THE EFFECT OF AFLATOXIN B1 AND OCHRATOXIN A ON TUMOR RELATED GENES IN IMMORTALIZED AND BREAST CANCER CELLS

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by

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LIST OF ABBREVIATIONS

AB1	Aflatoxin B1
AKT1	RAC-alpha serine/threonine-protein kinase
BAK1	*BCL2* antagonist/killer 1
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
BCL2	B-cell lymphoma 2
cMyc	Myelocytomatosis viral oncogene
CCND1	Cyclin D1
Casp8	Cysteine-aspartic acid protease 8
Casp9	Cysteine-aspartic acid protease 9
Casp3	Cysteine-aspartic acid protease 3
cDNA	Complementary Deoxyribonucleic acid
CO_2	Carbon dioxide
DAPK1	Death-associated protein kinase 1
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
dATP	2'-deoxyadenosine 5'- triphosphate
dCTP	2'-deoxycytodine 5'- triphosphate
dGTP	2'-deoxyguanosine - triphosphate
dTTP	2'-thymidine - triphosphate
dH_20	Deionized water

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EtBr	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
GADD45A	Growth Arrest and DNA Damage Inducible Alpha
HCL	Hydrochloric acid
hEGF	Human epidermal growth factor
HER1	Human epidermal growth factor receptor 1
HER2	human epidermal growth factor receptor 2
JAK1	Janus kinase 1
MAPK1	Mitogen-Activated Protein Kinase 1
MAPK3	Mitogen-Activated Protein Kinase 3
MCL1	Myeloid cell leukemia 1
MDM2	Mouse double mint 2
MIZ1	Zinc finger and BTB domain containing 17
MCF7	Michigan Cancer Foundation-7
mRNA	Messenger RNA
MCF10A	Michigan Cancer Foundation-10A
MgCl2	Magnesium Chloride
OTA	Ochratoxin A
Oligo	Oligonucleotide

p16	Cyclin-dependent kinase Inhibitor 2A
p21	Cyclin-dependent kinase inhibitor 1
p53	Tumor protein 53
PCNA	Proliferating cell nuclear antigen
RB1	Retinoblastoma 1
rRNA	ribosomal ribonucleic acid
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
RT	reverse transcription
RT-qPCR	Real time quantitative polymerase chain reaction
siRNA	short-interfering RNA
TGFA	Transforming growth factor alphaprovided
Tris	Tris (hydroxymethyl)aminomethane
tRNA	transfer RNA
WNT3A	Wingless-related integration site

LIST OF SYMBOLS

™	Trademark
®	Registered trademark
ºC	Degree Celsius
%	Percentage
nM	Nano Molar
M	Molar
µg	Microgram
mg	Milligram
g	Gram
kg	Kilogram
mmol/L	Millimole per liter
µL	Microliter
mL	Milliliter
µm	Micrometer
mm	Millimeter
cm	Centimeter
cells/mL	Cells/milliliter

KESAN AFLATOKSIN B1 DAN OCHRATOKSIN A PADA SEL KANSER PAYUDARA

ABSTRAK

Pencemaran mikotoksin di dalam komoditi makanan yang disebabkan oleh kulat seperti *Aspergillus* dan *Penicillium* adalah perkara biasa di negara-negara yang mempunyai cuaca tropika seperti Malaysia kerana keadaan ini dapat mengekalkan pertumbuhan dan perkembangan fungus tersebut. Mikotoksin dilaporkan boleh menyebabkan keracunan makanan yang teruk, kerosakan hati dan terbukti karsinogenik pada sel-sel buah pinggang dan hati. Sifat karsinogen Aflatoxin B1 dan Ochratoxin dapat meningkatkan risiko kanser payudara dan kajian molekular perlu dilakukan untuk mengesahkannya. Objektif kajian ini adalah untuk mengkaji kesan AB1 dan OTA terhadap aktiviti kesitotoksikan sel, ekspresi gen yang berkaitan dengan tumor dan peningkatan sel-sel kanser payudara di kepekatan yang rendah dan tinggi. Stok larutan 100 μg/mLAflatoxin B1 dan Ochratoxin A telah disediakan menggunakan DMSO sebagai pelarut dan AB1 dengan kepekatan 1.2 μg/mL dan OTA dengan kepekatan 3.4 μg/mLdigunakan untuk merawat MCF7. Di samping itu, AB1 dengan kepekatan 2.3 μg/mLdan OTA dengan kepekatan 5.7 μg/mLdigunakan untuk merawat sel MCF10A. Sel-sel dibiakkan dalam ketumpatan $0.3x10^6$ dan apabila sel mencapai 90% konfluensi, sel-sel telah dirawat dan diinkubasi selama 48 jam untuk melaksanakan prosedur- prosedur seperti sitotoksik XTT, morfologi dan penghijrahan sel, kitaran sel, apoptosis dan spesis oksigen reaktif. Di samping itu, anotasi gen dan kuantifikasi ekspresi gen menggunakan RT-qPCR dijalankan untuk menyiasat urutan gen berkaitan tumor di kedua-dua jenis sel. Di samping itu, p53 di dalam sel MCF10A dan cMyc di dalam sel MCF7 telah diubah menggunakan siRNA

dan RT-qPCR digunakan untuk menentukan kuantiti gen-gen tumor tersebut setelah diubah dan dirawat (sebelum/selepas) dengan AB1 dan OTA. Akhirnya, kepekatan AB1 dan OTA yang tinggi digunakan untuk merawat sel-sel MCF7 dan MCF0A dan implikasi terhadap daya tahan sel terhadap kepekatan toksin yang tinggi dan tahap ROS disiasat. Rawatan MCF7 (kepekatan AB1 1.2μg / mLdan OTA dengan kepekatan 3.4μg / ml) dan MCF10A (dengan kepekatan AB1 2.3μg / mLdan OTA dengan kepekatan 5.7μg / ml) menunjukkan perubahan ketara dalam ekspresi gen tumor yang berkaitan di dalam sel MCF7 dan MCF10A dan seterusnya menyebabkan kerosakan DNA, menahan kitaran sel, meningkatkan pergerakan dan meningkatkan saiz sel. AB1 dan OTA meningkatkan keagresifan MCF7 dan meningkatkan risiko dalam meningkatkan tumor dalam MCF10A.

