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**and Scientific Research**
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**College of Science**
**Biology Department**

# Antioxidant Enzymes Activities and Lipid Peroxide Levels in Blood of Women Effected with Breast Cancer

A thesis
Submitted to the Council of the College of Science / University of Babylon in a Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

**By**

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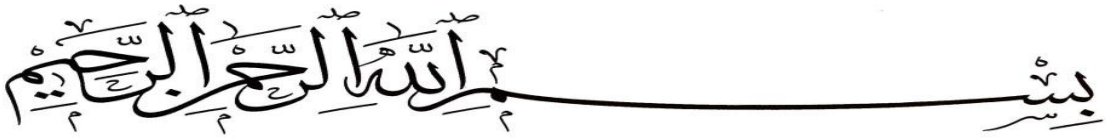


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# Dediction

**To whom Allah sent as mercy to the world …….prophet Mohammed**

**To My Father ….**

**Who taught me the ways of progressing ,and whose name I proudly carry …**

**TO My Mother God rest her soul….**

**To my heaven and God's pleasure be upon me To the one who fashions his tears into necklaces I brand them to fill me Determination and strength to the one who gave his whole life and lit him as a candle to light my path with joy...**

**To My Brothers…..**

**To My sisters…..**

**To those who were my support and the strength of my dependence To whom I am proud to carry their blood in my veins and my affiliation with them..**

**To My friends…..**

**To my dream and companion of my long journey and you are the best companion and bond....**

**To every patient who inspired me with determination from her suffering and her patience for what God, the Mighty and Sublime, has afflicted her.Glory be to God, to every family that pinned its hopes on me as it prayed for someone to save its dear...**

**To all of you I dedicate the grace of God to me**

**Raghad**

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## Summery

Breast cancer is the commonest cancer affecting women worldwide. The present study investigated the relationship between the genetic polymorphisms of Glutathione S-Transferase pi 1(GSTP1) and Glutathione S-Transferase Alpha 1 (GSTA1) genes and decrease of antioxidant enzyme among breast cancer patients and effect chemotherapy of it. This investigation was carried out on 70 patients (all were females) who were confirmatory for breast cancer by histopathological examinations attended from tumors center in Mirjan Medical City in Babel Provence and 30 of apparently healthy women were used as a control.

The study period was between September 2020 to may 2021at Babylon University College of Science, Biotechnology laboratory, this research was case control study , blood samples were collected from 70 breast cancer patients , 30 samples were collected as control group.

Blood samples were collected from 70 breast cancer women and 30 control women for determination lipid profile and lipid peroxidation level. Results have showed a significant increase in malondialdehyde (MDA) levels in breast cancer patients , while lipid profiles {Triglyceride (TG), Total cholesterol (TC) and High density lipid (HDL) and low-density lipoprotein (LDL) and Very-low-density lipoprotein (VLDL) showed decrease association with breast cancer.

In this study was determined of antioxidant marker such as Superoxide dismutase (SOD) Activity, Catalase (CAT) Activity, Hydrogen peroxide ($H_2O_2$) , Total antioxidant enzymes capacity(TAC) and lipid profile and Lipid peroxidation marker such as malondialdehyde (MDA). The present study was revealed the polymorphisms of genes *GSTPI* (rs1695) and *GSTAI* (rs3957357) in breast cancer patients with control.

The demographic characteristics of breast cancer patients were divided into three groups according to the number of chemotherapy doses with the control group.

The results of the present study showed differences between control and case with breast cancer in some of biochemical markers , the Superoxide dismutase (SOD) activity in the control group was 64.40±23.76 U/ml while its activity in the case groups were significantly decreased. The first group (1-5 doses) was 40.89±28.55 U/ml , the second group (6-10 doses) was 40.51±27.55 U/ml and the third group(>10 doses) reach to 43.65±28.75U/ml. The catalase (CAT) activity in control were 16.08±8.38 U/ml while its activity in the case groups were significantly decreased; the first group was 9.51±6.59 U/ml, the second group reach to 9.90±4.66U/ml and the third group was 9.25±4.62 U/ml. GSH concentration in control was 17.57±9.09 µmol/ml while its activity in the case groups were significantly decreased to reach 11.01±5.55 µmol/ml in the first group, the second group was 13.03±8.29 µmol/ml and third group reach to 11.07±5.55umol/ml. Whereas, the $H_2O_2$ concentration in control group was 1.22±1.18 nmol/ml while its concentration in the case groups were significantly decrease to reach 2.36±1.27 nmol/ml in the first group, the second group was 1.94±0.79 nmol/ml and increase in third group to reach 2.39±1.18 nmol/ml.

Lipid peroxidation marker such as the malondialdehyde (MDA) concentration in control was 2.06±1.39µmol/ml while its activity in the case were significantly decreased to reach 2.07±0.88 µmol/ml in the first group, the second group was 2.34±1.65µmol/ml and in third group reach to 1.28±0.18 µmol/ml. The total antioxidant capacity (TAC) in control was 83.17±50.77 U/ml while TAC in the case were significantly increased to reach 74.55±42.83U/ml in the first group, the second group was 67.24±22.73U/ml and in third group was 96.78±61.01U/ml. Lipid profile of the total cholesterol

in control was 507.86±178.62 mg/dl and the case groups were 260.21± 153.72 mg/dl and high density lipid (HDL) in control was1083.96 ±326.02 mg/dl and case was 423.99±109.54 mg/dl. Whereas the observed triglycerides(TG) in control was1065.12±588.85 mg/dl and the case was 1045.93±851.62 mg/dl , and low density lipoprotein (LDL) in case was 412.62±237.24 mg/dl and the control was 789.12±414.32 mg/dl. VLDL in case group was 209.19±170.32 mg/dl and the control group was 213.03±117.77 mg/dl.

The genetic polymorphisms of the Glutathione S-Transferase pi1(*GSTP1*) gene (*rs*1695) have been conducted using PCR RFLP technique. The polymorphism of the Glutathione S-Transferase Alpha1 (*GSTA1*) gene (rs3957357) was performed using Single-strand conformational polymorphism (SSCP –PCR) .

The genotype of *GSTP1* (*rs*1695) was homozygote AA (80%) followed by GA heterozygote genotype (16.7%) and homozygote genotype GG (3.3%) in control group. Also the wild homozygote AA was 82.8% followed by GA heterozygote genotype (8.6%)and GG homozygote genotype (8.6%) in case group. The results indicate there is no association of (*rs*1695) with breast cancer risk.

The present study found that the *GSTA1* (rs3957357) gene had two variant bands were detected by PCR-SSCP including one band (group A) and two band (group B),subsequently the DNA sequencing technique appeared two single mutation of A>G and T>G which had observed association with breast cancer.

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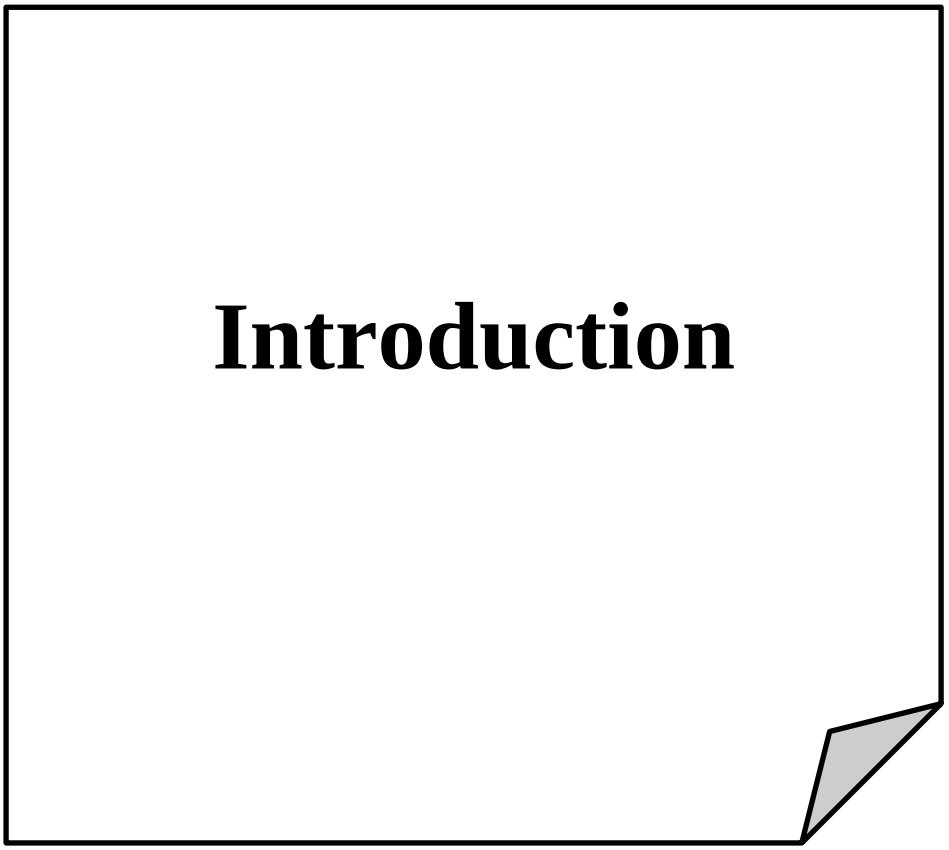
# List of Abbreviations

| Symbol | Definition |
|---|---|
| **BC** | Breast Cancer |
| **EDTA** | Ethylene Diamine Tetra Acetic Acid |
| **ROS** | Reactive Oxygen Species |
| **SOD** | Superoxide Dismutase |
| **CAT** | Catalase |
| **GSH** | Glutathione |
| **MDA** | Malondialdehyde |
| **SNPs** | Single Nucleotide Polymorphisms |
| **D.W** | Distilled Water |
| **GST** | Glutathione S-transferase |
| **ROMs** | Reactive Oxygen Metabolites |
| **WHO** | World Health Organization |
| **PCR** | Polymerase chain reaction |
| **MBI** | Body Mass Index |
| **HDL** | High-Density Lipoprotein |
| **TC** | Total Cholesterol |
| **TG** | Triglycerides |
| **LDL** | Low- Density Lipoprotein |
| **VLDL** | Very Low -Density Lipoprotein |
| **BRCA1** | Breast cancer gene 1 |
| **BRCA2** | Breast cancer gene 2 |
| **GPx** | glutathione peroxidase |
| **GR** | glutathione reductase |
| **CF** | cystic fibrosis |
| **GSSG** | glutathione disulphide |
| **JNK** | N-terminal kinase |

| | |
|---|---|
| **ASK1** | apoptosis signalregulating kinase 1 |
| **Val** | Valine |
| **Ile** | Isoleucine |
| **ITCs** | Isothiocyanates |
| **GSTA1** | Glutathione –S-Transferase alpha |
| **GSTP1** | Glutathione –S-Transferase pi |
| **CP** | Cyclophosphamide |
| **DFS** | disease-free survival |
| **OS** | overall survival |
| **EGFR** | Growth Factor Receptor |
| **lμ** | Microliter |
| **molμ** | Micromole |
| **M** | Molar |
| **Mg** | Miligram |
| **mM** | Milimolar |
| **Min.** | Minutes |
| **nm** | Nanometer |
| **NaOH** | Sodium Hydroxide |
| **O.D.** | Optical Density |
| **rpm** | Rotation Per Minutes |
| **TCA** | Trichloroacetic Acid |
| **U** | Unit |
| **TBE** | Tris-Borate-EDTA |
| **TAC** | Total Antioxidant Capacity |
| **NADH** | Nicotinamide Adenine Dinucleotide (NAD) + Hydrogen (H) |
| **$PI_3K$** | Phosphoinositide 3-kinase |
| **ERK** | Extracellular signal-regulated kinases |
| **PTEN** | Phosphatase and tensin homolog |

| | |
|---|---|
| **MAPEG** | Membrane-Associated Proteins in Eicosanoid and Glutathione |
| **BSO** | Buthionine sulfoximine |
| **AKT** | AK strain transforming |
| **RAS** | Reticular activating system |
| **MEK** | Mitogen-activated protein kinase |

# ***Chapter One***



## Introduction

Cancer is one of the most flourishing diseases all over the world. Cancer incidences and death rates are rapidly increasing world widely and specially in Pakistan (Javed *et al*.,2011). Breast Cancer (BC) is one of the most important cancers in women worldwide, according to the last global cancer statistics, and it was the second-leading cause of cancer-related deaths in 2018 (Bray *et al*.,2020).

To protect themselves, body maintains complex systems of multiple types of antioxidants, such as glutathione, vitamin C and vitamin E as well as enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST)( Champe et al.,2007) .These components or enzymes are involved in multiple biochemical reactions to prevent the harmful oxidative damage. Enzymes are proteins that act as biological catalysts (biocatalysts). Catalysts accelerate chemical reactions, The molecules upon which enzymes may act are called substrates, and the enzyme converts the substrates into different molecules known as products, Enzymes are known to catalyze more than 5,000 biochemical reaction types(Schomburg *et al*.,2013),Certainly, the genetic polymorphisms of these enzymes and their expression levels are correlated to the individuals susceptibility to DNA damage and cancer risk( Klusek *et al*.,2014).

Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) and antioxidants reaction capacity which stimulate the development of a disease such as breast cancer (Aghvami *et al*.,2006, Kostryk ina *et al*.,2009). Antioxidant defenses protect against free radicals, but these defenses are not completely adequate and systems that repair damage by ROS are also necessary (Russo *et al*.,2000). While some ROS are necessary and play important physiological roles, ROS can also cause harm. Excess oxidative species can directly damage DNA, proteins and lipids. Furthermore, reactive

oxygen species (ROS), such as superoxide anions and hydrogen peroxide induced lipid peroxidation, play a major role in malignant transformation and tumor cell proliferation and invasion (Tatiane *et al*.,2009). Antioxidants can be divided into two systems: enzymatic and non-enzymatic. The enzymatic system involves enzymes produced by the organism itself, as superoxide dismutase (SOD), catalase (CAT).

Genetic variations in the antioxidant genes coding for the SOD and CAT may lead to decreased or impaired regulation of their enzymatic activity and alter ROS detoxification. Therefore, genetic variations among enzymes that protect the cell against ROS may modulate disease risk (Forsberg *et al*.,2001). A single nucleotide polymorphism in the *GSTP1* gene causes substitution of isoleucine to valine at amino acid codon 105 (Ile105Val ). The valine allele is associated with a decreased activity of the enzyme compared with isoleucine allele (Allan *et al*., 2001). The polymorphism in *GSTA1* consisting of four mutational sites are apparently linked on the proximal promoter. The variants named as: *GSTA1**A (-631G/T, -567T, -69C, -52G) and hGSTA1*B (-631G, -567G, -69T, -52A) (Ping *et al*., 2006).

Chemotherapy is seldom used for treating BC, but in specific cases, it may be recommended (Loboda *et al*.,2020). Usually, BC is classified into molecular subtypes, and for some of them, chemotherapy is an option. Among the molecular subtypes, triple-negative BC is considered one of the most aggressive, and its chemotherapy response rate is considered higher when compared to the others. However, despite adjuvant chemotherapy, the overall survival of these patients is still poor (Anders *et al*.,2009) Since chemotherapy is usually used for triple-negative, inflammatory and advanced-stage BC, new strategies and molecular predictive markers are required to increase the patient's prognosis (Cleator *et al*.,2007). New predictive and prognostic markers can provide valuable information regarding the identification of patients that could benefit from chemotherapy. Besides that, di_erent strategies can be used to

increase drug delivery into tumor cells, including nanoparticles. Di_erent systems of nano structured carriers can be effective in cancer chemotherapy and overcome drug resistance. In this scenario, this manuscript aimed to critically review the previous literature regarding BC chemoresistance, elucidating its molecular features and providing the perspective of nano carrier structure use to reduce tumor chemoresistance. Chemotherapy regimens can decrease the antioxidants in the body. However, some drug combinations can promote the antioxidant status (Wakabayashi *et al*.,2014).

