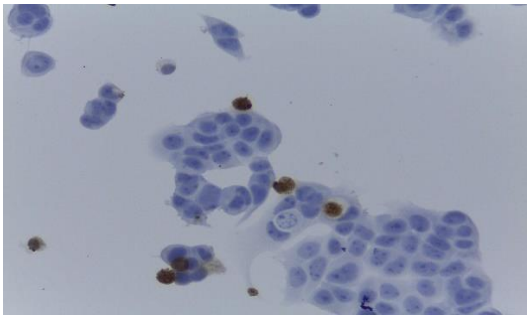


*Figure **C:** MIX OF SAPONINS*

Figure 11.1 MCF-7 cell lines after 24 hours of exposure CQ5 and MIX saponins

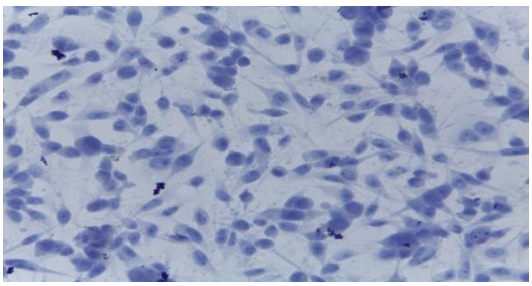


Figure A: control

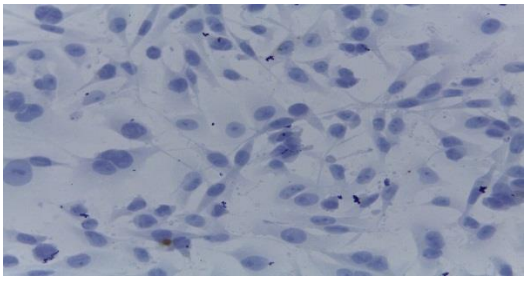


figure B: CQ5

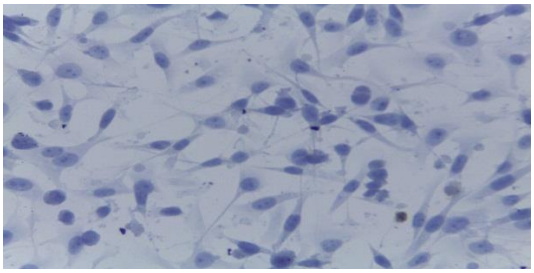


Figure C: MIX OF SAPONINS

Figure 11.2 MDA-MB231 cell lines after 24hours of exposure CQ5 and MIX saponin

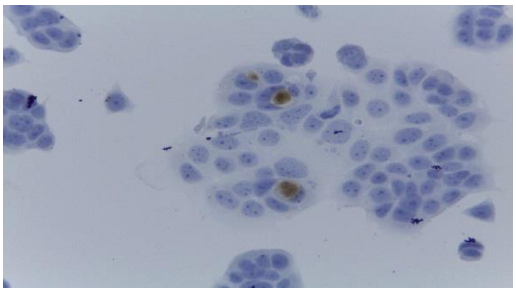


Figure A: control

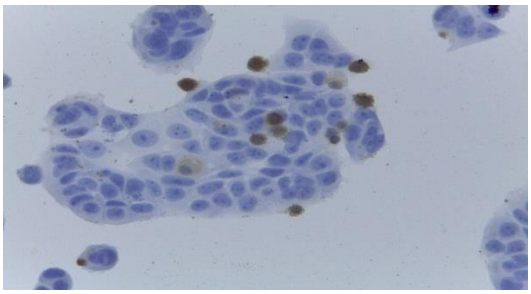


figure B: CQ1

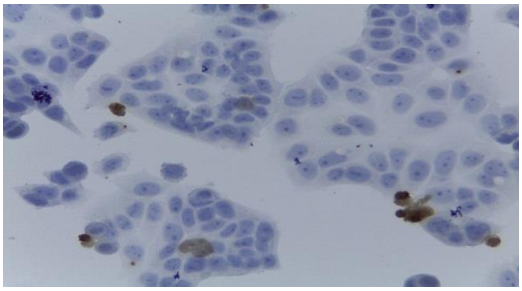


Figure C: CQ3

Figure 11.3 MCF-7 cell lines after 48 hours of exposure CQ1 and CQ3

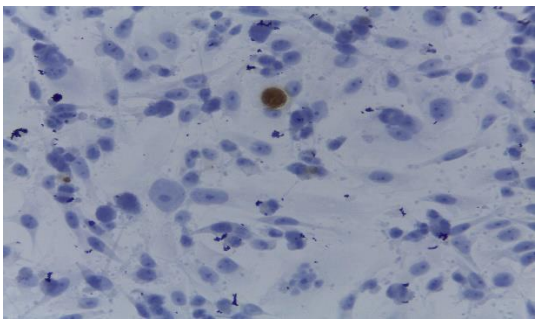


Figure A: control

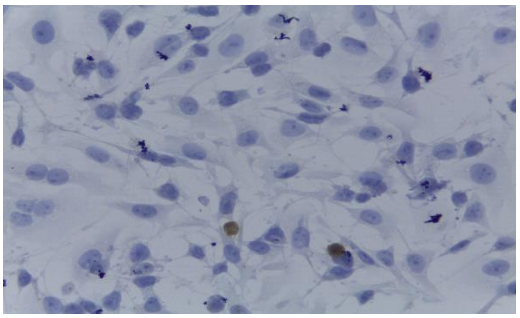


figure B: CQ1

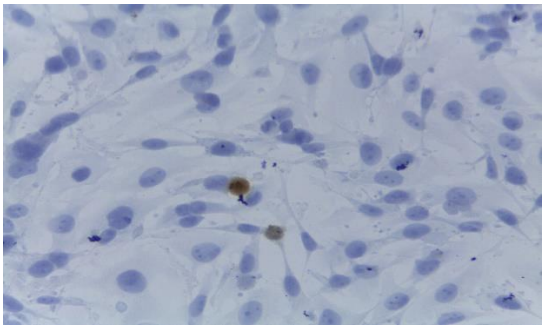


Figure C: CQ3

Figure 11.4 MDA-MB231 after 48 hours of exposure CQ1 and CQ3

6.0 DISCUSSION AND CONCLUSION

Breast cancer has been one of the major causes of death in women, in both developed and underdeveloped countries. In 2012, it was estimated that there are over 1.6 million diagnosed new cases of breast cancer in women globally (WHO 2012). There are different types of treatment approaches, ranging from chemotherapy in which formulated chemical compounds are used to kill cancer cells, or extraction of chemical constituents from potential medicinal plant to induce cellular apoptosis, immunotherapy in which immune cells are enhanced using drugs to induce cytotoxicity effects on cancer cells, the removal of tumor using surgery, to the use of radiation to destroy tumor tissues. However, all these treatment approaches have limitations such as: Cost, Side effects, and High Risk. Chemical components extracted from plants have been shown to have less side effects, low risk and cheap cost.

Saponins are a class of triterpene glucosides, they consist of a linear arrangement between 1 to 6 pentose-glycoside, or hexose-glycoside units, linked to sapogenin aglycone which exists in two forms (triterpenoid aglycone or steroidal). Several studies have reported the presence of saponins in more than 100 different families of plants, and 150 plants were found to poses anticancer properties (Man et al 2010).