THE IMPLICATION OF AFLATOXIN B1 AND OCHRATOXIN A EXPOSURE ON TUMOR RELATED GENES IN NORMAL AND CANCEROUS BREAST CELLS

ABSTRACT

Mycotoxin contamination of food commodities caused by fungal strains such as *Aspergillus* and *Penicillium* is common in countries with tropical weather such as Malaysia due to the conditions that sustains their growth and development. Mycotoxins have been reported to cause severe food poisoning, liver damage and were proven carcinogenic to kidney and liver cells. The carcinogenic nature of Aflatoxin B1 and Ochratoxin A could increase the risks of breast cancer and an investigation on a molecular level need to be carried out to confirms it. The objectives of this study are to investigate the effect of AB1 and OTA on cell activities cytotoxicity, tumor related genes expression and the proliferation of breast cancer cells at a low and a high concentration. Stock solutions of 100 ug/mL of Aflatoxin B1 and Ochratoxin A were prepared using DMSO as a solvent and AB1 in final concentration of 1.2 µg/mL and OTA in final concentration 3.4 µg/mL were used to treat MCF7. In addition to that, AB1 in final concentration of 2.3 µg/mL and OTA in final concentration 5.7 µg/mL were used to treat MCF10A cell. Cells were seeded in the density of 0.3 x 10^6 and once 90% confluent, cells were treated and incubated for 48 hours to perform XTT (2H-Tetrazolium, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-hydroxide) cytotoxicity assay, cell morphology and cell migration assay, cell cycle assay, apoptosis assay and reactive oxygen species assay. In addition to that, gene annotation and quantification of gene

expression using RT-qPCR was carried out to investigate the expression of tumor related genes in both cell lines post treatment. Next to that, p53 in MCF10A and cMyc in MCF7 were knocked down using siRNA and gene expression of tumor related genes after the knockdown and (pre/post) treatment with AB1 and OTA was quantified using RT-qPCR. Finally, high concentration of AB1 and OTA were used to treat MCF7 and MCF10A cells and the implication of these high concentrations on cell viability and ROS levels were investigated. Treating MCF7 (AB1 final concentration of 1.2 µg/mL and OTA in final concentration 3.4 µg/ml) and MCF10A (AB1 final concentration of 2.3 µg/mL and OTA in final concentration 5.7 µg/ml) showed a significant change in gene expression of tumor related genes in MCF7 and MCF10A and that in turn caused DNA damage, cell cycle arrest, increased motility and increase cell size. Result showed that AB1 and OTA increase the invasiveness of MCF7 and increase the risk in developing tumorigenicity in MCF10A.

CHAPTER 1 : INTRODUCTION

1.1 Overview

Breast cancer is the most common cause of death to women worldwide. Approximately 1 in 20 women in this country reported develop breast cancer (Yip, Taib and Mohamed, 2006). Genetics factors due to mutations of *BRCA1* and *BRCA2* are well known causes of breast cancer (Venkitaraman, 2001). The alteration *p53* gene in breast carcinomas and highly expressed of *HER1* (Human epidermal growth factor receptor 1), *HER2* (human epidermal growth factor receptor 2) and the *c-Myc* (Myelocytomatosis viral oncogene) oncogenes that leads to the synthesis of new protein also enhanced the activation of oncogenes. Mycotoxins have been reported to influence breast tumor suppressor genes *p53* (Tumor protein 53), *BRCA1* (Breast cancer 1) and *BRCA2* (Breast cancer 2), the human oncogenes *HER1, HER2* and *c-Myc*. Moreover, mycotoxins may inhibit the proliferation of cells, protein synthesis and initiating apoptosis. Aflatoxin B1 and Ochratoxin A are the most dangerous mycotoxins for their lethal affect to human and animal (El Golli-Bennour *et al.*, 2010). Aflatoxin B1 has been reported as the highest carcinogenicity among all mycotoxins capable of penetrating cell membrane and attaches to its DNA where it makes changes to the genome or causes irreversible mutations to become more stable (Smela *et al.*, 2001). Ochratoxin A involved in covalent DNA adduction and involved in oxidative DNA damage and considered as genotoxic carcinogen because of their ability to oxidase DNA lesions and the direct DNA adducts through Quinone formation (Liu *et al.*, 2012). Therefore, further study needs to be carried out to determine the effect of these mycotoxins on cancerous cells based on their ability to either kill or causes the breast cancer.

To achieve this, cytotoxicity, cell morphology and migration, cell cycle and reactive oxygen species (ROS) levels detection assays were conducted to investigate the toxic effect of these toxins on MCF7 and MCF10A. The interest of connecting mycotoxins contamination to the occurrence or the increase in the risk of breast cancer required the study of these toxins on normal and cancerous breast cells and since working with primary cells was hard to sustain, the use of MCF7 and MCF10A was the best option. Both MCF7 and MCF10A represent the best model to study normal and cancer breast cells *in vitro* (Simstein *et al.*, 2003; Kenny *et al.*, 2007) and they were used extensively in many researched and due to that these cells were selected. The use of these cells in this study was due to the face that one of the risk facto of In addition to that, the quantification of tumor suppressing genes (*BRCA1, BRCA2* and *p53*), oncogenes (*HER1, HER2* and *cMyc*), cell cycle genes *CCND1* (Cyclin D1), *WNT3A* (Wingless-related integration site), *MAPK1* (Mitogen-Activated Protein Kinase) and *MAPK3* (Mitogen-Activated Protein Kinase 3) and apoptosis genes *BCL2* (B-cell lymphoma 2), *MCL1* (Myeloid cell leukemia 1), *DAPK1* (Death-associated protein kinase 1), *Casp8* (Cysteine-aspartic acid protease 8) and *Casp9* (Cysteine-aspartic acid protease 9) gene expression in both immortalize and cancerous cells was conducted by using RT-qPCR. Finally, the cancerous and immortalized cells will be treated with high concentration of AB1 OTA to induce apoptosis and further investigate the morphological and the molecular changes of the cells. Next to that, this study conducted gene annotation to understand the regulation of gene expression upon treatment by comparing it with well-established biological pathways. Finally, gene knockdown of cMyc in MCF7 cells and treatment with AB1, knockdown of p53 in MCF10A cells and treatment with OTA was conducted and the implication of knocking down these genes and treating the cells with the respective

toxins on different tumor suppressing genes and oncogenes. The understanding of the regulation of these mycotoxins on normal and cancer breast cells may lead to new knowledge of prevention and awareness in human kind.

1.2 Hypothesis

Many chemical and physical mutagens were proved to cause mutation and inactivation of the cancer suppressor genes such as *p53, BRCA1* and *BRCA2* and the activation of the oncogenes such as *HER-1, HER-2* and the *cMyc*. Some of the important chemical compounds that could cause a mutation in these genes included mycotoxins. Mycotoxins are carcinogenic toxins that are produced by many Aspergillus and Penicillium species growing on food commodities. Therefore, this study hypothesized that there was impact of mycotoxins such as aflatoxins and ochratoxins on the regulation of breast cancer cells *p53, BRCA1, BRCA2, HER-1, HER-2* and *cMyc* and alter cell growth and might initiate apoptosis.