**Aim of study**

Role of antioxidant enzymes and Biochemical parameters as biomarker for breast cancers and detection of the effect of chemotherapy on antioxidant enzymes in breast cancer patients**.**

**Objectives**

1-Estimation of SOD,CAT and GSH activity,MDA,H2O2 concentration and TAC.

2-Biochemical parameters(Lipid profile , Lipid peroxidation).

3-physiological condition and risk factor such as (Chemotherapy).

4-DNA extraction and purification.

5-Molecular techniques (PCR ) of common SNPs for GSTP1(rs 1695), GSTA1(**rs3957357 A/G/T (52803891)** )genes and (PCR- RFLP and PCR-SSCP) for the SNPs.

6-Sequencing Technique for GSTA1 gene

7- Statistical Analysis.

# ***Chapter Two***

# 2.Literature Review

## 2.1.Breast Anatomy:-

The breast lies between the second and sixth ribs, from the sternal edge to the edge of the axilla, and against the pectoral muscle on the chest wall. Breast tissue also projects into the axilla as the tail of Spence. For clinical purposes, the breast is divided into four quadrants: upper inner, upper outer, lower inner and lower outer quadrants. Also it is composed of 15-20 lobes that radiate from the nipple. Each lobe is surrounded by fat and fibrous connective tissue and is divided into many lobules. The lobule (sometimes called the ductal-lobular unit) is the basic structural unit of the breast and is lined by epithelial cells. Each lobule is subdivided into 10 to 100 alveoli, the milk producing units of the breast. Milk flows from the alveoli of the lobules into the ducts. The ducts gradually coalesce into 10 to 15 major ducts; each lobe containing one major duct terminating in the nipple as shown in figure (1- 1) (American Cancer Society, 2006).

As well as the Breast contains blood and lymphatic vessels. Most lymphatic vessels within the breast lead to axillary lymph nodes, some also connect to supra- or infraclavicular nodes, and internal mammary nodes. They may enter lymphatic vessels and spread to lymph nodes. Beneath the tissues of the breast lie the muscles of the chest wall and between the two is the fascia (a layer of connective tissue). Two layers of suspensor ligaments (Cooper's ligaments) link the breast to the fascia, providing support. As these ligaments stretch with age or weight gain, the breast loses some of its firmness(Rehnke *et al*., 2018).

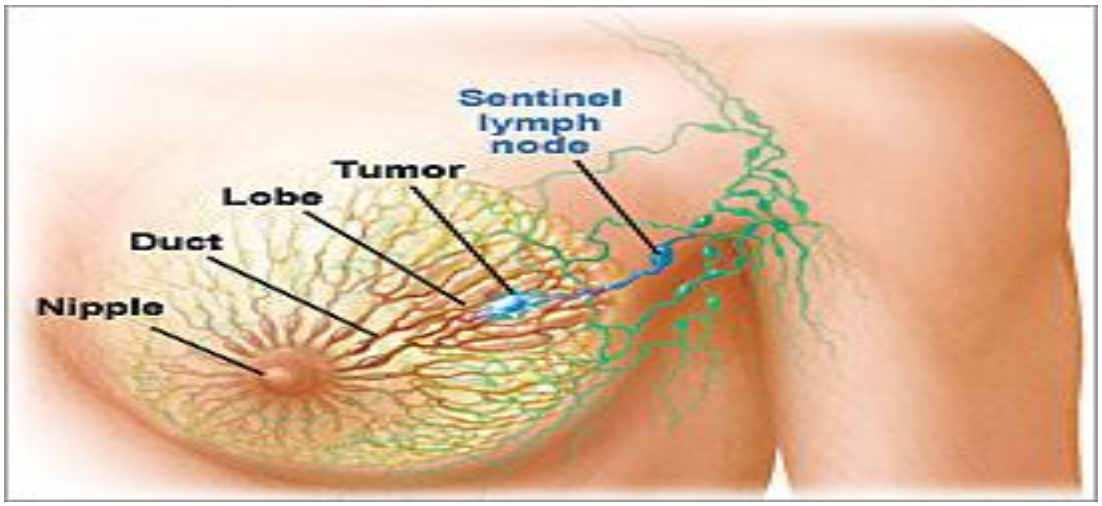



**Figure (2 – 1) primary anatomical features of the breast indicating the ducts and lobules (Brandy, 2004).**

## 2.2.Breast cancer

Breast tumor occurs when cells in the breast begin to grow out of control and then invade nearby tissues or spread throughout the body (Armstrong *et al.*, 2005). Large collections of this out of control tissue are called tumors (Cooper, 2000). A tumor may be either benign or malignant. A benign tumor remains confined to its original location, invading neither surrounding normal tissue nor spreading to distant body sites. A malignant tumor, however, is capable of both invading surrounding normal tissue and spreading throughout the body via the circulatory or lymphatic systems (metastasis). Only malignant breast tumors are properly referred to as cancer. Even in the countries in the Arab region, breast cancer occupies the number one position in terms of prevalence (Salim *et al.*, 2009).

## 2.3. Symptoms

- When breast cancer first develops, there may be no symptoms at all. But as the cancer grows, it can cause the following changes:

1. A lump or thickening in or near the breast or in the underarm area or in the neck.

2. A change in the size or shape of the breast.

3. Nipple discharge or tenderness, or the nipple pulled back (inverted) into the breast (Pam, 2007).

4. Ridges or pitting of the breast skin (like the skin of an orange).

5. A change in the way the skin of the breast, areola, or nipple looks or feels (for example, warm, swollen, red, or scaly) (World Health Organization (WHO), 2003).

### 2.4. Major Causes of Breast Cancer

Breast cancer is mainly caused by inherited mutations in genes which include BRCA1 and BRCA2. Family history is mainly involved in pathophysiology of breast cancer. The main cause of breast cancer is related with a personal or family history of the disease and inherited genetic mutations in the breast cancer susceptibility genes BRCA1 and BRCA2. The mutations in gene expression contribute approximately 5-10% among all cases of breast cancers (Natalia et al., 2013). Reporting of variation in incidence of breast cancer in different population of different parts of Asian continent may be due to multiple factors, including geographic variation, racial/ethnic background, genetic variation, lifestyle, environmental factors, the presence of known risk factors, and utilization of screening mammography, stage of disease at diagnosis, and the availability of appropriate care (Rajneesh *et al*.,2008, Suzana *et al*.,2008).

Other known factors involved in breast cancer may include obesity, use of hormone therapies (progestin and estrogen), increased breast tissue density, alcohol use and physical inactivity (Emens and Jaffee, 2005).

Normally cells undergo apoptosis after completion of their life cycle when they are not further required for body. Before apoptosis they are protected by different pathways and proteins. These pathways include PI3K/ AKT pathway and RAS/MEK/ERK pathway. Sometimes genes associated with these pathways

become mutated and these mutations cause permanent opening of these pathways which leads to continuous cell division and proliferation and prevents cell suicide after completion of their life span. Normally PTEN protein is responsible for turning off the PI3K/AKT pathway at the time of cell apoptosis. In some cases mutations occur in PTEN protein which leads to uncontrolled proliferation of tumor cells. In breast adipose tissues over expression of leptin is also responsible for breast cancer. However, breast cancer directly linked with levels of estrogen in body (Natalia *et al*., 2013).

### 2.4.1.Epidemiology:-

Breast was the site of cancer accounted for as many as 17.6% in patients registered in Basra, Iraq (Habib *et al*., 2007). The incidence of breast cancer has been reported to be higher in developed countries than in the developing countries, but this difference might be due to the availability of better detection methods in the developed countries. In fact, a recent epidemiological study from Iraq concluded that the incidence of breast cancer is quite high in Iraq, and has been increasing over the past few years (Habib *et al*., 2016).

Other observation has been noted in many other developing countries lying within the Eastern Mediterranean region (Alwan, 2016). In those areas reported rates of 31 percent, 30 percent and 26 percent have been documented in hospital based cancer registries of Egypt, Lebanon and Jordan respectively (Omar and Contesso, 1988). The annual incidence of breast cancer in the United states increases dramatically with age (5 per 100 population at 25 years of age, rising to 15 per 100 at 50 years of age and to more than 20 per 100 at 75 years of age).

Today, breast cancer, like other forms of cancer, is considered to be the final outcome of multiple environmental and hereditary factors. Some of these factors include:

1. Lesions of DNA such as genetic mutations. Mutations that can lead to breast cancer have been experimentally linked to estrogen exposure (Goldgar *et al*.,2004). Beyond the contribution of estrogen; research has implicated viral oncogenesis and the contribution of ionizing radiation in causing genetic mutation.

2. Failure of immune surveillance, a theory in which the immune system removes malignant cells throughout one's life (Linnea *et al*., 2001).

3. Abnormal growth factor signaling in the interaction between stromal cells and epithelial cells can facilitate malignant cell growth. For example, tumors can induce blood vessel growth (angiogenesis) by secreting various growth factors further facilitating cancer growth.

4. Inherited defects in DNA repair genes, such asBRCA1, BRCA2and P53 (Hedau *et al.,* 2004).

**2.5.Antioxidant Enzymes**

Oxidation occurs in over one-quarter of the known chemical reactions catalyzed by enzymes in living cells. In many cases this is accomplished by the transfer of hydrogen atoms or electrons from one molecule to another. Metabolic reactions of this type are the major source of energy for life processes(Champe *et al* .,2007). A paradox in metabolism is that although the vast majority of complex life on Earth requires oxygen for its existence, oxygen is a highly reactive molecule that damages living organisms by producing reactive oxygen species including hydrogen peroxide (H2O2), hypochlorous acid (HOCl) and free radicals such as the hydroxyl radical (·OH), the superoxide anion ($O2^-$) and lipid peroxides(Valco *et al* .,2007). Directly or indirectly, these chemical species of oxygen can transiently or permanently damage nucleic acids, lipids, and proteins. Oxidative damage to these cellular macromolecules is implicated in the genesis of several diseases, including cancer(Mayne.,2003).

**Superoxide dismutase** :-

Superoxide dismutase (SOD, EC 1.15.1.1) is an enzyme that alternately catalyzes the dismutation (or partitioning) of the superoxide ($O^{-}_{2}$) radical into ordinary molecular oxygen ($O_2$) and hydrogenperoxide ($H_2O_2$).

$$2O_2^- \xrightarrow{SOD} H_2O_2+O_2$$

Superoxide is produced as a by-product of oxygen metabolism and, if not regulated, causes many types of cell damage(Hayyan *et al*.,2016),catalyzes the dismutation of superoxide radical to hydrogen peroxide and oxygen in the interstitial spaces of tissues and in extracellular fluids (plasma, lymph, and synovial fluid), It eliminates superoxide radicals from the cell environment and prevents the formation of reactive oxygen species and their derivatives. EC-SOD is a secretory, tetrameric glycoprotein containing copper and zinc, with a high affinity to certain glycosaminoglycans, such as heparin and heparan sulfate. It plays an important role in maintaining vascular tone, lung function, and the metabolism of NO, and in the pathology of such diseases as atherosclerosis, diabetes, and arthritis, This paper describes EC-SOD structure, function in tissues, and possibilities of therapy with application of this enzyme (Skrzycki and Czeczot ,2004). constitute a very important antioxidant defense against oxidative stress in the body,Several studies have been performed that reveal the therapeutic potential and physiological importance of SOD ( Landis and Tower.,2005). The enzyme can serve as an anti-inflammatory agent and can also prevent precancerous cell changes( Noor *et al*.,2002).( Yasui and Baba.,2006) Natural SOD levels in the body drop as the body agesand hence as one age, one becomes more prone to oxidative stress-related diseases(Inal *et al*.,2001). SOD is used in cosmetics and personal care products as an anti-aging ingredient and antioxidant due to its ability to reduce free radical damage to the skin, therefore preventing wrinkles, fine lines, and age spots, and it also helps with wound

healing, softens scar tissue, protects against UV rays, and reduces other signs of aging.( Luisa Corvo *et al*.,2002) It has been reported that SOD has an important link in several human health problems including RBC-related disorders, cystic fibrosis (CF), postcholecystectomy pain syndrome, malignant breast disease, steroid-sensitive nephrotic syndrome, amyotrophic lateral sclerosis, neuronal apoptosis, AIDS, and cancer, Furthermore, a strong association between the activity of SOD and Alzheimer's disease has been suggested by some researchers,It has also been reported that treatment with SOD helps recovery from mustard gas burns. In many animal models having myocardial ischemia-reperfusion injury, inflammation, and cerebral ischemia-reperfusion injury, etc., SOD enzymes are found to be very effective ( Noor et al.,2002). SOD mimetics (small molecule catalytic antioxidants) offer a potential for treating diseases resulting from oxidative stress. SOD mimetics are synthetic compounds that mimic the native SOD by effectively converting O2 - into H2 O2 , which is further converted into water by catalase. They are of prime interest in therapeutic treatment of oxidative stress because of their smaller size, longer half-life, and similarity in function to the native enzyme. Several attempts have been made to use SOD as a therapeutic agent against the ROS-mediated diseases. The present review describes the various therapeutic potentials of SOD( Salvemin and Riley.,2000).