Hederagenin, Hydroxy-Hederagenin and Phytolaccagenic Acid, are saponins extracted from chenopodium quinoa, Hederagenin also called Hederagenic acid. Hederagenol is a chemical compound which belongs to the Triterpenoid family, and is one of the chemical components of many plants' biome. The most common plants that poses high amounts of Hederagenin is common ivy *(Hedera helix*) and *Chenopodium quinoa*. Hederagenin saponins possess diverse biological activities such as anticancer, or cytotoxic properties, as a result of their interaction with cell membrane, antifungal, antiviral, hemolytic and molluscidal activities. Phytolaccagenic acid belongs to the Triterpenoids class (organic compounds known as "terpene").

Hu et al (2017) evaluated the chemical characterization, antioxidant anticancer activity, and immune regulating activities, of bioactive polysaccharide components isolated from

Chenopodium quinoa seeds. Evaluation of anticancer activity was carried out using C. Quinoa Polysaccharide (CQP) on MCF-7 breast cancer cell lines and human liver cancer SMMC 7721. The result has shown that CQP possesses cytotoxic activity against breast cancer cells and liver cancer cells, with no effects on normal cells. Moreover, the result indicated that the bioactive components present in Chenopodium quinoa have a potential for use as anticancer, antioxidant and immune regulating agents.

A study conducted by Chwalek et al (2006) focused on the cytotoxicity of Hederagenin (i.e. α-hederin) on keratin forming tumor cell lines (KB cells). The research explored the sugar moieties of the compound Hederagenin diglycosides, the result has shown some saponins induced apoptosis to cells and anticancer activities due to the sugar moiety present in the compounds.

This study evaluated the cytotoxic effect of saponins extracted from Chenopodium Quinoa Plant on MCF-7 cell line in vitro. IC50 values for MCF-7 cell line after 24 hours, 48 hours, 72 hours' respectively, of exposure to different concentrations of saponins were found as (**0.006223 to 14.05**) for all time periods. R^2 values for 24 hours ,48 hours,72 hours respectively of exposure to saponins were calculated as (**0.3655,0.9193,0.5499)** respectively. Two way Anova multiple comparisons test (Dunnett's multiple comparisons) was performed for MCF-7 cell line to determine the significance of mean difference between control groups and other concentrations of saponins for 24 hours,48 hours,72 hours respectively, there was a significant change in the following cases:
Between control group and 25μM, 50μM and 200μM groups at 24 hours of exposure. After 48 hours of exposure there was a significant change between control group and 25μM group only. And between control group and 10μM, 25μM, 50μM and 100μM groups at 72 hours of exposure.