1.3 General objective

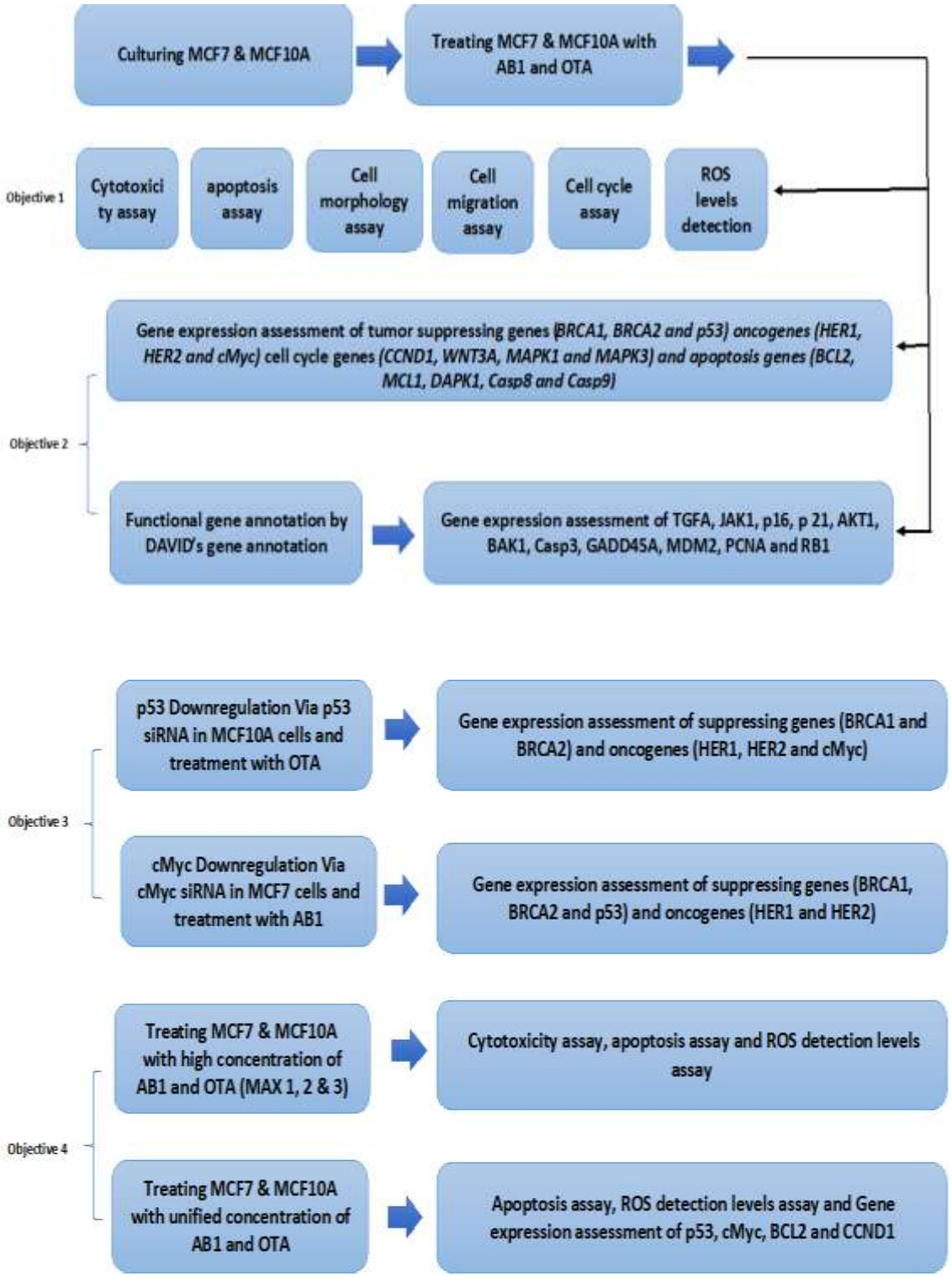
The main aim of this study was to assist the effect of Aflatoxin B1 and Ochratoxin A in increasing the risk of developing breast cancer by influencing the gene expression of tumor related genes.

1.4 Objectives

The objectives in this study include:

- To investigate the effect of AB1 and OTA on cell activities of MCF7 and MCF10A.
- To investigate the effect of AB1 and OTA on gene expression of tumor suppressing genes (*BRCA1, BRCA2* and *p53*), oncogenes (*HER1*, *HER2* and *cMyc*), cell cycle genes (*CCND1*, *WNT3A*, *MAPK1* and *MAPK3*) and apoptosis genes (*BCL2, MCL1, DAPK1, Casp8* and *Casp9*) in MCF7 and MCF10A.
- To investigate the effect of AB1 and OTA on tumor suppressing genes (*BRCA1, BRCA2* and *p53*), and oncogenes (*HER1*, *HER2* and *cMyc*) in MCF7 with knocked down *cMyc* and in MCF10A with knocked down *p53* and to investigate the mechanisms regulated by aflatoxin B1 and ochratoxin A on the proliferation of the breast cancer cells.
- To investigate the implication of high and uniformed concentration of AB1 and OTA in MCF7 and MCF10A.

1.5 Study flow chart



CHAPTER 2 : LITERATURE REVIEW

2.1 Mycotoxins

Mycotoxins can be defined as carcinogenic toxins that produced by many fungal species such as *Aspergillus* and *Penicillium* that can grow easily on food commodities where they produce these toxins as secondary metabolites (Turner, Subrahmanyam and Piletsky, 2009). There are several mycotoxins discovered up to date but among all of them, aflatoxin B1 has been distinguished as the strongest carcinogenic mycotoxin (C. Renzulli *et al.*, 2004; Nguyen *et al.*, 2007; Villa and Markaki, 2009). Aflatoxin B1 capable of penetrating the cell membrane and combine with cellular DNA where it can cause damage and mutations (Adam *et al.*, 2017). The ability of mycotoxins to move across the cell membrane was due to its chemical nature that makes mycotoxins highly liposoluble compounds that can be absorbed from the most common sites of exposure like gastrointestinal and respiratory tract and eventually it can reach the bloodstream and dissimilate throughout the body (Godfrey *et al.*, 2013). There are many portals of exposure to these toxins and that include ingestion of the contaminated food, drinking contaminated water, direct contact with the skin and (Godfrey *et al.*, 2013). The action of mycotoxin inside the cell cytoplasm usually takes place by the action of cytochrome P450 which metabolize mycotoxins to mycotoxin-8, 9-epoxide through detoxification metabolic pathway. These mycotoxin-8, 9-epoxide are highly reactive and unstable so they tend to attach themselves to more stable cellular components such as DNA or protein to regain their stability (Godfrey *et al.*, 2013). In the case of Aflatoxin B1, one of the strongest oncogenes, metabolization of that toxin will produce aflatoxin-8, 9-epoxide will bind to the DNA molecule with enormous affinity forming aflatoxin-N7-guanine

which cause a transmutation in the DNA molecule where guanine (G) with me transverse to thymine (T). This type of mutation would lead to altering cell cycle by affecting the activation of *p53* genes and inhibiting the production of *Tp53*, an essential protein for the control of tumor progression and development (Ricordy *et al.*, 2002; Bbosa *et al.*, 2013; Adam *et al.*, 2017).