**Catalase:**

**Catalase**:- is a common enzyme found in nearly all living organisms exposed to oxygen (such as bacteria, plants, and animals) which catalyzes the decomposition of hydrogen peroxide to water and oxygen ,It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS),Hydrogen peroxide ($H_2O_2$) is harmful to cells and it is converted to extremely reactive hydroxyl radical (•OH) in the presence of cuprous and ferrous ions by a Fenton reaction, Catalase, a first line antioxidant enzyme located in the peroxisomes (catalase is absent in mitochondria of mammalian

cells), utilizes either iron or manganese as cofactor for converting hydrogen peroxide ($H_2O_2$) into water and molecular oxygen ($O_2$) (Chelikani et al.,2004). The enzyme functions in two steps (i) oxidation of heme to an oxyferryl species by hydrogen peroxide ($H_2O_2$) and (ii) generation of a porphyrin cation radical following oxidation of iron and the porphyrin ring. Catalase consists of four subunits molecular weight 240 Kilodalton encoded by the gene *ctt1* located in chromosome 11. Thus, it exists as a tetrameric protein with each subunit containing a heme group and a molecule of NADPH. Genetic polymophisms as well as mutations of *ctt1* is responsible for the onset of various diseases related to the alterations in the cellular oxidative status including cancer (Radi *et al*.,1991, Chelikani *et al*.,2004). However, there is contradictory evidence concerning the role of catalase enzyme on neoplasms of various types of tissues(Marengo et al.,2016) .

$$\mathbf{2H_2O_2 \longrightarrow 2H_2O + O_2}$$

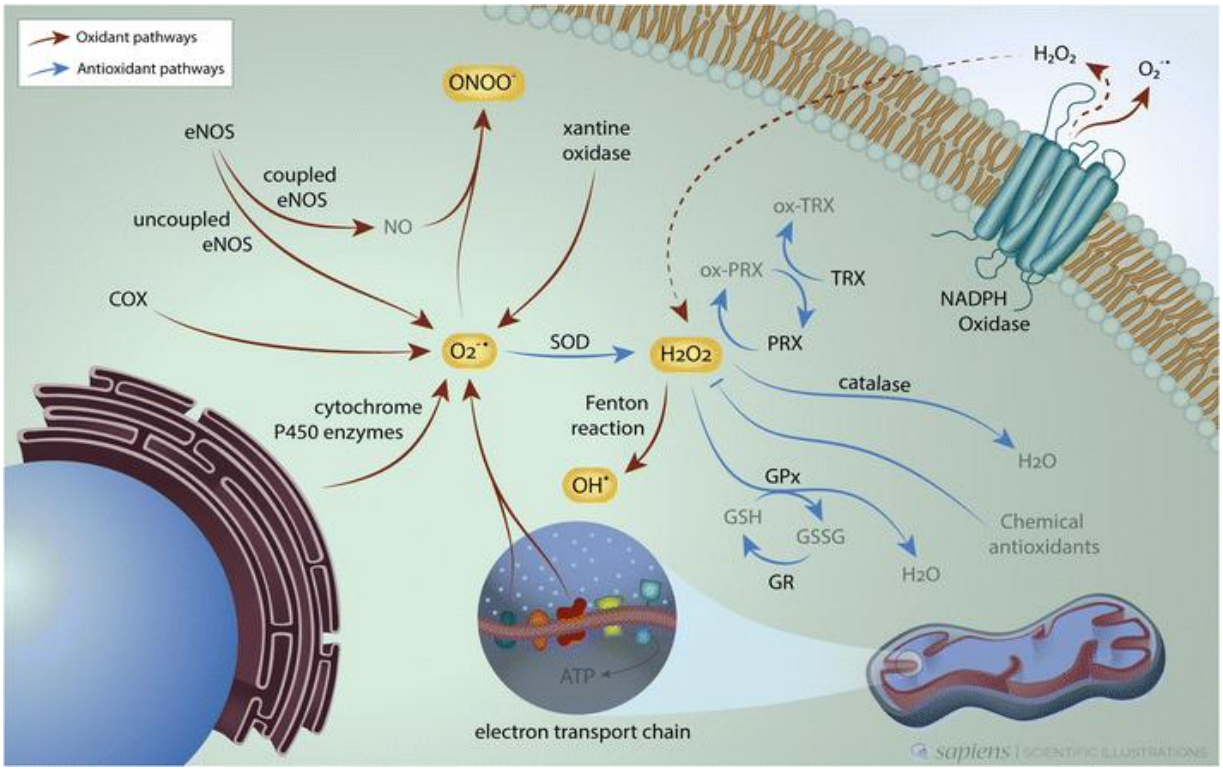
**Glutathione (GSH):-**

A major antioxidant defence molecule existing in all cellular compartments which is composed of amino acids cysteine, glutamine, and glycine. It is one of the key antioxidants which play a greater role in the synthesis of nucleic acids, proteins as well as in the detoxification of xenobiotics (*Diaz et al*.,2010). GSH is synthesized mainly by the liver and converted as oxidized glutathione by the enzymes glutathione peroxidase (GPx) as well as glutathione reductase to neutralize the free radicals and resides in the cytoplasm of all metabolically active cells. GSH performs amino acid transport across plasma membranes, functions as a co-factor for several enzymes, regenerate vitamin C and E after utilization as antioxidants and scavange various oxide radicals in the cell (Birk *et al*.,2013). In fact, the sulfhydryl group present in the glutathione allows it to function as a cellular antioxidant. A decline in the glutathione/glutathione disulphide (GSH/GSSG) ratio is responsible for the increase in oxidative stress leading to carcinogenesis. Thus, an increased

GSH/GSSG ratio due to the elevated GSH levels is desirable for reducing oxidative stress and preventing cancer progression, facilitating cells to achieve a state of homeostasis (Traverso *et al*.,2013). Further, decreasing the levels of extracellular cysteine (two linked cysteine molecules) uptake or the use of glutaminase inhibitors is a therapeutic strategy in cancer cells to induce excessive ROS and cell death (Li *et al*.,2019).

### 2.6. Oxidative damage to DNA and cancer

Oxidative stress plays an important role in carcinogenesis because of induction of DNA damage and its effects on intracellular signal transduction pathways(Frederiks *et al* .,2009). Reactive oxygen species (ROS) induce almost all forms of DNA damage, including base modifications, base-free (apurinic and apyrimidinic) sites, strand breakage and DNA-protein cross-links, but the specific spectrum of products depends on the reactive species involved. These types of mutation are reported in genes whose dysfunction is involved in the genesis of cancer(Hussain *et al* .,2000). ROS may also play a key role in cancer development by inducing and maintaining the oncogenic phenotypes of cancers(Gargouri *et al* .,2009). The highly significant correlation between consumption of fats and oils and death rates from leukemia and malignant neoplasia of the breast, ovaries and rectum among persons over 55 years may be a reflection of greater lipid peroxidation(Kulbaelca *et al* ., 2009). Currently, oxidative stress has been increasingly postulated as a major contributor to carcinogenesis. The assessment of damage in various biological matrices, such as tissues and cells, is vital to understand the mechanisms of carcinogenesis and subsequently devising intervention strategies. Study on genetic polymorphisms, gain or loss of functions of several antioxidant enzymes such as SOD, CAT, GR, GPx and GST has become important way to understand the development of cancer and its therapies(Sengottuvelan *et al* .,2009).




**Figure(2-2):The role of Oxidative stress on breast cancer development and therapy (Hecht *et al*.,2016)**

**2.7.Expression of glutathione S-transferase in cancer**

Another glutathione related important enzyme family is GST,GST family catalyzes the conjugation of reduced glutathione via a sulfhydryl group to electrophilic centers on a wide variety of substrates, This activity detoxifies endogenous compounds such as peroxidized lipids(Leaver and George .,1998). *GSTM1* has been extensively studied as a cancer risk factor. Houlstone reported that *GSTM1* status has no effect on the risk of lung cancer(Houlstone *et al* ., 1999). Also, the results of another study indicated that *GSTM1* genetic polymorphisms are not associated with breast cancer risk, even in an environment low in antioxidant defenses(Ambrosone *et al* .,1999). The *GSTM1* null genotype has also been reported as showing no association with breast cancer by( Curran *et al*.,2000, Smith *et al*.,*2001)*found no association of *GSTZ1* variant and reported that *GSTZ1* does not appear to play a significant role in the development of sporadic breast cancer. However, the *GSTM1* null genotype has been reported as showing significant association in breast cancer risk by (Park *et*

*al*.,2000). A potential effect modification by the *GSTP1* Ile105Val polymorphism genotype on smoking and the risk of prostate cancer was reported by( Mao *et al*.,2004 Nomani *et al.,2005)*suggested GST measurement as a marker in colorectal cancer, as they found plasma GSTs activity significantly higher in colorectal cancer patients than those obtained from normal individuals. alterations in serum total GST level may have a role in cancer progression (Prabhu and Bhat.,2007).

## 2.8.Glutathione *S*-transferases (GSTs)

**Glutathione *S*-transferases** (**GSTs**), previously known as **ligandins**, are a family of eukaryotic and prokaryotic phase II metabolic isozymes best known for their ability to catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification. The GST family consists of three superfamilies: the cytosolic, mitochondrial, and microsomal—also known as MAPEG—proteins (Allocati *et al*.,2009). Members of the GST superfamily are extremely diverse in amino acid sequence, and a large fraction of the sequences deposited in public databases are of unknown function( Atkinson ana Babbitt.,2009).The Enzyme Function Initiative (EFI) is using GSTs as a model superfamily to identify new GST functions. GSTs belong to the family of intracellular isoenzymes that mediate the conjugation of reduced glutathione to exogenous or endogenous compounds. Thus, oxidative stress products, prostaglandins, chemical carcinogens and therapeutic drugs are detoxified by GSTs (Ramsay and Dilda .,2014). The nucleophilic attack of reduced glutathione on electrophilic substrates, catalyzed by GST enzymes, represents a defense mechanism in the cell. Indeed, glutathione conjugation reduces the toxic effects of strongly reactive products on proteins and DNA (Ramsay and Dilda.,2014). Therefore, one of the roles of GSTs is to protect DNA against oxidative damage, which may lead to mutations, and in consequence, favor carcinogenesis (Ramsay and Dilda.,2014). More recently, it has been shown that GSTs also play important roles in

regulating signaling pathways in a catalytic-independent manner through direct interaction with kinases, such as *c-jun* N-terminal kinase (JNK) and apoptosis signalregulating kinase 1 (ASK1) to modulate their phosphorylation activities (Pajaud *et al*.,2012)**.**

Glutathione *S*-transferases (GSTs) are enzymes that primarily catalyze the detoxification of exogenous and endogenous electrophilic compounds by conjugation with glutathione. Their substrates include known and suspected carcinogens, such as benzo(*a*)pyrene and other polycyclic aromatic hydrocarbons, reactive intermediates derived from estrogen metabolism, and byproducts of oxidative stress. These enzymes also function as peroxidases, isomerases, and thiol transferases and act in cellular processes unrelated to detoxification reviewed by (Hayes *et al* .,2005)Specific to breast cancer, the roles of the cytosolic GST class μ and π enzymes have been of particular interest, since both enzymes are frequently expressed in normal and tumor breast tissue (Haas *et al*.,2006) and are encoded by genes that contain common, functional polymorphisms. In the *GSTP1* gene (chromosome 11q13), which encodes the GST class π enzyme, a base change (A>G) polymorphism (rs1695) causes an amino acid substitution of isoleucine (Ile) with valine (Val) at codon 105 that results in decreased levels of enzymatic activity (Zimniak *et al*., 1999). Therefore, it has been hypothesized that differences in the metabolic capacity of either GST-specific enzyme might affect susceptibility to breast cancer, such that reduced GST activity, due to inheritance of the *GSTM1* null genotype or *GSTP1* Val allele, would predispose to increased cancer risk. Evidence to support an association between *GSTM1* and *GSTP1* polymorphisms and breast cancer risk, however, has been weak. Numerous studies have detected no overall association between the *GSTM1* null genotype and risk of breast cancer (Wu *et al*.,2006) although a few have suggested the association might differ by menopausal status (Park *et al*.,2004), smoking (Terry and Goodman *et al*.,2003), alcohol use (Zheng *et al*.,2003), consumption of well-done meat (Zheng *et*

*al*.,2002), and a combination of parity and age at first full-term pregnancy (Park *et al*.,2003). The majority of studies examining the *GSTP1* Ile105Val polymorphism and breast cancer risk have also yielded null results (Spurdle *et al*.,2007). Few studies to date have examined this potential interaction between *GST* variation, diet, and breast cancer risk (Ambrosone *et al*.,2004). In a study of Chinese women, an inverse relationship between urinary ITC levels and risk of breast cancer was found to be slightly more evident among *GST* null than non-null carriers (Fowke *et al*.,2003). To our knowledge, neither *GST* polymorphism has been examined in relation to the risk of benign fibrocystic breast conditions. Although the etiology of these conditions is not well understood, women with proliferative lesions of the breast with or without atypia have a greater risk of developing breast cancer than women with non-proliferative lesions (Hartmann *et al*.,2005). Therefore, by also investigating whether either *GST* polymorphism is associated with fibrocystic breast conditions, particularl those characterized as proliferative, insight may be gained into whether GST-specific activity is influential early in mammary carcinogenesis. In a case-control study of Chinese women enrolled in a randomized trial of breast selfexamination, we examined whether the *GSTM1* deletion and *GSTP1* Ile105Val polymorphisms are associated with the risk of breast cancer and fibrocystic breast conditions, and whether these associations differ by either menopausal status or presence of proliferation in the extratumoral epithelium or benign lesions. We also explored whether the associations between *GST* genotype and risk of breast cancer and fibrocystic breast conditions varied by level of fruit or vegetable intake (Seow *et al*.,2005).

Human *GSTA1-1* enzyme catalyzes the GSH dependent detoxification of electrophiles showing highly promiscuous substrate selectivity for many structurally unrelated chemicals, including environmental carcinogens (e.g. benzo (a) pyrene diol epoxides), several alkylating chemotherapeutic agents (such as busulfan, chlorambucil, melphalan, phosphoramide mustard,

cyclophosphamide, thiotepa), as well as, steroids and products of lipid degradation. GSTA1-1 is the most highly expressed GST of the liver and could therefore, be critical for "systemic" detoxification of electrophilic xenobiotics including carcinogens and drugs (Coles and Kadlubar, 2005). In addition to enzymatic detoxification, GSTA1 acts as modulator of mitogen-activated protein kinase (MAPK) signal transduction pathway via amechanism involving protein-protein interactions. Namely, *GSTA1* forms complexes with c-Jun N terminal kinase (JNK), modifying JNK activation during cellular stress (Adnan *et al*., 2012). Thus, it is possible that *GSTA1* confer drug resistance by two distinct means: by direct inactivation (detoxification) of chemotherapeutic drugs and by acting as inhibitors of MAPK pathway.

## 2.9.Genetic Molecular

### 2.9.1. *Glutathione S-Transferase pi 1*(*GSTP1*) Gene

The GSTP1 gene has a single base change of an A to G that results in an amino acid change of Ile to Val, resulting in reduced enzyme activity (Harries *et al*., 1997). This variant has been associated with increased risk of cancer at various sites (Hirvonen, 1999). The *GSTP1* gene is involved in a wide range of detoxification reactions which protect cells from carcinogens (Pongtheerat *et al*.,2011,Lu *et al*.,2011). GSTs provide protection against the electrophilic metabolites of carcinogens and reactive oxygen species. GSTP1 is a biotransformation enzyme expressed in normal breast epithelial cells. High levels of GSTP1 have been associated with a poor prognosis in breast cancer (Pongtheerat *et al*.,2011). GSTs provide protection against the electrophilic metabolites of carcinogens and reactive oxygen species. GSTP1 is a biotransformation enzyme expressed in normal breast epithelial cells. High levels of GSTP1 have been associated with a poor prognosis in breast cancer (Pongtheerat *et al*.,2011).

*GSTP1* has a polymorphic site at codon 105 in exon 5, where an adenosine to guanosine (A>G) transition results in an Ile to Val substitution (*I105V*), giving rise to the *GSTP1*B* allele,Individuals with the valine allele exhibit significantly lower enzyme activity and a reduced detoxification ability (Sakoda *et al*.,2007).There are five classes of GST enzymes (α, μ, π, σ and θ) in humans. Studies have been published concerning the potential effects of the changes to the activation and detoxification abilities of GST class π enzymes on an individual's risk of breast cancer and have established an association between the GSTP1 Ile105Val polymorphism and breast cancer risk (Antogneli *et al*.,2009,MARLE-GENICA,2010). Previously (Saxena *et al*.,2009,Zhang *et al*.,2009), that homozygous mutant individuals have a significantly higher risk of breast cancer. Therefore, we investigated the hypothesis that epigenetic modification in homozygous mutants with reduced enzymatic activity increases the risk of breast cancer, which is further modified by various clinicopathological parameters, lifestyle factors and dietary habits. Silencing of tumor-suppressor genes through the hypermethylation of their promoter regions is a frequent event in carcinogenesis (Jones *et al*.,2007). Hypermethylation of CpG islands in the gene promoter regions of numerous tumor suppressor and DNA repair genes has been reported to be associated with events such as chromatin condensation, replication delay and gene silencing (Fraga,2004,Esteller *et al*.,2008). Identification of epigenetic changes and their correlation with clinical factors may lead to improvements in breast cancer diagnosis and treatment. The 5' region of *GSTP1* is rich in CpG islands and its methylation causes changes in expression levels in neoplastic cells, as has been reported in a number of published studies (Zhong *et al*.,2002). *GSTP1* promoter hypermethylation is also associated with a loss of GSTP1 expression (Chan *et al*.,2005,Zhong *et al*.,2002). Studies have investigated the methylation status of *GSTP1* in invasive breast cancer (Arai *et al*.,2006,Shinozoki *et al*.,2005) and a different study revealed *GSTP1* promoter methylation to be an early event in

breast cancer (Lee.,2007). *GSTP1* promoter methylation has also been reported to be associated with a poor prognosis in breast cancer (Arai *et al*.,2006).