Tukey's multiple comparison test was performed for MCF-7 cell line to determine the significance of mean difference within each group of concentration of saponins and means obtained after 24h, 48h and 72h were compared to produce the following results:
There were significant differences between means when 24h-48h, 24h-72h, 48h-72h values were compared in control group, there were significant differences between means

when 24h-48h and 24h-72h values were compared in 200μM group, there were significant differences between means when 48h-72h values were compared in 100μM group, while there were significant differences between means when 24h-72h values were compared in 25μM group.

This study also evaluated the cytotoxic effect of saponins extracted from Chenopodium Quinoa Plant on MDA-MB231 cell line in vitro. IC50 values for MDA-MB231 cell line after exposures of 24 hours ,48 hours, and 72 hours' respectively to different concentrations of saponins were found as (**2.511 to 70.37**) for all time periods. R^2 values for 24 hours, 48 hours, and 72 hours' exposures to saponins were calculated as **0.1997, -0.002739, 0.3835,** and respectively.

Two way Anova multiple comparisons test (Dunnett's multiple comparisons) was performed for MDA-MB231 cell line to determine the significance of mean difference between control groups and other concentrations of saponins for 24 hours,48 hours, and 72 hours respectively, revealing the following:
There was a significant change between control group and 25μM and 50μM groups at 24 hours of exposure, after 48 hours of exposure there was a significant change between control group and 10μM and 200μM groups and there was a significant change between control group and 10μM and 200μM groups at 72 hours of exposure.

Tukey's multiple comparison test was performed for MDA-MB231 cell line to determine the significance of mean difference within each group of concentration of saponins and means obtained after 24h, 48h and 72h were compared. There were significant differences in the following cases:
Between means when 24h-48h, 24h-72h, 48h-72h values were compared in control group, between means when 24h-72h values were compared in 200μM group, between means when 24h-48h and 24h-72h values were compared in 100μM group, between means when 24h-48h and 24h-72h values were compared in 50μM group, between means when 24h-72h values were compared in 25μM group and between means when 24h-72h values were compared in 10μM group.

In conclusion, this study was examined the cytotoxic effect of saponins extracted from the Chenopodium Quinoa seeds on MCF-7 and MDA-MB 231 breast cancer cell lines. And according to the above investigations, the best concentration that significantly affected the MCF-7 cells proliferation was 25µM at 48 hours, while the best concentration found to significantly affect the MDA-MB231 cells proliferation was 50µM at 24 hours.

Additionally, experiments which showed saponins activity in cellular models (in vitro) and with animals (in vivo) began in 90s of the last century. Since then, the numbers of newly isolated triterpene saponins has been increasing constantly, many of these compounds exert impressive anticancer effects and may help to develop novel anticancer therapy (Koczurkiewicz et al 2015). Alam et al (2017) extracted saponins from Zanthoxylum armatum. L and examined the effect of eight concentrations (10, 25, 50, 100, 200, 300, 400 and 500 µg) of saponins on (MCF-7 and MDA-MB468) and colorectal (Caco-2) cancer cell lines, the saponins showed growth inhibition against MCF-7 and MDA MB-468 by 79.89 (±7.45) % and 95 (±2.64) % respectively, and the growth inhibition against Caco-2 by 75.88 (±8.41) %, 61.82 (± 4.07) and 68.62 (± 2.48) respectively.

Lin cheng et al (2018) isolated a hederagenin saponins from Clematis ganpiniana to induce the apoptosis in MCF-7 and MDA-MB-231 breast cancer cell lines via the mitochondrial pathway, the cells were treated with four concentrations (0.08, 0.4, 2, or 10 µg/ml) of hederagenin saponin and the results showed that a strong cytotoxic effect on MCF-7 and MDA-MB231 breast cancer cell lines by 2.0 µg/ml CHS for 24h and showed an early apoptosis rate of 29.3 and 19.8% respectively. Li-Hua Mu et al (2019) extracted Triterpenoid Saponins from Ardisia gigantifolia and examined the inhibiting proliferation on MCF-7, T47D, MDA-MB-231 and SK-BR-3 breast cancer cell lines by using seventeen 13,28-epoxy triterpenoid saponins, results showed that the best inhibitory activity to MCF-7 cells (IC50 0.73 µM), to T47D cells (IC50 0.88 µM), for MDA-MB231 cells (IC50 0.76 µM) and SK-BR-3 cells (IC50 0.95µM), and showed that Saponin 7 showed significant and selective activity on triple-negative breast cancer MDA-MB-231.

This research could add to the state of art in this field, and demonstrate clear results regarding the effects of using saponins on breast cancer cell lines. The differences between this study and other similar studies which investigated the cytotoxic effects of saponins on breast cancer cell lines were the use of a MIX of saponins and the examination of their effects on proliferation of breast cancer cell lines showing effective results.

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