2.1.1 Mycotoxins and their natural habitats

In an experiment conducted by El-Banna and colleagues (El-Banna, Pitt and Leistner, 1987) 1400 *Penicillium* species and isolates were collected from different cultures and sources and some of them were even isolated from food, food commodities, and animal feed. Each isolate was identified by the help of Pitt's classification to determine their mycotoxins production and from this study, more than 18 different mycotoxins were isolated. For the extraction of the different mycotoxins, each of the isolates was grown on malt extract agar and cultures were incubated for one to three weeks at room temperature. Once the fungal growth reach maximum and mycotoxins concentration in the media become high, mycotoxins were extracted by chloroform and mycotoxins were concentrated and finally they were classified and characterized by TLC (van der Gaag *et al.*, 2003). The result of this experiment was outstanding and a total of 18 mycotoxins where classified and identified from several *Penicillium* species namely: Citreoviridin, Brevianamid A, Fumitremorgin B, Citrinin, Cyclopiazonic acid, luteoskyrin, Griseofulvin, Ochratoxin A, Patulin, Penicillic acid, PR-toxin, Penitrem A, Verrucosidin, Verruculogen, Xanthomegnin, Roquefortine, Rugulosin and finally Viridicarumtoxin (El-Banna, Pitt and Leistner, 1987; Pitt and Hocking, 2009).

According to Kozakiewicz and colleagues, an additional important group of highly carcinogenic mycotoxins was found to be produced by *Aspergillus* species and they are called Aflatoxins (Pitt and Hocking, 2009). Aflatoxins are mainly produced by the fungi *Aspergillus Flavus* and *Aspergillus Parasiticus* and its carcinogenicity and its involvement in cancer was studies and confirmed by many studies (Smela *et al.*, 2001; Cui *et al.*, 2015; Kim *et al.*, 2016; Zeng *et al.*, 2016). Aflatoxins are classified based on the producing organism, *Aspergillus Flavus* will produce Aflatoxin B1 & B2, and *Aspergillus parasiticus* will produce Aflatoxin G1 & G2 (Turner, Subrahmanyam and Piletsky, 2009; S.-P. Zhao *et al.*, 2016) and researchers indicated that Aflatoxin B1 and G1 are the most carcinogenic and were reported in many human and animal diseases and cancers. Aflatoxin contamination can be found largely in food and feed products such as cereal such as maize, sorghum, pearl millet, rice, and wheat. Another group of food was the oilseeds that include groundnut, soybean, sunflower, and cotton. Additionally, research showed that even spices such as chilies, black pepper, coriander, turmeric, and zinger can be contaminated by aflatoxins. Another group of food that can be easily contaminated by aflatoxin was the tree nuts such as almonds, pistachio, walnuts, and coconut and aflatoxin contamination was reported many times in milk and dairy products (Rasooly *et al.*, 2013; Mohd Azaman *et al.*, 2015; Nierman *et al.*, 2015; Prakash *et al.*, 2015; Gizachew *et al.*, 2016; Ngoma *et al.*, 2017; Singh and Cotty, 2017). Recent findings showed that aflatoxins can also contaminate tobacco leaves (Kedia *et al.*, 2015; Zitomer *et al.*, 2015; Qi *et al.*, 2017) and it can also be present in dry soil (Ortega-Beltran, Jaime and Cotty, 2015; Starr, Rushing and Selim, 2017; Thathana *et al.*, 2017; Pereyra *et al.*, 2018) and the underground water (Oliveira *et al.*, 2016). Aflatoxin B was reported to contaminate milk and dairy products and many

techniques were developed to accomplish that. Some of the effective methods to reduce Aflatoxin B1 includes heat and irradiations but the most effective method reported was treatment with bisulfate, strong acids and bases. In milk, Aflatoxin B1 was reduced by treating milk with Hydrogen peroxide and riboflavin (Kabak *et al.*, 2006; Bhat *et al*., 2010; Afsah-Hejri et al., 2013).

Ochratoxin was found to be as important and as dangerous as Aflatoxin group (Heussner and Bingle, 2015). According to the studies performed on Ochratoxins, the mode of action and how they affect humans and animals are similar to Aflatoxin (Li *et al.,* 2012; Hibi *et al.,* 2013; Ahmed, 2015). The main producer of Ochratoxins was *Aspergillus* species namely *Aspergillus Ochraceus* and *Aspergillus Niger* in addition to some *Penicillium* species namely *Penicillium Verrucosum* and *Penicillium Carbonarius* (El Golli-Bennour *et al.,* 2010). Among all types, Ochratoxin A was widely studied due to the high degree of carcinogenicity (Khatoon *et al.,* 2018) and usually, it was found in the food commodities favored by *Aspergillus* and *Penicillium* species such as coffee, dried fruit, and cereals (Cabañes and Bragulat, 2018).

2.1.2 Nature of mycotoxins (physical and chemical)

Among the different mycotoxins, it was reported that the most important mycotoxins are Aflatoxin B1 and Ochratoxin A (El Golli-Bennour *et al.,* 2010) and normally, both toxin co-exist in the same habitat (Nguyen *et al.,* 2007; Bircan, 2009; El Golli-Bennour *et al.*, 2010).