### 2.9.2. *Glutathione S-Transferase Alpha 1*(*GSTA1*) Gene

is abundantly expressed in A549 cells, located in the cytoplasm and/or membranes (Pan *et al*.,2014). Furthermore, its expression is associated with an increased risk in colorectal, breast and gastric cancer (Cordoba *et al*.,2016,Eichholzer *et al*.,2012). The alpha class, *GSTA1-A5* are not only expressed in normal human tissues, but also in human cancer (Pan et al.,2014). *GSTA1* are widely expressed in human tissues,the genetic polymorphism of *GSTA1* is characterized by two alleles, *GSTA1*A* and *GSTA1*B* (Pan et al.,2014). However, the roles of *GSTA1* in NSCLC cells remain to be elucidated. We hypothesize that downregulation of *GSTA1* serves a functional role in inhibiting proliferation and inducing apoptosis in the A549 cell line.

*GSTA1* and other GSTs of the _ class are the predominant GSTs in human liver,the major site of drug metabolism, and are also expressed in other tissues (Morel *et al*.,2002). Other studies have shown that among human GSTs, *GSTA1* has the highest catalytic activity for glutathione conjugation of nitrogen mustard chemotherapy agents,3 including metabolites of cyclophosphamide (CP),( Dirven *et al*.,1994) ,which is used in combination chemotherapy for breast cancer. A polymorphism that influences the hepatic expression of GSTA1 has recently been described.( Morel et al.,2002, Coles *et al*.,2001) Liver cytosols from individuals who carried the variant *GSTA1*B* allele, which consists of several linked SNPs in the proximal promoter region of the *GSTA1* gene, had reduced levels of *GSTA1* enzyme.( Coles et al.,2001)Because of the importance of the *GSTA1* enzyme in metabolism of chemotherapeutic drugs, it can be hypothesized that individuals carrying the low expression *GSTA1*B* allele may have altered responses to chemotherapy. (Board et al.,2002).To consider the possible influence of the reduced-expression *GSTA1*B* allele on cancer patient

survival, we have conducted a pilot study of breast cancer patients treated with CP-containing combination chemotherapy.

## 2.10.Neoadjuvant Chemotherapy for Breast Cancer

In general, BC treatments focus on the cure of the disease, higher disease-free survival (DFS) and overall survival (OS) time and quality of life. Di_erent types of modalities can be associated, such as local, systemic and support treatments. Local treatments include surgery and radiotherapy, and systemic therapies can be divided into chemotherapy, immunotherapy and target and hormone therapy (Cardoso et al.,2019 , National Cancer Institute ,2020) The choice of the treatment must consider the tumor location and size, lymph node commitment, histopathology, molecular subtype and presence of metastases. Moreover, the patient's health condition, age, hormonal status and preferences should be also discussed (Cardoso *et al*.,2019). In patients with nonmetastatic tumors, it is recommended to perform local therapy, with surgical removal of the tumor and, in some cases, of the axillary lymph nodes with subsequent radiotherapy. Radiation therapy is recommended for most patients after local surgery with breast conservation. In addition, systemic therapy can be used as a neoadjuvant and/or adjuvant tool for surgery (Burstein *et al*.,2019 , Waks *et al*.,2019). In luminal tumors A and B, tumors ER+ and HER2-, the standard treatment is with adjuvant endocrine therapy. Some patients with this type of tumor can have some benefits adding chemotherapy. Women with stage III tumors and patients with commitments of four or more lymph nodes, even if it is a lobular carcinoma and/or grade 1 tumor or luminal A, should receive chemotherapy (Burstein *et al*.,2019).

HER2+ tumors should be treated with chemotherapy and targeted monoclonal antibody therapy, and triple-negative tumors are treated mainly with chemotherapy (Burstein *et al*.,2019 , Waks *et al*.,2019).

Alkylator and taxane-based regimens with anthracycline are chemotherapy drugs recommended for Luminal A and B BCs. HER2+ tumors, in stage 2 or 3,

should be treated with anthracycline-, alkylator- and taxane-based chemotherapy in combination with trastuzumab or pertuzumab and stage 1 with paclitaxel and trastuzumab (Burstein *et al*.,2019) . The most used treatments for triple-negative breast cancer in women are doxorubicin in combination with cyclophosphamide, doxorubicin with cyclophosphamide and paclitaxel, and in cases of recurrence and resistance to doxorubicin, docetaxel can be used together with cyclophosphamide (Waks and Winer .,2019, Balic *et al*.,2019). In patients with triple-negative tumors and metastasis at the time of diagnosis, the first line of treatment is chemotherapy with taxanes (paclitaxel and docetaxel), platinum compounds (cisplatin) or anthracyclines (doxorubicin) as a monotherapy (Waks *et al*.,2019, Curigliano *et al* .,2017 , Rangarao *et al*.,2018).

Triple-negative BC treatment is based on the molecular characteristics of the tumor, and some proposed treatments are chemotherapy with anthracyclines, taxanes, alkylators and platinum-based, PARP1 inhibition when the tumor has an absence of or reduced BRCA1 function, antibody treatment when the tumor overexpresses Epidermal Growth Factor Receptor (EGFR), c-KIT tyrosine kinase inhibitor when it overexpresses c-KIT and multikinase inhibitors when overexpressing EGFR (Cleator *et al*.,2007, Burstein *et al*.,2019, , Balic *et al*.,2019).

Metastatic breast tumors are treated like nonmetastatic breast tumors; however, the focus is on prolonging the patient's survival using palliative therapy (Waks *et al*.,2019). Triple-negative tumors do not have specific targets for therapies, nor do they have estrogen and progesterone receptors for hormone therapy. Thus, treatment options are more restricted. Therefore, even in cases without metastasis to distant organs or lymph nodes, chemotherapy is always used. In the case of triple-negative breast tumors with metastases, other treatment options can be used, in conjunction with chemotherapy, such as immunotherapy and PARP inhibitors and cisplatin or carboplatin (American CancerSociety,2020).

# *Chapter Three*

## Materials and Methods

# 3.Materials and Methods

## 3.1.Materials

### 3.1.1.Equipment and Apparatus

**Table (3.1) showed equipment and apparatus supplied by different origins**

| Name of Equipment | Manufacturer | Origin |
|---|---|---|
| **Autoclave** | 4Labtech | China |
| **Balance** | Sartorius | Germany |
| **Cooling Centrifuge** | Hettich | Germany |
| **Gel Documentation Vision** | EBOX CX5 | UK |
| **Horizontal Gel Electrophoresis** | Clever Scientific | UK |
| **Incubator** | Memmert | Germany |
| **Bio-Digital Clean Bench** | BCB-160D | Korea |
| **Magnetic Stirrer With Hot Plate** | Philekorea | Korea |
| **Micropipette (Automatic) (10µl, 50µl, 100µl and 1000µl)** | Gilson | France |
| **Micropipette tips (10 µl,100 µl,1000 µl)** | Bio-Basic | Canada |
| **Micropipette tips with filter (10 µl,100) µl,1000 µl)** | Bio-Basic | Canada |
| **Nanodrope spectrophotometer** | Cambridge | UK |
| **Power Supply** | Clever Scientific | UK |
| **Sensitive Balance** | Sartorius | Germany |
| **Thermal cycler** | Clever Scientific | UK |
| **Vortex Tube Shaker** | Fischer Scientific | Germany |
| **Water Bath** | Fischer Scientific | Germany |
| **Water Distillatory** | 4Labtech | China |

### 3.1.2 Biological and Chemical Materials

**Table (3.2) showed Biological and chemical materials supplied by different origins**

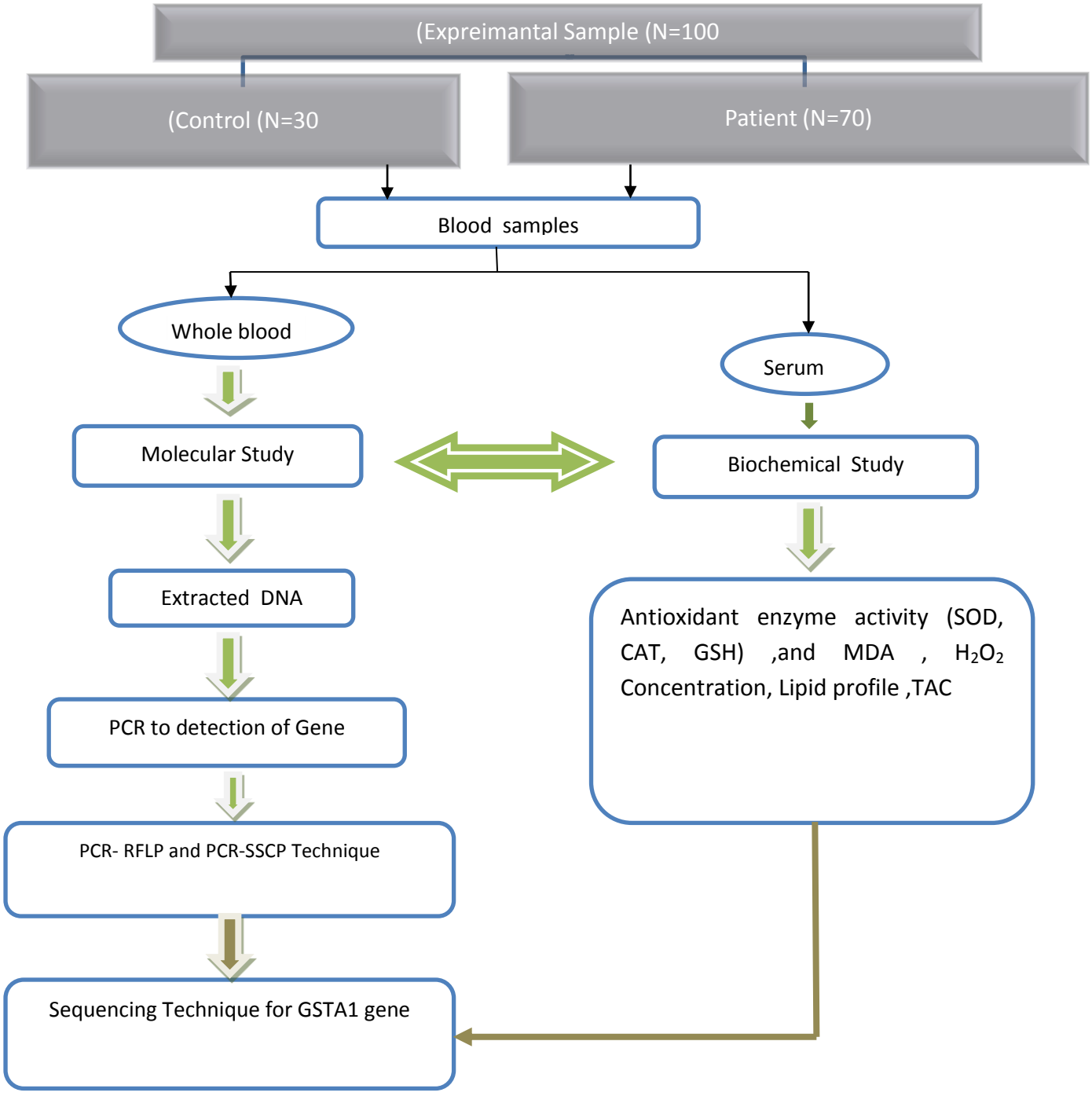
| Type of Material | Manufacturer | Origin |
|---|---|---|
| **Absolute Ethanol (96%)** | Fluka | USA |
| **Agarose** | Bio-Basic | Canada |
| **Ethidium Bromide (EtBr)** | Bio-Basic | Canada |
| **Loading Solution (Bromophenol Blue) (6x)** | Bio-Basic | Canada |
| **Nuclease Free Water** | Promega | USA |
| **Proteinase K** | Promega | USA |
| **Tris Borate EDTA Buffer(10XTBE)** | Bio basic | Canada |

**Table (3.3) showed Kits supplied by different origins and Molecular tools**

| Type of kit | manufacturer | Origin |
|---|---|---|
| **DNA extraction kit from blood** | Genaid | **Taiwan** |
| ***GoTaq*® PCR master mix** | Promega | **USA** |
| **Lipid Profile Kit** | Biolabo | **France** |
| **50 bp DNA ladder** | Promega | **USA** |

### 3.1.3. Experimental Design

The experimental design of the study group is schematically presented as the following:




**Figure(3.1):- Experimental Design of this study**

## 3.2.Subjects and Methods:

### 3.2.1. Study Population

The study subjects comprised from 70 patients selected from Merjan Teaching Hospital all were female as patients group with age range (26-80 years). The control group study included 30 people apparently healthy that also were females with age range (20–71)years. All subjects in this study were taken written consent before participation in this study .

### 3.2.2. Data Collection:

A questionnaire was taken from people included in the study. It included : Age, marital status, residence, Occupational status, education level, family history, previous delinquency , smoking , body mass index (BMI).

### 3.2. 3. Blood Samples

About four milliliters of venous blood sample was collected from each subject in the study. Each blood sample was divided into two parts: The first one (2 ml) was collected into EDTA containing tubes to use for genetic analysis, the second parts of blood (2ml) was used to separate the serum by centrifugation at 3000 rpm for 15 min then kept in eppendorf tubes at -20 ºC until used.

### 3.2.4.Body Mass Index (BMI)

Body mass index (BMI) is a ratio of a person weight to height; it commonly used to classify weight as healthy or unhealthy. BMI calculated as follow (Whitlock*et al*., 2005):

BMI ($kg/m^2$) = weight (kg) / $(height)^2$ ($m^2$)

There are four BMI categories (Sturm, 2007):

1- BMI fewer than 18.5 are considered underweight.
2- BMI values between 18.5 and 24.9 are considered normal or healthy weight.
3- BMI values between 25 and 29.9 are considered overweight.
4- BMI 30 and above are considered obese (BMI 30-39.9 considered severely obese, BMI 40-49.9 considered morbidly obese, BMI>50considered super obese).

## 3.3. Biochemical Markers

### 3.3.1. Antioxidants Enzymes

#### 3.3.1.1. Superoxide dismutase SOD

The activity of superoxide dismutase was determined by autoxidation of Pyragallol according to Marklund and Marklund, (1974) SOD activity determination:

1. Tris buffer (pH 8.0): was prepared by dissolving 0.258 gm of tris and 0.111 gm of Ethylenediaminetetraacetic acid (EDTA) in $dH_2O$ and completing the volume to 100 ml.
2. Pyragallol solution (0.2 mM): was prepared by dissolving 0.0252 gm of pyragallol with 10 ml of HCl and completing the volume to 100 ml with $dH_2O$.

**Procedure**

According to Marklund and Marklund (1974), reaction mix is consisting of 50 µl crude enzyme extract with 2 ml of tris buffer and 0.5 ml of pyragallol (0.2 mM) which absorbs light at 420 nm. Control solution contains the same materials except for the enzyme extract that was replaced by dH2O. As a blank, dH2O was used. One unit of enzyme is defined as the amount of enzyme that is capable of inhibiting 50% of pyragallol oxidation. SOD activity was calculated using the following

equation:

$$SOD\ Activity\ (unit) = \frac{\frac{\%P}{50\%} \times R}{T}$$

Where:

• %P: percentage of the inhibition of pyragallol reduction

*Note: %P of every sample is calculated by comparing Δabs of the sample (X%) with Δabs of control (100%)

• R: Total reaction volume (2.55 ml)

• T: Time of reaction in minutes (2 minutes)

### 3.3.1.2.Catalase (CAT)

Catalase assay was measured according to procedure of Aebi, (1974, 1984). Taken 2ml buffer of phosphate solution and 1ml $H_2O_2$ 30% then taken 10 µl from serum and for blank 2ml from phosphate buffer solution with 1ml D. water for each sample, then measured absorbance at 240nm.

$$\textbf{Catalase U/ml Activity} = \frac{\Delta\ \boldsymbol{Absorbtion/\ \min} \times \boldsymbol{reaction\ valume}}{\mathbf{0.01}}$$

Whereas A1 for initial absorbance in 5 seconds and A2 for final absorbance in 2 min, reaction volume =2.4ml.

### 3.3.1.3.Glutathione Concentraition

Glutathione activity was determined according to the method of Beutler *et al.*, (1963) and Moron *et al.*, (1979). The acid soluble sulfhydryl groups form a yellow colored complex with dithionitrobenzene (DTNB).Taken 100µl of serum, was mixed with 0.7ml of 0.2M sodium phosphate buffer (pH 8) and 2ml of 0.6 mM DTNB ( prepared in 0.2M buffer , pH 8 ) . The yellow colored obtained was measured after 10 min at 412nm against a blank which contained 0.1 ml of 5%TCA in place of the supernatant. A standard graph was prepared using different concentrations (10-50 nmols) of GSH in 0.3 ml of 5% TCA as in figure

(3.2). The GSH content was calculated with the help of this standard graph and expressed as micromoles/ml for blood.

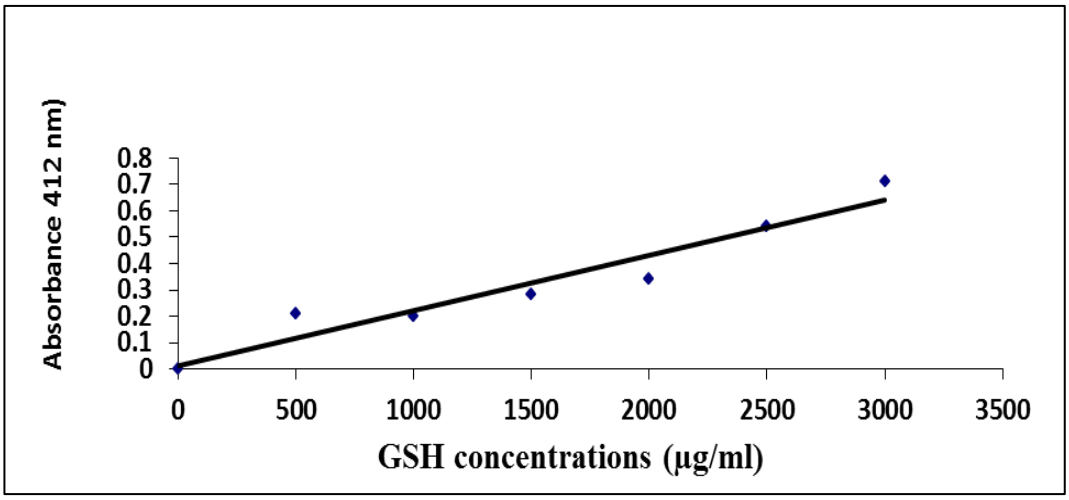



**Figure (3.2): standard curve of of GSH .**

### 3.3.1.4.Lipid peroxidation

Lipid peroxidation had been estimated by the Thiobarbituric acid assay for Malondialdehyde (MDA) concentration according to Burtis and Ashwood, (1999) .

The serum of lipid peroxidation was determined by (Ohkawa *et al* .,(1979), through take of 0.5 ml of serum and then added 2.5 ml of 0.02% trichloroacetic acid TCA and tube is left to stand for 10 min at room temperature. After centrifugation at 3500 rpm for 10 min, the precipitate was washed once with 0.05 M $H_2SO_4$. The precipitate was suspended in distilled water and estimated the TBARS by taken 100 µl of serum was mixed with 1000 µl (TCA 20%), and 1000 µl (TBA 0.6%). Tubes are mixed by vortex type (Cenco Instrument.Ltd. Netherlands). Then incubated in water bath (100°C) for 15 minutes at 4°C , and left for cooling to room temperature, centrifuged for 15 minutes at 4000 rpm ( 4°C ), and then absorption was read at the wavelength of 532 nm against blank ,the result was expressed as nmol/ml of serum.

**Concentration of Malondialdehyde (MDA)** = $\frac{Absorbtion\ at\ 532nm}{E \times b}$

Whereas E= Extinction coefficient (153mmol/cm), and b = light bath (1cm)

### 3.3.1.5. Hydrogen Peroxide concentration measurment by KI(Velikova *et al*.,2000)

1- The first step was prepared TCA (0.1%) by adding 0.1 gm of TCA in 100 ml of distilled water.

**2-** 0.5 ml of serum was added and 200 µl(0.2ml of TCA) was the prepared in the first step.

2-Centrifugal action for samples at 14000 rpm for 10 minutes.

4-Were taken 0.1 ml of the filtrate, then add 0.2 ml of phosphate (pH = 7) and finally add 0.5 ml of potassium iodide.

4-Measurement of concentration of $H_2O_2$ by a spectrometer at wavelength 390nm against reagent blank, Where iodide is converted to iodine ion(Velikova *et al*.,2000).

### 3.3.1.6.Total Antioxidant Capacity (TAC)

#### 3.3.1.6.1.Principle

Total antioxidant capacity was measured using the ferric reducing ability (FRA) of serum , in which ferric to ferrous ion reduction at low pH causes a blue ferrous-tripyridyltriazine (TPTZ) complex. FRA values were obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration . The reaction is non-specific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferrous (Fe III to Fe II ) ion formation. The change in absorbance is, therefore, directly related to the combined or total reducing power of the electron donating antioxidant present in the reaction mixture (Iris et al., 1996).

### 3.3.1.6.2.Reagents

**1-Acetate Buffer (0.3 M) pH 3.6**

A weight of 3.1 gm sodium acetate trihydrate was added to 16 ml of glacial acetic acid and the volume was completed to 1 liter with DW.

**2- 2,4,6-Tripyridyle-s-triazine (TPTZ) (10 Mm) in (40Mm) HCl**

A weight of 0.03123 gm of TPTZ was dissolved in 100 ml of (40mM) HCl, prepared by dilution of 0.334 ml of 37% concentrated HCl in 100 ml of DW.

**3- $FeCl_3.6H_2O$ (20 mM)**

A weight of 0.324 gm of $FeCl_3.6H_2O$ was dissolved in 100 ml of DW.

**4- Working FRA Reagent**

The working FRA reagent was prepared by mixing buffer,TPTZ solution and FeCl3.6H2O solution in the ratio of 10:1:1 at the time of use.

**5- Standard**

A weight of 0.0417 gm of ferrous sulfate $FeSO_4.7H_2O$ was dissolved in 100 ml of acetate buffer to get stock solution of 1500 **microM.** Calibrators were prepared by dilution with acetate buffer.

**Procedure**

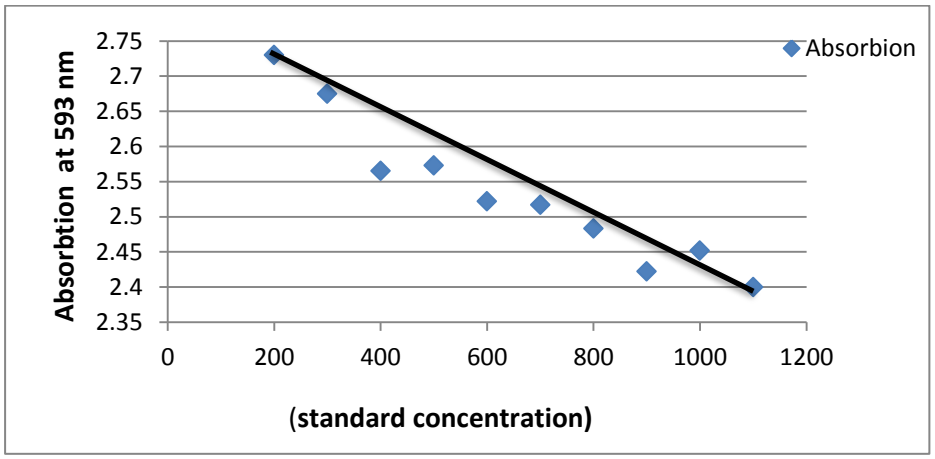
Reagent blank, standard and sample test tubes were prepared, and then pipetted into test tubes.

| Reagent | Sample | Standard | Reagent Blank |
|---|---|---|---|
| **Serum** | 100 | - | - |
| **Standard** | - | 100 | - |
| **Buffer** | 1000 | 1000 | 1100 |
| **FRA Reagent** | 1000 | 1000 | 1000 |
| **Total Volume** | 2100 | 2100 | 2100 |

Tubes were mixed in vortex mixture and incubated at 37c for 15 minutes. Absorption was read at 593 nm against reagent blank .

**3.3.1.6.1.**Calculation of TAC Using FRA (Ferric reducing ability)

TAC was obtained from the calibration curve in (Figure 3-1 ).




**Figuer(3-3):-Calibration curve of total antioxidant capacity using ferric reducing ability**

From the curve extract activity of TAC from the following equations:-

$$\textbf{Slope} = \frac{\sum \text{absorbance}}{\sum \text{concentration of standard}}$$

$$\text{Activity of Total antioxidant capacity enzyme U/ml(TAC )} = \frac{\text{Absorbance for sample}}{\text{Slope}}$$

### 3.3.1.7. Lipid Profile Kit

Depending on the method of work in the kit for the lipid profile(TC,TG,HDL-C), which belongs to the company biolabo, But the only difference in good cholesterol was the volume used 25 ml instead of 10 ,and mix let stand for 5 minutes at 37 c or 10 minutes at room temperature ,and record absorbance at 500 nm(480-520) against reagent blank ,colour is stable for 1 hour. The following table shows how it works**:-**

**Table (3.4) showed Lipid profile (TC ,TG, HDL-C,LDL,-C,VLDL-C) Kits**

| **Pipette in well identified test tube** | **Blank** | **Standard** | **Assay** |
|---|---|---|---|
| **Reagent** | 1ml | 1ml | 1ml |
| **Demineralized water** | 10ml | | |
| **Standard 100mg/dl** | | 10ml | |
| **Sample** | | | 10ml |

## 3.4.Molecular Analysis:

### 3.4.1.Extraction of DNA

Genomic DNA of frozen blood was extracted according to DNA extraction kit protocol ( Geneaid ) as the following:

**1-** A volume of 200 µl blood was added to 1.5 ml a sterile micro centrifuge tube.

If the sample volume was less than 200 µl then appropriate volume of PBS was added.

2. A volume of 20 µl Proteinase K (10 mg/ml) was added to the sample and mixed briefly. The mixture was incubated at 60 ˚C for 15 minutes .

3- A volume of 200µl of GSB Buffer was added to the sample and mixed by shaking vigorously.

4. The mixture was incubated at 60 ºC for 15 minutes to lyse the sample. During incubation, the sample was inverted every 2 minutes.

5- At this time the required volume of elution buffer was pre-heated (200 µl/sample ) to 60 ºC (for step "13" DNA Elution)

6- A volume of 200 µl of absolute ethanol (96- 100 %) was added to the

sample lysate and mixed by shaking vigorously for 10 seconds .

7- A GS Column was placed in a 2 ml collection tube. The mixture was transferred (including any precipitate) carefully to the GS column and centrifuged at 14-16,000 rpm for 1 min then the GS column was placed in a new collection tube.

8- About 400 µl of W1 Buffer was added to the GS Column and centrifuged at 14-16,000 rpm for 30 sec then the flow-through was discarded .

9- Wash Buffer of 600 µl was added to the GS Column and centrifuged at 14-16,000 rpm for 30 sec then the flow-through was discarded .

10. Additional centrifuge at 14000 x rpm for an 3 minutes was done to dry the column.

11- The GS Column was placed to 1.5 ml micro centrifuge tube .

12- About 100 µl of pre-heated elution buffer was added to the membrane center of GS Column and let stand GS Column for 3 minutes. For effective elution, the elution solution was dispensed onto the membrane center and is absorbed completely.

13- Centrifuge at 14-16,000 rpm for 1 minutes to elute total DNA.

14- The DNA was stored at -20 °C.

### 3.4.2.Estimation of DNA Concentration and Purity

The DNA concentrations of samples were estimated by using a spectrophotometer (Nano drop) as the following:

1- Aliquot of 1µl of TE solution was added on the lens for empty – apparatus carefully without touching the lens.

2- Aliquot of 1µl of DNA sample was added in to the nanodrope to detect concentration in µg/ml ,the good quality of DNA sample should have A260/A280 ratio about 1.8 to2 .

### 3.4.3.Preparing the Primers:

The Bioneer® primers in tables (3.6),(3.9) ,were prepared depending o n manufacturer instruction. To create a stock of primers. The lyophilized primer dissolved with nuclease free water was added to each primer to obtain master stock that will be used again to obtain a working solution.

The following steps are followed for reconstituting and diluting the primers:

**1**- The tube was spine down before opening the cap.

**2-** A desired amount of water was added according to the oligos manufacturer to obtain a 100pmoles/ µl ( stock solution)

**3-** The mixture was vortex properly for re-suspend the primers evenly.

**4-** Ten µl of the master stock was transferred to a 0.2 ml Eppendorf tube that contains 90 µl of sterile ,nuclease –free water to obtain 10 pico moles/ µl ( stock solution)

**5-** The stock solution was stored at -20˚C.

**6-** The working solution was stored at -20C˚.

### 3-4-4-PCR Reaction Mixtures:

The PCR reaction mixtures were brought together according manufacture procedure of the master mix (Promega) . All the appending was done in laminar flow cabinet on the frozen cooling blocks and ice when it was necessary. The components of PCR working solution were given in table:

**Table (3.5): The working solution components of PCR:**

| Component | Amount (µl) | Concentration |
|---|---|---|
| **Master Mix 2X** | 12.5 | **2X** |
| **DNA Template** | 3 | **30-60 ng/µl** |

| **Primers** | 2 | **10p moles** |
|---|---|---|
| **DNas free water** | Up to 25µl | **-** |
| **Total volume** | **25µl** | **-** |

### 3.4.5. PCR Amplification

#### 3.4.5.1 Glutathione –S-Trancferase pi (GSTP1) genotyping

For GSTP1 genotyping , a set of primers as in table (3-6)

**Table (3-6) a set of GSTP1 primers**

| | Sequence 5'⟶ 3' | Product size | Tm | References |
|---|---|---|---|---|
| *GSTP1* gene | F= TCCCCAGTGACTGTGTGTTG | 224 bp | 59.8C° | Gatedee et al., 2007 |
| | R=GAAGCCCCTTTCTTTGTTCA | | 56.1 C° | |

**Table (3.7): Amplification conditions of GSTP1 gene**

| Stage | | Temp.(C°) | Time(min) | Cycles |
|---|---|---|---|---|
| 1 | Initial Denaturation | 94 | 5 | 1 |
| 2 | DNA denaturation | 95 | 30 sec | 30 |
| | Primer Annealing | 60 | 30sec | |
| | Extension | 72 | 30sec | |
| 3 | Final extension | 72 | 7 | 1 |
| 4 | Hold | 4 | | |

The conditions of the PCR and annealing temperature was determined as reported in (Gatedee *et al*., 2007) Amplified DNA fragments were electrophoresis on 2 % agaros(1 X) TBE buffer and the bands visualized after staining with ethidium bromide under UV light , A

50 base- pair ladder was used as a size marker for estimating product sizes.