2.1.2(a) Aflatoxin B1

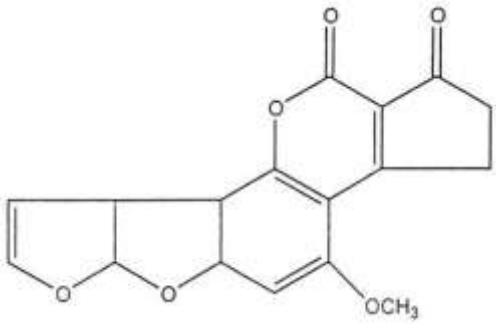


Figure 2.1: Chemical structure of Aflatoxin B1 (Bennett, Klich and Mycotoxins, 2003)

Aflatoxins were reported to be the most important mycotoxins and they ate produced mainly by A. flavus and A. parasiticus (Mokbel and Alharbi, 2015). Up to date, there are four types of aflatoxins known namely B1, B2, G1, and G2 and these types are classified based on the colour the omit under the UV light, either green or blue and their movement in thin layer chromatography (TLC) (Zhang, Liu and Chen, 2005; Li *et al.,* 2015; Qi *et al.,* 2017). The chemical composition of the aflatoxin was presented as $C_{17}H_{12}O_6$ (Wei *et al.,* 2017) mentioned in Figure 2.1. The biosynthesis of aflatoxins takes place by first the production of norsolorinic acid, anthraquinone precursor, and these precursors will join by the action of polyketide synthase. Next,

to that, there will be 15 post polyketide synthase steps which will result in yielding a series of toxigenic metabolites (Conradt *et al.*, 2015).

2.1.2(b) Ochratoxin A

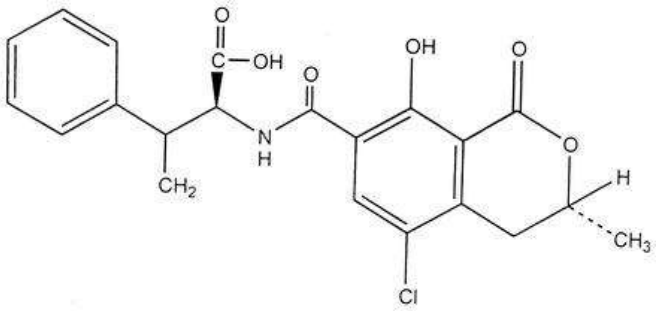


Figure 2.2: Chemical structure of Ochratoxin A (Bennett, Klich and Mycotoxins, 2003)

Along with Aflatoxin B1 and Ochratoxin A were considered to be the most important mycotoxin for their ability to induce mutation, altering cell growth and induce cell arrest, imitate apoptosis and cause kidney cancer (O'Brien and Dietrich, 2005; Rached *et al.*, 2006; Kim *et al.*, 2016). Ochratoxin A categorized as one of the Ochratoxin group of mycotoxins and produced by *Aspergillus* ochraceous (Z. Y. Zhao *et al.*, 2016) and chemically presented as $C_{20}H_{18}C_lNO_6$ (Santoro *et al.*, 2017) mentioned in Figure 2.2.

Due to mycotoxins chemical composition, they are reposted to be strong and heat stable which makes it very difficult and sometimes impossible to completely neutralize them during food processing procedure and sterilization and their contamination in food will eventually entered the human biological system (Bullerman and Bianchini, 2007; Kabak, 2009). The heat stability of mycotoxins was due to the presence heterocyclic, oxygen-containing bisdifuran ring system (Iram *et al.*, 2016) that make these toxins withstand high heat. From the structure of Aflatoxin B1 and Ochratoxin A described in Figure 2.1 and 2.2, we can see that both AB1 and OTA have 2 rings in which six carbon atoms are attached together and each carbon atom with a hydrogen atom attached to it in a perfectly regular hexagon that has single and double bonds distributed evenly around the ring structure and this structure was responsible for their thermal and chemical stability (Manahan, 2002; Tomašević-Čanović *et al.*, 2003; Weiss *et al.*, 2003; Yiannikouris *et al.*, 2006; Siegel *et al.*, 2010). Mycotoxins stability prevent most of them from being eliminated or destroyed by different sterilization protocols and that make them a very common contaminant in food and feed (Bhat, Rai and Karim, 2010; el Khoury and Atoui, 2010; Afsah-Hejri *et al.*, 2013; Nierman *et al.*, 2015; Gizachew *et al.*, 2016) which eventually reach the biological system of human and animals. Many researchers relate the chemical composition and the physical nature of mycotoxins to their carcinogenicity as many of them were reported in several cancers around the globe and that makes these toxins even more dangerous and hazardous (Pfohl-Leszkowicz and Manderville, 2007; Kim *et al.*, 2016; Adam *et al.*, 2017).

Many researchers conclude that the most mycotoxins found in cereals or grains are Fumonisins, Aflatoxins, Zearalenone, Ochratoxin A and Deoxynivalenol (Visconti and Pascale, 2010). Many food processes are reported to effect mycotoxins

and these include cleaning, cooking, roasting, canning, alkaline cooking, baking, frying, flaking, sorting, milling and brewing (Kabak, 2009). The increase in temperature will affect mycotoxin and the concentration of these toxins will decrease but it cannot be completely neutralized from food or animal feed because of the high temperature needed to completely degraded them (Bullerman and Bianchini, 2007; Kam, Bianchini and Bullerman, 2007). Aflatoxin B1 completely degraded at 160° C and beyond while Ochratoxin A will stay stable up to 180° C (Raters and Matissek, 2008).

2.1.3 The implication of mycotoxins on the genomic DNA

Aflatoxin B1 and Ochratoxin A considered as the most dangers mycotoxins because of their lethal affect to human and animal (El Golli-Bennour *et al.*, 2010). Among all mycotoxins, Aflatoxin B1 has the highest carcinogenicity and it could pass through mammalian cell membrane where it gets metabolized into unstable form and attached to cellular genomic DNA in order to become more stable and make different changes (Jarvis & Miller, 2005; Serra, Braga, & Venâncio, 2005; Moradi *et al.*, 2015). Aflatoxins B1 considered to be liposoluble compounds in nature that can be absorbed at the site of exposure and circulates in the blood stream and move across the body (Agag, 2004). Once Aflatoxins B1 are inside the cells, they are metabolized by the action of cytochrome P450 and get reduced by to aflatoxin-8, 9-epoxide, a highly reactive and unstable form of Aflatoxins B1 which requires binding to much more stable molecule such as DNA or protein in order to stabilize itself (Eaton & Groopman, 2013; He *et al.*, 2006). Upon binding of the unstable aflatoxin-8, 9-epoxide to the DNA molecule, aflatoxin-N7-guanine will be formed which was capable of transversion GC to TA mutations. Due to this

mutation, cell cycle will be altered by interfering *p53* gene expression and the production of T-*p53* protein which promotes the development of tumors and cancers (Yates *et al.*, 2006).

Ochratoxin A can disturbs cellular physiology according to many researches, but its primary effects was reported to be associated with phenylalanine metabolism that inhibits enzymes involved in synthesis of the phenylalanine-tRNA complex required for cell development (Marquardt & Frohlich, 1992). Cell exposure to Ochratoxin A can result in the increase in intracellular reactive oxygen species (ROS) which in turn cause a damage to the cellular DNA (Russo *et al.*, 2005; Liu *et al.*, 2012). The damage cause by OTA was mainly due to the regulating of FLT3 signaling (AbdulSalam, Thowfeik and Merino, 2016). FLT3, a tyrosine kinase receptor that upon binding to a ligand will dimerized and initiate an autophosphorylation that will be blocked by PTPRJ that in turn will leads to the activation of AKT, STAT5 pathways and NOX4 transcription. The transcript NOX4 and CYBA will sustain the signaling pathway responsible for generating O_2- which will convert to H_2O_2 by the SOD1 and by the oxidative iteration between PTPRJ and PTEN (AbdulSalam, Thowfeik and Merino, 2016). The produced ROS will attack DNA molecules and will impose modification in its bases causing DNA breaks, inter- and intra-strand crosslinks in addition to DNA-protein crosslinks (Jena, 2012). These modification and DNA damage would lead to mutation, cancer and several other diseases. Finally, the increase in ROS levels through OTA exposure will lead DNA lesions by the means of 8-oxo-7,8-dihydro-guanine, oxazolone, and hydantoins (Abdul Salam, Thowfeik and Merino, 2016). In addition to that, Ochratoxin A can inhibit ATP production in mitochondria and it enhanced lipid peroxidation (Ringot *et al.*, 2006).

2.1.4 Mycotoxins carcinogenicity

Based on the nature of mycotoxins, their effect on human and animals will vary. Many mycotoxins are considered to be mutagenic and it was concluded in many types of researches that Aflatoxin B1 and Ochratoxin A are strong carcinogens (El Golli-Bennour *et al.,* 2010). Aflatoxin B1 considered to be a well-known hepatotoxin (Groopman, Kensler and Wild, 2008) and AB1 outbreaks were linked to the incidents of liver cancer in many parts of the world (Yu and Yuan, 2004; Azziz-Baumgartner *et al.,* 2005; Chen and Zhang, 2011; El-Serag, 2012). Ochratoxin A considered as another important mycotoxin because of how it targets the kidney renal cells (Marin-Kuan et al., 2007). Ochratoxin A has a strong nephrotoxin ability that caused damage in animal and human. Additionally, Ochratoxin A also considered to be a very strong liver toxin, highly carcinogenic and it can cause immune suppression (Y.-M. Chu et al., 2002).

2.1.4(a) Aflatoxin B1

The ubiquitous nature Aflatoxin B1 made them considered as toxic metabolites causing serious public health concern and its contamination was involved in liver diseases and proven to be hepatotoxic (Kensler *et al.,* 2011). Aflatoxin B1 was believed to have a role in causing hepatic and extrahepatic carcinogenesis in human by causing single and double DNA breaks (Gradelet, Astorg, Le Bon, Bergès, & Suschetet, 1997). The bioactivation of Aflatoxin B1 will produce its epoxide metabolite which will bind to DNA molecules and eventually cause a neoplastic transformation of the cells (Massey, Stewart, Daniels, & Liu, 1995). The activation of Aflatoxin B1 could be accomplished by chemical and

enzymatic approach. In the chemical approach, chemical oxidation of dimethyldioxirane and enzymatic activation will take place by cytochrome P450 which will produce a mixture of exo and endo-8,9-epoxides (Iyer *et al.*, 1994). Exo-8,9-epoxides was known to be the most unstable and most dangerous because it will interact with the DNA molecule by attacking the nitrogen atom of the 7^{th} position of guanine of the C8 by S_n2 reaction and that will cause trans adduct that lead to the formation of a malignant cell (Johnson & Guengerich, 1997; Wilson *et al.*, 1999). Endo-8,9-epoxides could not bind to DNA molecules and not considered as carcinogenic agent (Iyer *et al.*, 1994). In many cases, the hepatocellular carcinoma was somehow related to high levels of AB1 contamination and further investigations showed that this toxin cased a mutation in 249th codon of the *p53* gene (Hollstein, Sidransky, Vogelstein, & Harris, 1991). AB1 will induce a transversion mutation in the third position 249th codon of *p53* gene and it will cause insertion of serine at the 249 codon mutant protein (Katiyar *et al.*, 2000). Aflatoxin B1 mutagenic affect can cause G→T and C→A transversions between adjacent codons but in less frequencies. AB1 transversions mutation in 249^{th} codon in *p53* gene and the production of mutant T*P53* protein was proven to be responsible for hepatocellular carcinoma in areas where Aflatoxin B1 contamination in the food was reported (Liu and Wu, 2010; Paget, Lechevrel and Sichel, 2011). Another study confirmed the carcinogenic effect of AB1 on liver cells and causing hepatocellular carcinoma in Mozambique and Transkei and several parts of Africa and Asia where food poisoning with AB1 was reported (Hollstein *et al.*, 1993; Montesano, Hainaut and Wild, 1997; Jackson and Groopman, 1999). Another work done by Kelly and colleagues studied the effect of AB1 on lung cells and its potential to cause lung cancer and the result showed that exo-8,9-epoxides was present in the patients

samples collected (Kelly, Eaton, Guengerich, & Coulombe, 1997) suggesting AB1 involvement in increasing the risk of developing adenocarcinoma of the lung (Divine *et al.*, 2001).

2.1.4(b) Ochratoxin A

Ochratoxin A nephrocarcinogenicity was discussed extensively and it was proven to be carcinogenic in kidney cells in human and animals. In an experiment on Male Fischer rats who kidney tumor was discovered within the first three to six months (M Marin-Kuan *et al.,* 2006). Ochratoxin A could alter gene expression of gene responsible of calcium homeostasis and affect the expression of HNF4α and Nrf2 in the kidney (Maricel Marin *et al.,* 2008). Additionally, Ochratoxin A can suppress genes responsible for DNA repair in the evens of damage and it was reported that it effects the initiation of apoptosis and might lead for the development of tumorgenicity (Dörrenhaus *et al.,* 2000; Maricel Marin-Kuan *et al.,* 2008). Finally, Ochratoxin A can affect Nrf2-regulated genes in the kidney which was required for the chemical detoxication and antioxidant defense mechanism within the cell and in the events of cellular oxidative stress (Boesch-Saadatmandi *et al.,* 2008). In a study done by Annie and colleagues, Ochratoxin A was found to have a renal carcinogenesis effect in rate kidney and it was proven to have genotoxicity by the means of covalent DNA adduct formation (Annie Pfohl-Leszkowicz & Manderville, 2011). When human and animal cells were treated with OTA, single cell death in kidney took place (Klarić *et al.*, 2008) and the toxin induce cell proliferation inhibition and enlarged cellular nucleus which indicates OTA role in altering cell cycle. In addition to that, the results of that experiment indicate the ability of OTA to cause increase in lactate dehydrogenase (LDH) activity, increase in Casp3 expression