### 3.4.5.1.1. BsmA1 restriction enzyme (Promega) :

Recognition sequence : 5'…G T C T C (N)1…3'

3'…C A G A G (N)5…5'

**Product Source:-**

An *E. coli* strain that carries the cloned BsmAI gene from *Bacillus stearothermophilus* A664 (Z. Chen)

### 3.4.5.1.2. Setting up a Restriction Enzyme Digestion

An analytical-scale restriction enzyme digestion is usually performed in a volume of 20µl with 0.2–1.5µg of substrate DNA and a two- to tenfold excess of enzyme. If an unusually large volume of DNA or enzyme is used, aberrant results may occur. The following protocol is an example of a typical RE digestion.

1. In a sterile tube, assemble the following components as in table 3.8.

**Table (3.8) The Restriction Enzyme Digestion reaction GSTP1 protocol**

| Component | Volume |
|---|---|
| Sterile, deionized water | 16.3µl |
| Restriction Enzyme 10X Buffer | 2µl |
| Acetylated BSA, 10µg/µl | 0.2µl |

| | |
|---|---|
| DNA, 1µg/µl | 1µl |
| Mix by pipetting, then add: | |
| Restriction Enzyme, 10u/µl | 0.5µl |
| Final volume | 20µl |

2. Mix gently by pipetting, close the tube and centrifuge for a few seconds in a microcentrifuge. Incubate at the enzyme's optimum temperature for 1–4 hours.

3. Add loading buffer to a 1X final concentration and proceed to gel analysis.

Note :Overnight digestions are usually unnecessary and may result in DNA degradation and different incubation times hours have been tested

This mixture was placed in PCR tube then moved to water bath for incubation at 37$^{o}$Cfor 45 minutes.

### 3.4.5.2 Glutathione –S-Trancferase alpha 1(*GSTA1*) genotyping

For Glutathione –S-Trancferase alpha (*GSTA1*) genotyping , a set of primers as in table (3-9)

**Table (3-9) a set of GSTA1 primers**

| | Sequence 5'⟶3' | Product size | Tm | References |
|---|---|---|---|---|
| *GSTA1* gene | F= GCATCAGCT TGC CCT TCA | 400 bp | 57.9C° | Ping et al., 2006 |
| | R= AAACGC TGT CACCGT CCTG | | 60.5 C° | |

**Table (3.10) Amplification conditions of GSTA1 gene**

| Stage | | Temp.(C°) | Time(min) | Cycles |
|---|---|---|---|---|
| 1 | Initial Denaturation | 94 | 5 | 1 |
| 2 | DNA denaturation | 95 | 30 sec | 30 |
| | Primer Annealing | 60 | 30sec | |
| | Extension | 72 | 30sec | |
| 3 | Final extension | 72 | 7 | 1 |
| 4 | Hold | 4 | | |

Theconditions of the PCR and annealing temperature was determined as reported in (Ping et al., 2006).Amplified DNA fragments were electrophoresis on 1% agaroe TBE buffer and the bands visualized after staining with ethidium bromide under UV light , A 50 base- pair ladder were as a size marker for estimation of fragment sizes.

### 3.5. The steps of SSCP:

#### a. Device Setup For Casting Polyacrylamide Gel:

The two glass plates (on which the gel is intended to be cast) were situated properly; the plain glass plate is placed outermost, while a notched glass plate with bonded spacers is positioned innermost. To keep away from leakage, the glass plates were assembled so that the bottom of the glass plates and the spacers were aligned on a flat surface. Then, the pair of glass plates was inserted into the slab gel insert that contains several the required anti-leakage accessories, such as bars and silicone pads, which surrounds both glass plates.

#### b. Polyacrylamide Gel Preparation For SSCP:

The following recipe was followed sequentially when preparing 8% neutral polyacrylamide gel (PAG) containing 7% glycerol and 1× TBE. The gel was prepared and poured into the medium-casting tray (20 cm × 20 cm × 0.1mm.; H×W×T) (Orita *et al*., 1989): as shown in the table (3-11).

**Table (3-11): The components of SSCP gel**

| step | Solution | Concentration | volume |
|---|---|---|---|
| 1 | Acrylamide/bisacrylamide (37.5 / 1) | 8% | 8 ml |
| 2 | TBE | 10X | 4 ml |
| 3 | Glycerol | 100% | 2.8 ml |
| 4 | TEMED | 100% | 40µl |
| 5 | 10% Ammonium persulfate | 10% | 400µl |
| 6 | Free $dH_2O$ | 100% | 24.8 ml |
| Total volume | | | 40ml |

Once, the gel mixed, it was loaded between the glass plates using a commercially available 5 ml medical syringe. When the gel reaches the upper limits of the two glass plate, a required comb was inserted carefully to avoid bubbles. About 30 min was enough to solidify the gel.

**c. Amplicons samples preparation for SSCP:** For each 10µl amplified fragments, 10µl of 2X SSCP loading dye was added to a microfuge tube. The contents were quietly mixed and placed into a 95°C PCR for 7 min and then on ice for 5 min. Then, samples were loaded into 8% polyacrylamide SSCP gel. SSCP reagents including denaturation solution (total volume 10 ml) (Al-Shuhaib *et al*., 2018).

- 95% (V/V) formamide.
- 0.05 % (W/V) of xylene cyanol.
- 0.05 % (W/V) of bromophenol blue.

- 20 mM of EDTA

**d.Running in Polyacrylamide Gel electrophoresis:** Electrophoresis was done in vertical slab gel unit (Mini VS10DSYS) provided by Cleaver Scientific - the UK) and performed at room temperature using pre-cold (1XTBE) electrophoresis buffer that store in the refrigerator. The running conditions were set at 200V, 100mA for about 120 minutes or until SSCP tracking dye reached the bottom of the gel.

**e.Staining SSCP Products Using Ethidium**

After the electrophoresis was completed, the gel was taken and placed in a container containing 100 ml of TBE buffer(1x) and 10 µl of ethidium dye was added,and moving the container every ten minutes to mix stain with gel .

**3.5.1.Sequencing of PCR Products**

Each unique SSCP samples' sets (15 samples, were 1 to 10 patients and 11-15 control) of each of the different migration patterns that observed by the SSCP technique for the fragments amplified by each primer pair were submitted to purification according to the protocol suggested by Macrogen sequencing corporation (Macrogen – Korea),and determining the nucleotide sequence of DNA by sanger method .The PCR product was sequenced with one primer (forward) by the dideoxynucleotide chain termination reaction. The sequences of both gene fragments analyzed were aligned by multiple sequence alignment program according to Bio Edit, with the sequences published in the Gen Bank database taken as a reference to identify the polymorphisms.

# *Chapter four*

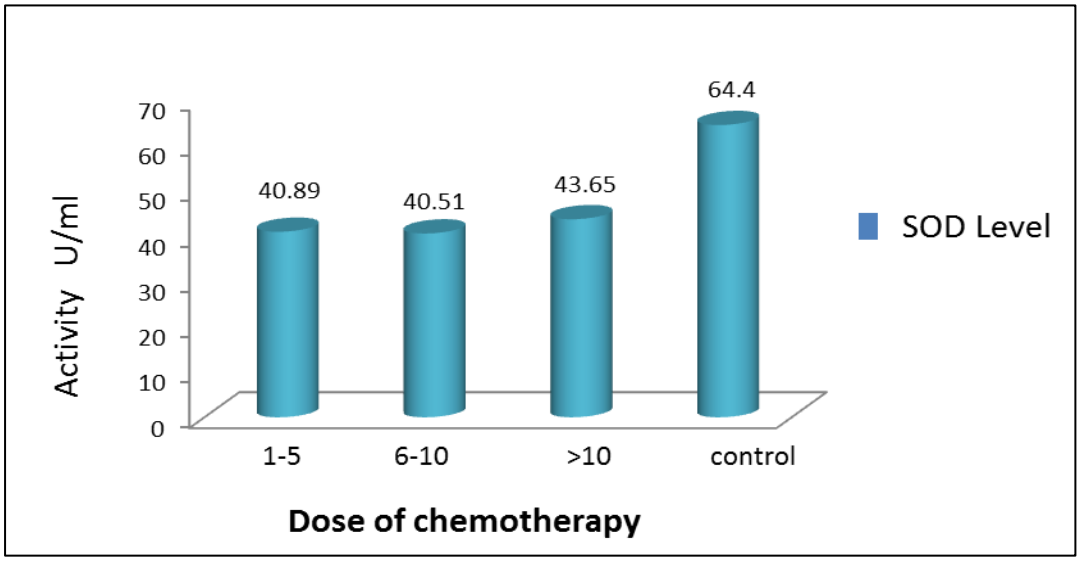
## Results and Discussions

# 4.Result and Discussion

## 4.1. Biochemical Markers

### 4.1.1 Superoxide Dismutase (SOD)

The results of the present study were showed differences between control and case with breast cancer in SOD activity , where these patients were divided into three groups according to the number of chemotherapy doses, the Superoxide Dismutase (SOD) activity in control were (64.40±23.76) U/ml while its activity in the case were significantly decreased to reach in the first group(1-5) doses (40.89±28.45)U/ml and in the second group(6-10) doses reach to (40.51±18.04)U/ml and in third group(>10) doses reach to (43.65±28.75)U/ml figure (4-1) .




**Figure(4-1) : superoxide dismutase (SOD) activity in serum of control and breast cancer patients**

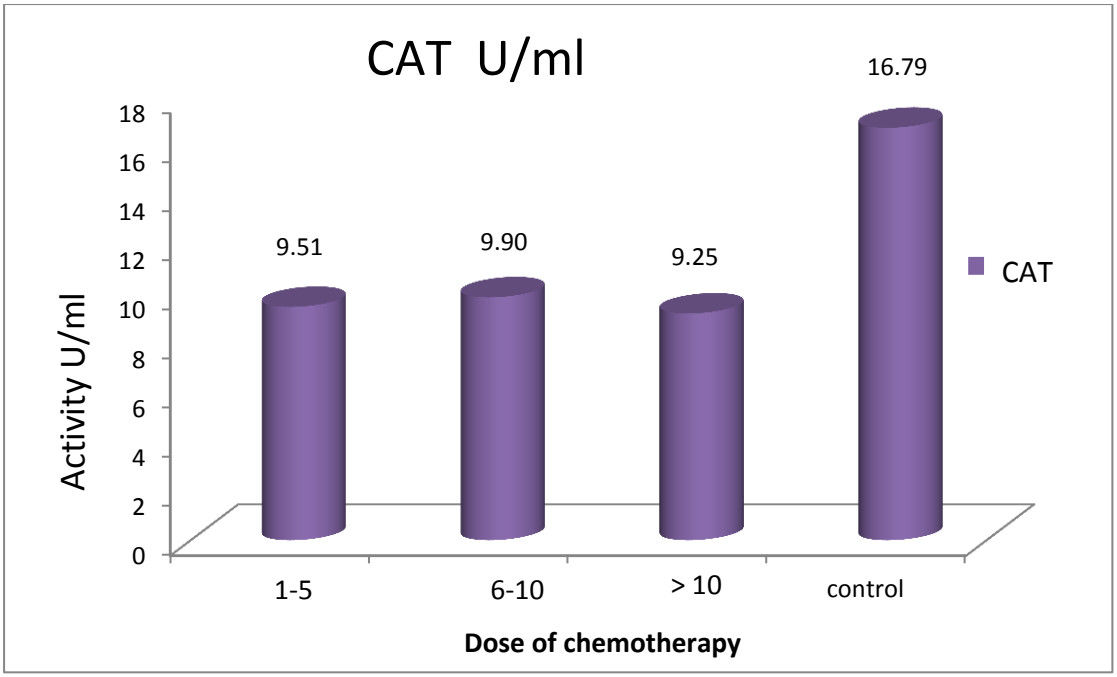
In other study SOD levels alteration might be due to direct effects of chemotherapy or indirect effects of tumor regression, The direct effect was that cisplatin–paclitaxel chemotherapy induced ROS either in the form of free radicals, reactive anions containing oxygen atoms or molecules containing oxygen atoms that could produce free radicals, or chemically activated by them. Those examples were hydroxyl radicals,

superoxide, hydrogen peroxide, and peroxide nitrite. The high ROS would directly affect SOD levels that become front-line defense against free radicals (Kaya *et al*.,2005).other suggested that there was another path than ROS pathway that caused apoptosis that was extrinsic pathways that caused apoptosis going on despite SOD levels increased, The extrinsic pathway involved the activation of tumor necrosing factor receptors (TNF), Such as Fas receptor activation by Fas ligand(Ghodsian *et al*.,2004). Superoxide anions are highly reactive and accumulation of these has also been reported in tumor cells (Devi *et al*, 2000). The over expression or the high levels of the SOD might be an adaptive response and it results in increased dismutation of the superoxide anions to hydrogen peroxide. In other study reported to Cisplatin is a platinum-containing chemotherapy agent that can react in vivo and cause cross-linking in the DNA chain that eventually leads to apoptosis (Malathi *et al*.,2011). Paclitaxel activates ROS which in turn also activates caspase cascade (Rubin *et al*.,2004) . DNA damage or malignant progression leads to a decrease in SOD levels because of continuous resistance to ROS (Salzman *et al*.,2006). The effects of necrosis and apoptosis of NPC cells after chemotherapy will lead to a decrease in proliferation with clinical manifestations in the form of reduced malignancy \ progress. The reduced malignant progression, SOD levels increases (*Kaya et al*.,2005). The study of ( Atukeren *et al*., in 2010) included 30 patients (before treatment and after first and second chemotherapy course) and 20 healthy cases. Their results showed the reduction of antioxidant enzyme activities, (Atukeren *et al*.,2010).( Kasapovic *et al*.2010) conducted chemotherapy with 5-fluorouracil, adriamycin and cytoxan (FAC) based on superoxide dismutase (SOD) levels in 58 cases of breast cancer and 60 healthy cases. Their studies showed that antioxidant enzyme activities decreased in response to the FAC chemotherapy. Overall, FAC

chemotherapy and radiotherapy increase the oxidative shift (Kasapovic *et al*.2010). They concluded that chemotherapy reduces CAT and SOD enzyme levels (EL-Bindarya *et al.*,2013). In our study, a decrease in the activity of the antioxidant enzyme SOD was observed, and this may be due to mutations in the gene that patients carry it, or it may be due to their taking chemotherapy as it may also cause mutations in the gene or this decrease in SOD activity may be due to the body not responding to chemotherapy This promotes an increase in the oxidative potential and thus an increase in free radicals that may attack the DNA,Where we showed in the third group (more than 10 doses) of patients a significant decrease in the level of SOD enzyme activity, which may be due to a decrease in the patient's immune system, Modulation of the immune response by antioxidants has long been proposed as a therapeutic target in cancer, but a direct relationship between them has not been established yet, Antioxidants play an important role in preserving cellular integrity and are critical in maintaining homeostasis of the host's immune system (Thyagarajan and sahu.,2018) **.**

### 4.1.2. Catalase

The results of the present study was showed differences between control and case with breast cancer in catalase activity , where these patients were divided into three groups according to the number of chemotherapy doses, the catalase (CAT) activity in control were (16.79±8.38) U/ml while its activity in the case were significantly decreased to reach in the first group(1-5) doses (9.51±6.59)U/ml and in the second group(6-10) doses reach to (9.90±4.66)U/ml and in third group(>10 ) doses reach to (9.25±4.62) U/ml figure (4-2).