and increase in apoptosis rates. OTA was reported to induce overexpression of mitosis regulatory genes such topoisomerase II, surviving, protein kinases Polo-like kinase 1, cyclin-dependent kinase 1, cyclins and cyclin-dependent kinase inhibitors (Adler *et al.*, 2009). Ochratoxin was proven to be genotoxic carcinogen and cause DNA damage due to their ability to oxidase DNA molecules because DNA adducts by quinone formation (Pfohl- Leszkowicz & Manderville, 2007). In human cells, Ochratoxin A can cause phosphorylation of atypical-PKC and that will lead to activation of *ERK1* and *ERK2* and their substrate *ELK1, ELK2* and *p90RSK* downstream where PKC and the MEK-ERK MAP-kinase pathways get activated by Ochratoxin A and that in turn result in altering cell proliferation, cell survival, apoptosis initiation and eventually, renal cancer development (M Marin-Kuan *et al.*, 2007). Ochratoxin A exposure was also suggested to be one of the environmental factors involved in increasing breast cancer risk to male and female rats (Wolff *et al.*, 1996) and it was also suggested that it can be involved in testicular cancer (Jennings-Gee *et al.*, 2010).

2.1.5 Mycotoxins and their implications on human tumor related genes

There are over 291 cancer and tumor related genes discovered and that represents almost 1% of the total genome in human (Futreal *et al.*, 2004; Santarius *et al.*, 2010) and researches have conclude the involvement of mycotoxins in regulating these genes (Wainfan and Poirier, 1992; Kim *et al.*, 2016; Adam *et al.*, 2017). The main effect of mycotoxin on tumor related genes was by the means of inducting mutation which would lead to changes in the cell function (Wang and Groopman, 1999) which eventually could lead to altering cell cycle, inhibition of apoptosis and developing cancer (Fink Grernmels, 1999). Another implication of mycotoxins on

tumor related genes was by regulating gene expression by interfering the different signaling pathways in the cell (Lemmer *et al.*, 1999; Pestka, 2008). Some of the genes reported to be targeted by the action of mycotoxins include *p53* (Bennett, Klich and Mycotoxins, 2003) where the action induce mutation in the gene and deactivation of its expression which might result in the development of cancer. In a study conducted by Carvajal and colleagues on Keshan disease, the effect of mycotoxin was investigated and the result showed that mycotoxins will affect the genes that responsible for cell differentiation, development and even apoptosis which indicate the role of mycotoxins in the development of Keshan disease (Carvajal-Moreno, 2015).

2.1.6 Aflatoxin B1 and Ochratoxin A and cancer in Malaysia

Malaysia weather and climate are favorable for the growth and the propagation of different fungal strains which will grow on food and produce mycotoxins as secondary metabolites. Some of the food found contaminated with mycotoxins in Malaysia include cereals, beans, and spices (Afsah-Hejri *et al.*, 2013). Mycotoxin exposure in Malaysia was first recorded in 1960 in an incident were AB1 outbreak caused liver damage to farm animal and in 1988 aflatoxicosis caused the death of 13 children in another AB1 outbreak in Perak and the attribute of liver cancer due to the exposure to these toxins was reported in many cases in this country (Mohd-Redzwan *et al.*, 2013).

Aflatoxin contamination in Malaysia was very common in food such as peanuts, spices, and cereals but surprisingly, the levels of contamination in some food exceeds the allowed limit designated by the Malaysian Food Regulation 1985 (Mohd-Redzwan *et al.*, 2013) and since Malaysians are moderately exposed to

Aflatoxins ingestion and since this toxin involvement in liver cancer was proven, many studies have reported the involvement in dietary Aflatoxin to liver cancer incident around the country. According to the International Agency for Research on Cancer (IARC), Aflatoxin ingestion can lead to the development of liver cancer (IARC, 1993) and Aflatoxin biomarkers in the biological sample of patients were investigated and results showed the presence of AFB1-albumin adduct in the hepatocellular cancer patients (Zulhabri *et al.* 2009). Another study showed that AFB1-lysine adduct was present in 97% of 170 subjects in Penang (Leong *et al.*, 2012). This study was performed on all different ethnics in Malaysia and results shows that Chinese were 3.05 and Indians were 2.35 times more likely to have higher levels of AFB1-lysine adduct when compare to the Malay (Leong *et al.*, 2012) and it was reported by the ministry of health in Malaysia that Chinese are reported to have the highest incident of liver cancer in compared to other main ethnicities (National Cancer Registry, Malaysia, 2011). Finally, Aflatoxin M_1, one of Aflatoxin B1 metabolite was detected in the urinary sample of 98 subject who consume milk and dairy product that can be contaminated with Aflatoxin B1 and the results showed that levels of this metabolite was detectable in their samples (Redzwan *et al.*, 2012). The presence of Aflatoxin metabolite in the liver cancer patient's biological samples was a strong indicator to the involvement of Aflatoxin B1 contamination and ingestion in the development of liver cancer in Malaysia (Mohd-Redzwan *et al.*, 2013).

Food contamination with Ochratoxin A was common in Malaysia and studies has confirm their presence in human food and animal feed and it known for being nephrotoxic in human and animals (Jalili, Jinap and Radu, 2010). Some of the food that was found to be contaminated with OTA in Malaysia include dried chili (Jalili and Jinap, 2012) and it was reported that A total of 65 out of 80 samples were found

to have OTA contamination ranging between 0.2 to 101.2 ng/g of the total weight and apparently the chilies sold in an open market has a higher chance of getting higher concentration of the toxin that the chili sold in the supermarket . In another study, an investigation carried out to quantify the levels of OTA in chili powder in Malaysia with limit of detection set at 0.10 ng g^{-1} and the results showed mean level, range and incidence of positive samples for OTA were 2.21 ng g^{-1}, 0.14–20.40 ng g^{-1} and 79%, respectively and fifteen positive samples for total for OTA exceeded the Malaysian limit which was 5 ng g^{-1} (Ali, Hashim and Shuib, 2015). There are no record of the involvement of OTA in causing kidney or other types of cancer in Malaysia so far as more attention was given to AB1 but the involvement of OTA in kidney cancer and their nephrotoxic ability was studied and proven extensively around the world (IARC, 1993; Pfohl-Leszkowicz and Manderville, 2007; el Khoury and Atoui, 2010).