**Figure(4-2) : Catalase (CAT) activity in serum of control and breast cancer patients**

In present study the results showed that in patients with breast cancer there was a significant decrease in CAT activities compared to the controls, This research is correspond with (Sahu, A. *et al*., 2015) ,a decrease in the activity of the antioxidant enzyme CAT was observed, it may be due to their taking chemotherapy as it may also cause mutations in the gene or this decrease in CAT activity may be due to the body not responding to chemotherapy This promotes an increase in the oxidative potential and thus an increase in free radicals that may attack the DNA,Where we showed in the third group (more than 10 doses) of patients a significant decrease in the level of CAT enzyme activity( Jayaraman et al.,2003).

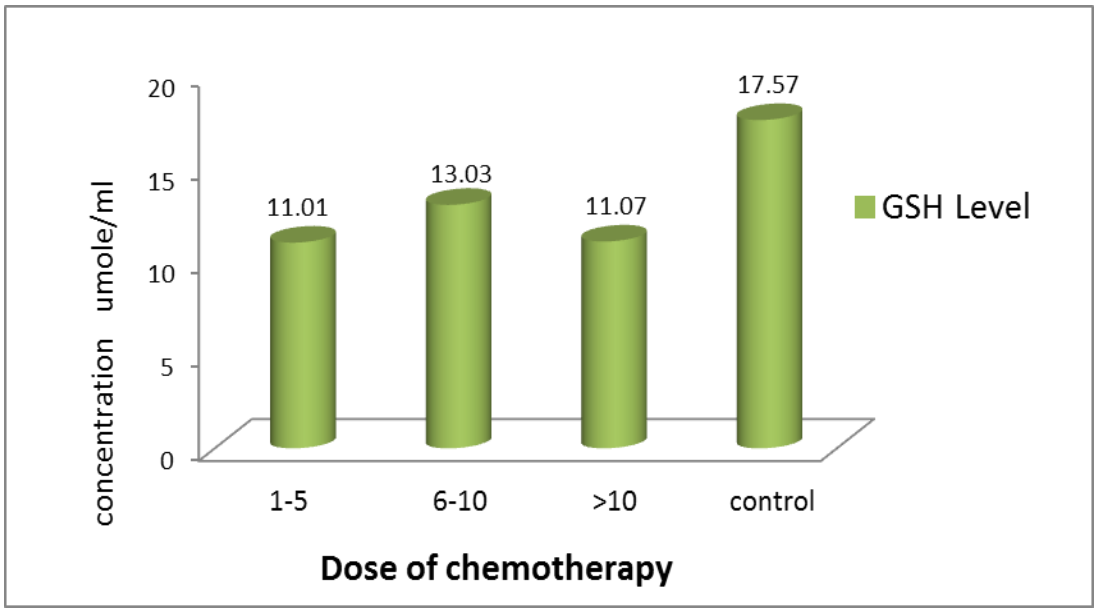
The decreased activity of CAT may be a compensatory regulation in response to increased oxidative stress,It has also been reported that the low levels of CAT are due to the inactivation of the enzyme by the superoxide anions (Keno Y, 1975). Other study reported a decreased

CAT activity in all stages of breast cancer and showed that lipid peroxidation in breast cancer tissue was enhanced compared to non-malignant tissue(Tas *et al*, 2005). Further they reported that higher oxygen free radical production and decreased catalase activity supporting oxidative stress hypothesis in breast carcinogenesis (Seth *et al*. , 2003). Other study CAT activity in category I and category II and category III patients was observed to be lower than the normal healthy women. Our results are also similar to the findings of( Punnonen *et al*, 1994) and these low levels might be due to treatment by anticancer drugs which reduces antioxidants and induces oxidative stress which increases with disease progression (Carmia Borek, 2004).

The decreased levels of CAT results in accumulation of large amounts of hydrogen peroxide resulting in higher production of OH• radicals resulting in oxidative stress in breast cancer,The increased activities of antioxidants enzymes may be a compensatory regulation in response to this increased oxidative stress( Carmia Borek, 2004) . Therefore, exogenous administration of antioxidants may be helpful in the management of breast cancer, so, the treatment with antioxidants in the initial stages of the disease may be useful as secondary therapy to prevent the oxidative damage. Different catalase mutations in patients can cause decreased catalase activity leading to increased H2O2 concentrations in the blood and tissues . Depending on the mutations, such patients may be subject to an increased risk of type 2 diabetes, vitiligo and increased blood pressure (Goth et al., 2004). And other study conducted a research in malignant and benign breast cancer patients, A significant decrease was also observed in CAT in patients as compared with the control group ( Ragab *et al*.,2014 ).

### 4.1.3.Glutathione concentration

The results of the present study was showed differences between control and case with breast cancer in GSH activity , where these patients were divided into three groups according to the number of chemotherapy doses, the Glutathione (GSH) concentration in control were (17.57±9.09) umol/ml while its activity in the case were significantly decreased to reach in the first group(1-5) doses (11.01±28.45)umol/ml and in the second group(6-10) doses reach to (13.03±8.29) umol/ml and in third group(>10) doses reach to (11.07±5.55) umol/ml figure (4-3).




**Figure(4-3) : Glutathione concentration (GSH) in serum of control and breast cancer patients**

In other study observed increase in GSH levels, is due not only to the higher levels of ROS production in most tumor cells, but it is again related to the fact that some of the classical tumor promoters also activate GSH synthesis and turnover mechanisms (e.g., NRF2) (Rahman et al.,2004). Chemoresistance is a multifactorial phenomenon and many studies show that a coordinated expression of efflux transporter proteins and phase II conjugating enzymes in tumor cells is linked to the

development of the multidrug resistance phenotype. In particular, the overexpression of GSH transferase and efflux pumps in tumors may reduce the reactivity of various anticancer drugs (Estrela et al.,2006) .

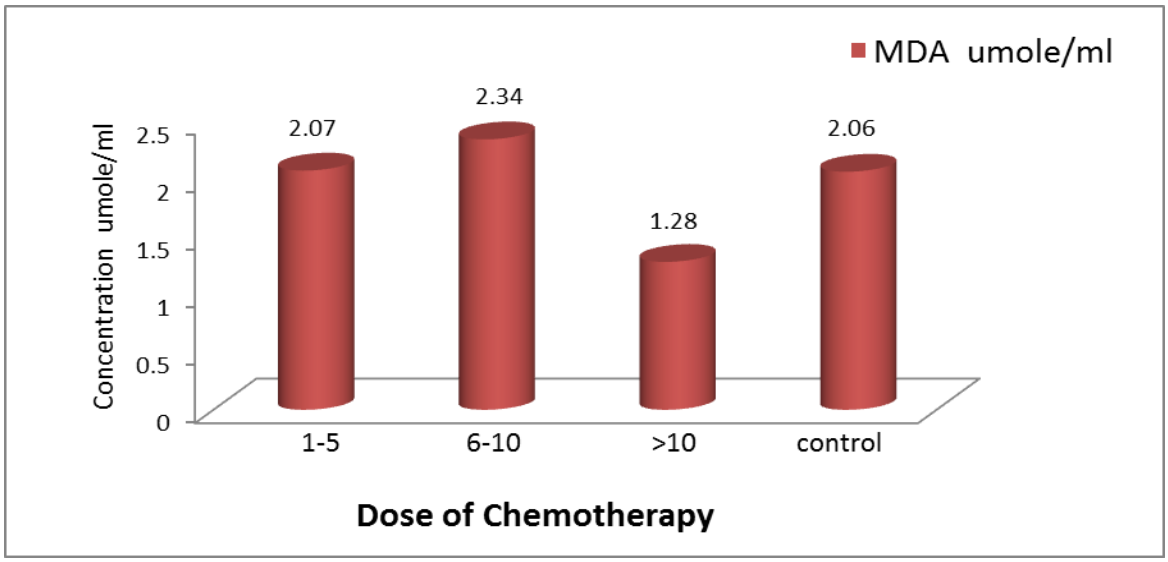
On the other hand, high levels of GSH improve antioxidant capacity and resistance to oxidative stress, and this is observed in many types of cancer( Hayes *et al*.,2005). And combined with the delivery of ROS generating endogenous radiation, to exploit the specific GSH levels in normal cells and cancer cells (Nogueira and Hay.,2013).Many studies using primary cancer tissue, however, have shown increased levels of ROS scavenging enzymes and antioxidant compounds (Oltra *et al*.,2001).This increase can result from an adaptive response to the intrinsic ROS stress.

The relationship between GSH depletion, chemotherapy, and/or the radiation response has been examined in many tumor cells after treatment with different drugs, including BSO, diethylmaleate, 2-oxothiazolidine-4-carboxylate, and different radiosensitizing agents (Estrela *et al*.,2006, Gatti and Zunino .,2005) However BSO (as well as other thiol-depleting agents) is non-specific and, besides promoting tumor GSH depletion *in vivo*, can cause irreversible damage in most normal tissues. Moreover, GSH depletion only appears to be therapeutically effective when very low levels of this tripeptide can be achieved within the cancer cells (Estrela et al.,2006). Thus, achievement of selective tumor GSH depletion under *in vivo* conditions appears as a superb pharmacological challenge.

In the present study showed that there is a decrease in the level of antioxidant GSH enzyme activity ,and this is evidence of disease progression , especially the third group of patients, the fact that GSH depletion can be deleterious for cancer cells and, potentially, enhance the effectiveness of chemotherapy and/or ionizing radiations, is known (Balendiran *et al*.,2004, Estrela *et al*.,2006) .

### 4.1.4.Malondialdehyde Concentration

The results of the present study was showed differences between control and case with breast cancer in MDA activity , where these patients were divided into three groups according to the number of chemotherapy doses, the malondialdehyde (MDA) activity in control were (2.06±1.39) umol/ml while its activity in the case were significantly increased to reach in the first group(1-5) doses (2.07±0.88) umol/ml and in the second group(6-10) doses reach to (2.34±1.65) umol/ml and decreased in third group(>10) doses reach to (1.61±0.18) umol/ml figure (4-4)




**Figure(4-4) : Malondialdehyde(MDA) concentration in serum of control and breast cancer patients**

Increased ROS levels in cancer cells are often regarded as adverse factors that cause genetic instability. In cancer cells there is an abnormal increase in ROS with high oxidative stress which makes the cancer cells to be more susceptible to further oxidative stress (Wang *et al*.,2017), therefore there was significant difference in MDA levels between breast cancer patients and healthy controls. Increased MDA levels in breast cancer sufferers might be due to induction of breast cancer cells to

increase ROS that can induce oxidative stress followed by molecular damage and including lipid peroxidation (Bhattacharjee *et al*.,2018).

ROS level elevation, redox balance alteration, and redox signaling deregulation are common hallmarks of cancer progression and treatment resistance. ROS generation is mainly contributed by mitochondria during oxidative phosphorylation. Elevated ROS levels detected in cancer cells might due to several aspects, such as high metabolic activity, cellular signaling, peroxisomal activity, mitochondrial dysfunction, oncogene activation, and increased enzymatic activity of oxidases, cyclooxygenases, lipoxygenases, and thymidine phosphorylases. Intracellular homeostasis is maintained by developing an immense antioxidant system including catalase, superoxide dismutase, and glutathione peroxidase. Besides these enzymes, important antioxidant glutathione and transcription factor Nrf2 also contribute to balancing oxidative stress. ROS-mediated signaling pathways activate pro-oncogenic signaling which promotes cancer progression, angiogenesis, and survival. Additionaly, to maintain ROS homeostasis and evade cancer cell death, cancer cells increase antioxidant capacity level.( Kumari *et al*.,2018) One of the most produced lipid peroxidation aldehydes is MDA. It can react with proteins and DNA causing gene mutations that will trigger the formation of cancer cells besides increasing MDA levels as a marker of cancer cell development(Hauck *et al*.,2016) Increased MDA in breast cancer patients is associated with excessive ROS production and deficiency of antioxidant defenses. Excessive ROS production is triggered by exposure to chemical, biological and physical carcinogenic substances. A significant increase in MDA in cancer along with a decrease in antioxidants indicates the higher levels of oxidative stress and lower levels of antioxidant defenses. This event plays an important role in tumor development and the pathogenesis that results

from gene mutations caused by increased levels of MDA(Bhattacharjee *et al*.,2018) .

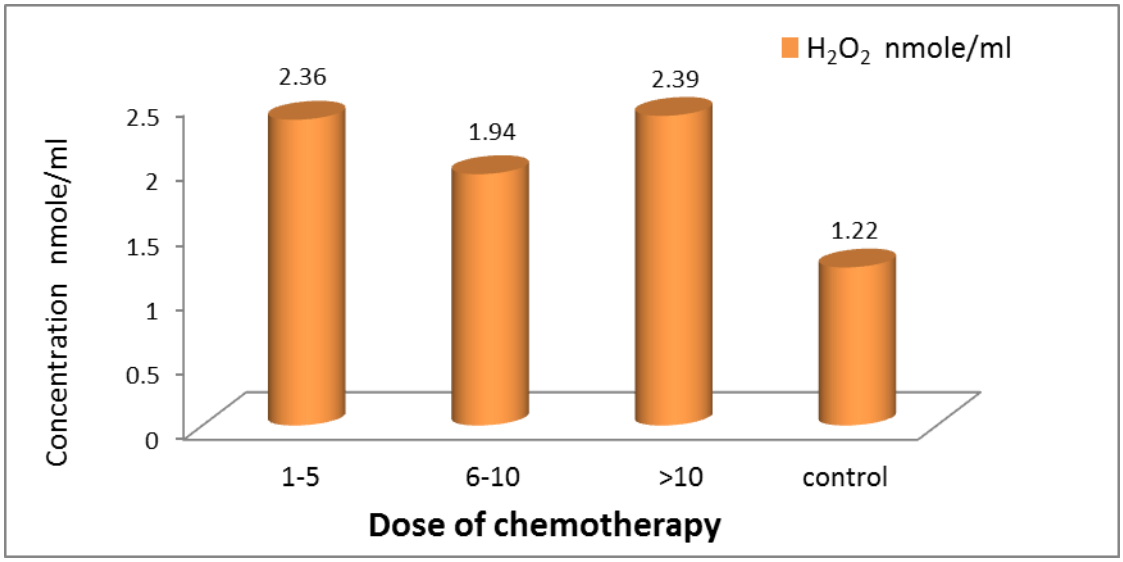
A similar research showed the increase in MDA levels in breast cancer patients with an average of 5.8 + 3.2 nmol/ml and a control group of 1.9 + 0.28 nmol/ml with p-value = 0.01 ($p < 0.05$) so that there were statistically significant differences between the breast cancer group and the control group. MDA is a product of lipid peroxidation caused by an increase in ROS in the body, which can lead to the development of breast cancer cells ( Sahu *et al*.,2013).

In the present study, we observed an decrease in MDA levels, which is evidence of high lipid peroxidation and a reduction in the action of the peroxidase enzyme , which works to break down free radical $H_2O_2$ into water and oxygen for respiration , and this rise may lead to mutations as MDA is one of the natural products of lipid peroxidation and this compound has the ability to bind with proteins and DNA and causes mutations ,but in third group showed MDA was decreased may be reason was immune system of pateints, Malondialdehyde is a natural product from lipid peroxidation capable of DNA interaction to form different adducts, including Malondialdehyde-1- deoxyguanosine (M1dG), Malondialdehyde-1-deoxyguanosine is mutagenic and triggers carcinogenesis ( Ma *et al*.,2010).