2.1.7 Aflatoxin B1 and Ochratoxin A involvement in human cancer and their possible involvement in Breast cancer

Aflatoxin B1 and Ochratoxin A were classified as the most dangerous and carcinogenic mycotoxins and they were reported to be involved in many cancers. Some of the cancers that was associated with AB1 and OTA exposure include liver (Gradelet *et al.*, 1998; Lemmer *et al.*, 1999; ALI, 2000; Meki, Abdel-Ghaffar and El-Gibaly, 2001), kidney (Christoph *et al.*, 2006; Pfohl-Leszkowicz and Manderville, 2007), urinary track (Pfohl-Leszkowicz and Manderville, 2007; el Khoury and Atoui, 2010; Sulaiman, Jamaluddin and Sabran, 2018), esophageal (Gelderblom *et al.*, 1991; Sydenham *et al.*, 1991) and lung (Cui *et al.*, 2015). The mutagenic effect of AB1 and OTA on *p53* gene was demonstrated in many studies (El Golli-Bennour *et al.*, 2010; Kim *et al.*, 2016) and a mutation in *p53* was reported in many breast cancer cases (Spandidos *et al.*, 1992; Lacroix, Toillon and Leclercq, 2006; Liu *et al.*, 2007). In addition to that, the mutagenic effect of AB1 and OTA was reported on *BRCA1* and *BRCA2* genes (Bennett and Davis, 2002; Hibi *et al.*, 2013; Pogribny and Rusyn, 2013) and a mutation in *BRCA1* and *BRCA2* was reported in most of breast cancer cases (Venkitaraman, 2001; Xu *et al.*, 2001; Gudmundsdottir and Ashworth, 2006). From the effect of AB1 and OTA on tumor suppressing genes *BRCA1*, *BRCA2* and *p53*, a hypothesis can be made which suggest the possibility of AB1 and OTA involvement in developing or affecting the development of breast cancer.

2.3 Human breast

Breast defined as the organ projected outside the human body and located on the upper chest in both males and females (Falk Dahl *et al.*, 2010). Breast in females considered of great importance since it serves as the mammary gland and it's the major component in the process of lactation and it produced milk to feed infants. Breasts developed from the embryological tissues in both males and females but is has no significant importance in males as it was in females (Kent *et al.*, 1999). The development of breast in females starts during puberty where estrogens hormones along with human growth hormone result in breast development and maturation. During the development of breast, a subcutaneous fat will cover breast tissue and will cover a network of ducts that meet at the nipple and breast tissues along with subcutaneous fat and ducts shaped the breast and gives it its size (Wiseman and Werb, 2002). In the breast, the milk produced in the lobules, the small divisions of the breast lobes, a glandular tissue arranged like the petals of a flower. Lobules located at the end of the ducts or clusters of alveoli, and it will be stored there and excreted in response to hormonal signals. Breast plays and important function during pregnancy where it responds the interaction between estrogens, progesterone, and prolactin which helps in the completion and the development of lobules in a process called lobuloalveolar maturation, to prepare the best for lactating and breast feeding (Li *et al.*, 2002).

2.3.1 Normal breast cells and tissue

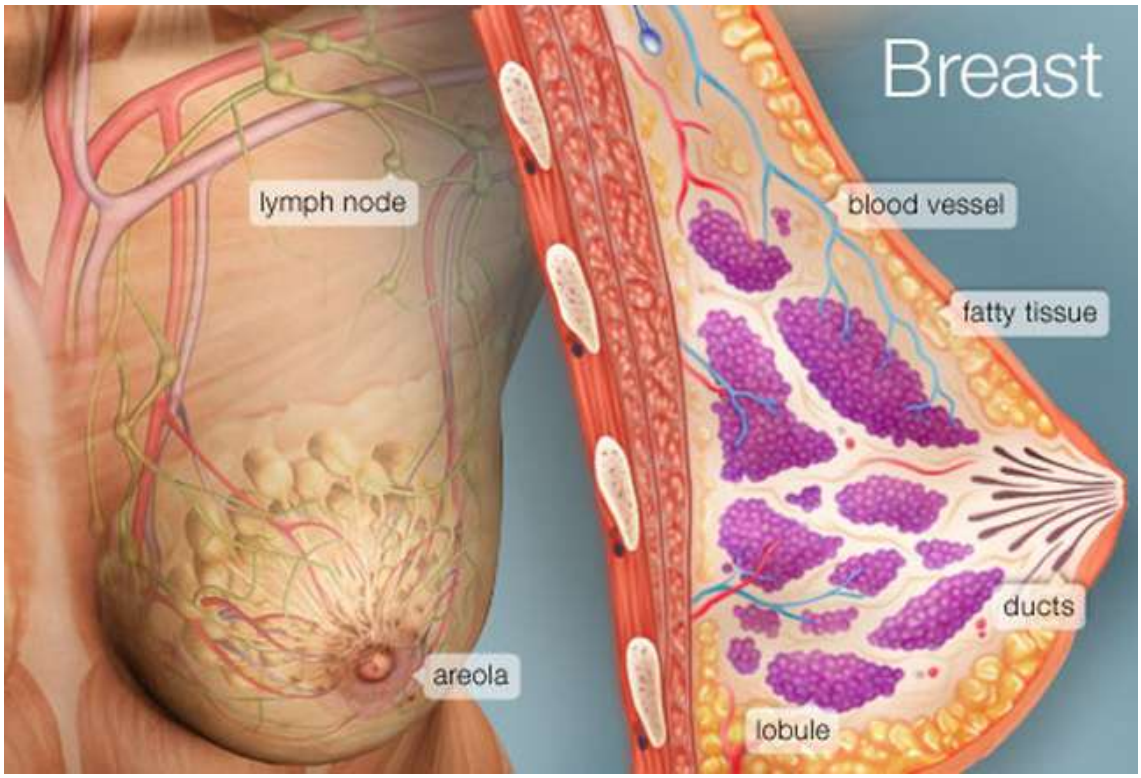


Figure 2.3: Normal human breast. The source of the picture was retrieved from https://www.webmd.com/women/picture-of-the-breasts#1

In the normal breast, breast tissue will cover the chest muscles and they are made up from specialized tissue capable of producing milk and these tissues are called the glandular tissue, in addition to some fatty tissue. The fat of the breast considered to be important in determining the shape and the size of the breast and milk usually produced through 15 to 20 sections called as lobes. The milk in the normal breast will travels through network small ducts and these ducts come together forming larger ducts and milk will exit the body through the nipple on the skin. The nipples were surrounded by a dark area called the areola and the connective tissue and ligaments in the breast will provide support and form breast chap. In addition to that, normal breast will have network of nerve that gives the breast its sensation, it also contains many lymph vessels, blood vessels and lymph nodes (Sahiner *et al.*, 1996; Yan *et al.*, 2006; Lazebnik *et al.*, 2007; Krause *et al.*, 2010)