### 4.1.5. Hydrogen Peroxide ($H_2O_2$) Concentration

The results of the present study was showed differences between control and case with breast cancer in H2O2 concentration, where these patients were divided into three groups according to the number of chemotherapy doses, the hydrogen peroxide (H2O2 ) concentration in control were (1.22±1.18) nmol/ml while its activity in the case were significantly

increased to reach in the first group(1-5) doses (2.36±1.27) nmol/ml and in the second group(6-10) doses reach to (1.94±0.79) nmol/ml and in third group(>10) doses reach to (2.39±0.89) nmol/ml figure (4-5).




**Figure(4-5) : Hydrogen Peroxide ($H_2O_2$) Concentration in serum of control and breast cancer patients**

In the present study, showed increase in the level of hydrogen peroxide ,especially in the third group ,and this indicates that there is a decrease in the level of antioxidant activity ,especially catalase enzyme ,as it is responsible for analyzing hydrogen peroxide into water and oxygen and ridding the body from free radicals( Carmia Borek, 2004).. Here ,a rise also may be due to the effect of chemotherapy in patients ,as it lead to damage to DNA ,also caused a decrease in the level of catalase activity. Oxygen free radicals which were generated through several enzymatic and non enzymatic biological reactions in aerobic organisms have the ability to attack a wide variety of macromolecules such as lipid, protein, carbohydrate and DNA (Rao *et al.*,1996).(Batra *et al.* 2004), demonstrates that there were increases of reactive oxygen metabolites (ROMs) production in various path physiological conditions (Tatiane *et al.*,2009). In addition hypothesized that mutagenecity of oxygen led to

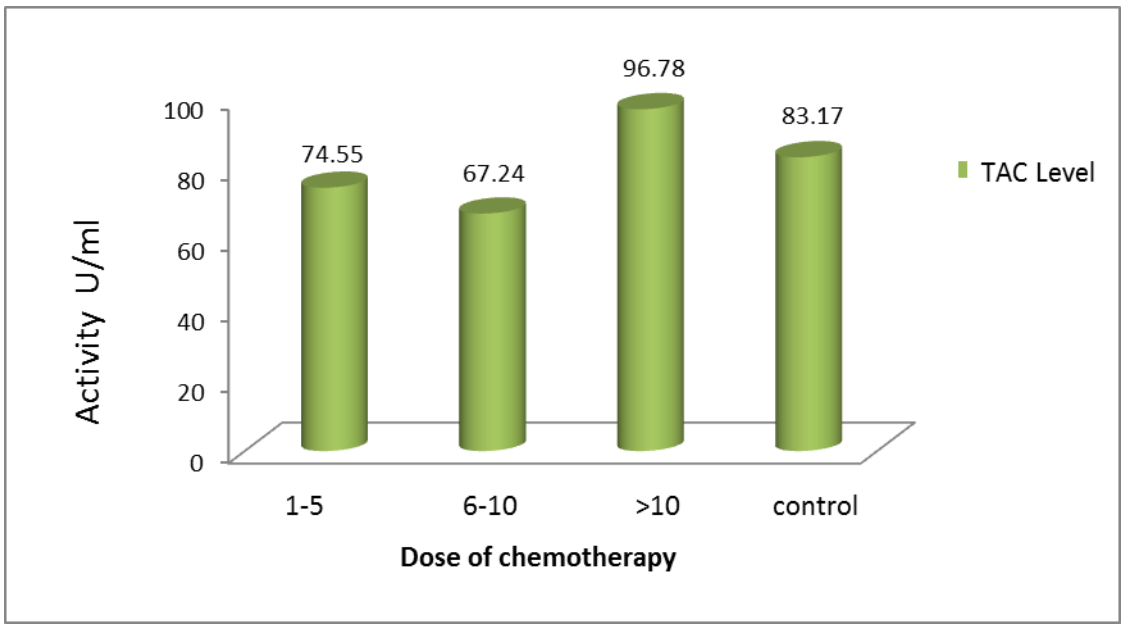
chromosomal damage resulting from an increase in the free radical production ( Negahdar *et al.*, 2005)**.**

Oxygen-free radicals generated by a number of processes in vivo are highly reactive and toxic( Marnett *et al*.,2000). Excess generation of oxygen free radicals can cause oxidative damage to biomolecules resulting in lipid peroxidation, mutogenesis and carcinogenesis. Oxygen free radicals induced lipid peroxidation has been implicated in neoplastic transformation ( Hristozov *et al*.,2001). Reactive oxygen species are involved in initiation, promotion and progression of carcinogenesis, where inactivation or loss of certain tumor suppressor genes is occurred.( Haris *et al*.,1989). The role of reactive oxygen species in breast carcinoma may not be limited to early mutagenic events, however, carcinoma cells are frequently under persistent oxidative stress. Human tumor cell lines in vitro produces reactive oxygen species at a far greater rate than do non transformed cell lines15 and markers of constitutive oxidative stress have been detected in samples from in vivo breast carcinoma( Rajneesh *et al*.,2008).Oxidative stress can induce apoptosis by causing damage to cellular components (e.g, DNA), and some studies suggest that ROS are downstream mediators of apoptosis ,However, there is considerable evidence that apoptosis does not require ROS and that their generation is a late event after cells are already committed to programmed cell death( Johnson *et al* .,1996).

### 4.1.6.Total Antioxidant Capacity

The results of the present study was showed differences between control and case with breast cancer in TAC activity , where these patients were divided into three groups according to the number of chemotherapy doses, the total antioxidant capacity **(TAC)** activity in control were (83.17±50.77) U/ml while its activity in the case were significantly

increased to reach in the first group (74.55±42.83)U/ml and in the second group reach to (67.24.69±22.73)U/ml and in third group reach to (96.78±61.01)U/ml figure (4-6).




**Figure(4-6) : Total Antioxidant Enzyme Capacity (TAC) activity in serum of control and breast cancer patients**

Other studies concerning antioxidant status in women with breast cancer showed a higher levels of antioxidant substances,( Portakal *et al*.,2000 ) showed that the activities of mitochondrial and total superoxide dismutase, glutathione peroxidase and catalase in tumor tissues significantly increased compared to the controls.( Rajneesh *et al*.,2008 ) showed a significant elevation in both enzymic and non-enzymic antioxidants (superoxide dismutase, catalase, reduced glutathione, glutathione peroxidase and glutathione S-transferase) in serum samples of 40 breast cancer patients. In a recent study made by( Sharhan *et al*.,2008) revealed that poor antioxidant status and high oxidative stress are associated with breast cancer risk as evident antioxidants as compared with the controls.

Another recent study reported by ( Kasapovic *et al.*,2008) showed that women with breast cancer have low plasma levels of the antioxidant substances including: superoxide dismutase, catalase and glutathione reductase enzymes were measured in blood cells of breast cancer patients. (Yuvaraj *et al*.,2008) showed that the various circulating enzymatic and non enzymatic antioxidants were low in a group of women with breast cancer.

The results of the above studies are in consistence with the results obtained from our study which indicate a low level of antioxidant status in the women with breast cancer,observed increased in TAC activity may be due to the types of non-enzymatic antioxidants( vitamin C, vitamin E, plant polyphenol, carotenoids, and glutathione, etc)( Maria et al.,2013).

### 4.1.7.Lipid profiles

Serum levels of various lipid parameters i.e. triglycerides (TG); total cholesterol (TC) and high density lipoprotein (HDL) were measures in a 70 cases of breast cancer along with 30 control women. The mean levels of serum triglycerides(TG), total cholesterol(TC) and high density lipoprotein(HDL) and low density lipoprotein (LDL) and very low density lipoprotein(V-LDL) were found to be significantly different in breast cancer cases as compared to controls, In the present study, it was observed that cases had decrease serum total cholesterol, LDL-cholesterol ,triglycerides and and HDL-cholesterol,and V-LDL as compared to controls showed in table(4-1).

**Table (4-1); Lipid Profile (Total clolesterol, Triglycerides, HDL-Cholesterol, LDL-Cholesterol,VLDL-Cholesterol in breast cancer patient**

| Lipid Profile | | Total Cholesterol | Triglycerides | HDL-Cholesterol | LDL-Cholesterol | VLDL-Cholesterol |
|---|---|---|---|---|---|---|
| Patient | Mean | 260.21 | 1045.93 | 423.99 | 412.62 | 209.19 |
| | St.D | 153.72 | 851.62 | 109.54 | 237.24 | 170.32 |
| Control | Mean | 507.86 | 1065.12 | 1083.96 | 789.12 | 213.03 |
| | St.D | 178.62 | 588.85 | 326.02 | 414.32 | 117.77 |
| Sig | | 0.000 | 0.897 | 0.000 | 0.000 | 0.897 |

It has previously been hypothesized that cholesterol plays role in carcinogenesis (Cruz *et al*.,2013).Mammary tissue metabolizes lipids from plasma under the influence of gonadal hormones. Malignant proliferation has been shown to be associated with aberrations in plasma lipids and lipoproteins. The elevated serum LDL-cholesterol, as noticed has been shown to be more susceptible to oxidation, and may result in high lipid peroxidation in breast cancer patients. This may cause oxidative stress leading to cellular and molecular damage, thereby resulting in cell proliferation and malignant conversions, which may be true with breast tissue. The proinflammatory microenvironment induced by high cholesterol levels, as seen in atherosclerosis, in which LDL-C is the most important causative factor (,Ross *et al*.,1999, Buchwald *et al*.,1992) can also play an effect on breast cancer initiation and progression. The use of statins before cancer diagnosis reduces cancer related mortality . (Owiredu *et al*., 2009)reduction of LDL-C is supposed to be the main mechanism through which statins exert effect (Llaverias *et al*.,2011, Mishra *et al*.,2004).In other study, values of T-C, LDL-C, and T-C/ HDL-C ratio were significantly increased in all the four stages of breast cancer (*P* value <0.05), while the values of HDL-C were not significantly changed. These findings are in agreement with( Florenza *et*

*al.*,2000) and (Michael *et al.*, 2009),and agreement with (Patricia *et al.* 2007),who found that HDL-C levels were significantly decreased, and also disagrees with(Kiran *et al.* 2005).

This decline in total cholesterol level among women with breast cancer was due to a combination of fat fill and this because of lack of blood cholesterol due to increased use by the tumor cells to configure the dynamic membrane , The possible mechanisms for these alterations in lipid profile in breast carcinoma may be due to interplay of these lipid metabolisms by cells and influence of female sex steroid hormones on breast tissue physiology. Several methodological aspects may explain the diverse conclusions, but the influence of cholesterol in BC risk remains to be clinical demonstrated.

## 4.2. Genetic polymorphisms of some Antioxidant genes associated with breast cancer patients

The genomic DNA (Fig.4- 7) was extracted from the blood samples as a first step to amplify the target region of some Antioxidant genes (GSTP1 and GSTA1 ).

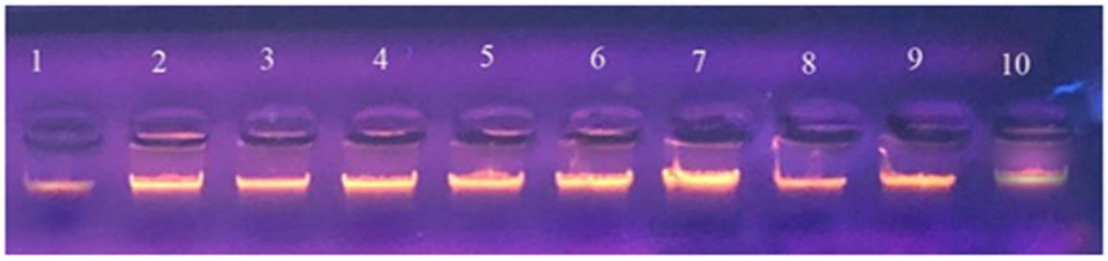



**Figure (4- 7): The electrophoresis pattern of gnomic DNA extracted from blood samples of breast cancer patients and healthy control groups.**

lanes 1 - 5 breast cancer patients and lanes 6 - 10 healthy control groups refer to genomic DNA from blood samples; Electrophoresis conditions, 1% agarose, 75 V, 20 mA for 1h , stained with ethidium bromide.

### 4.2.1 Genotyping of *GSTP1* (rs1695) Gene Polymorphisms

For GSTP1 (rs1695) genotyping, the genomic DNA was amplified using specific primers and accomplished by the Thermo-cycler apparatus

under the optimal conditions The results revealed that the presence a one bands (224bp) of the target sequence of *GSTP1* (rs1695) gene in agarose gel (Fig. 4-8).

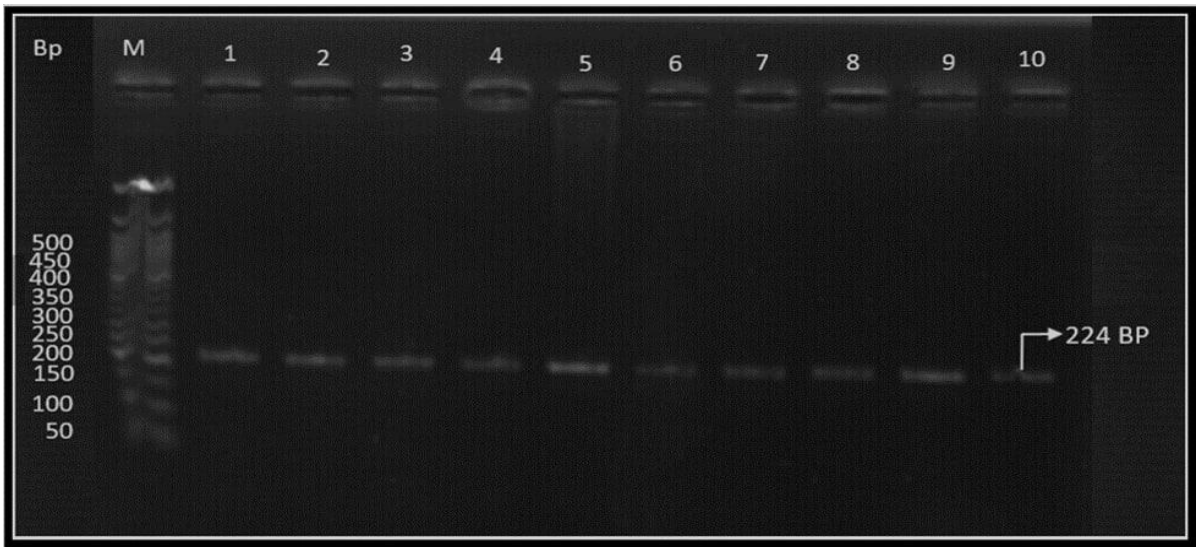



**Figure (4-8) Agarose gel electrophoresis of an amplified product patterns of Glutathion S-Transferase Pi 1 (*GSTP1*) with specific primer**.

M: refers to DNA size marker(50bp); lanes 1 - 7 refer to PCR products of ***GSTP1*** (224bp) of breast cancer patients and lanes 8 - 10 healthy control groups. Electrophoresis conditions: 1% agarose concentration 1%; 75 V, 20 mA for 120 min. Staining method; ethidium bromide.

After that, the PCR products of the *GSTP1* (rs1695) target sequences were digested with *BsmAI* (5' GTCTCN$^{\downarrow}$… 3') restriction enzyme to detect the rs1695 SNP in *GSTP1* gene (Fig. 4- 9). The genotypes of the studied subjected has been distributed into three groups based on the presence or absence of the Polymorphisms : A/A homozygous 224 bp , A/G heterozygote demonstrated 224 bp,146 bp,78 bp and G/G homozygous 146 & 78 bp figure(4-9).

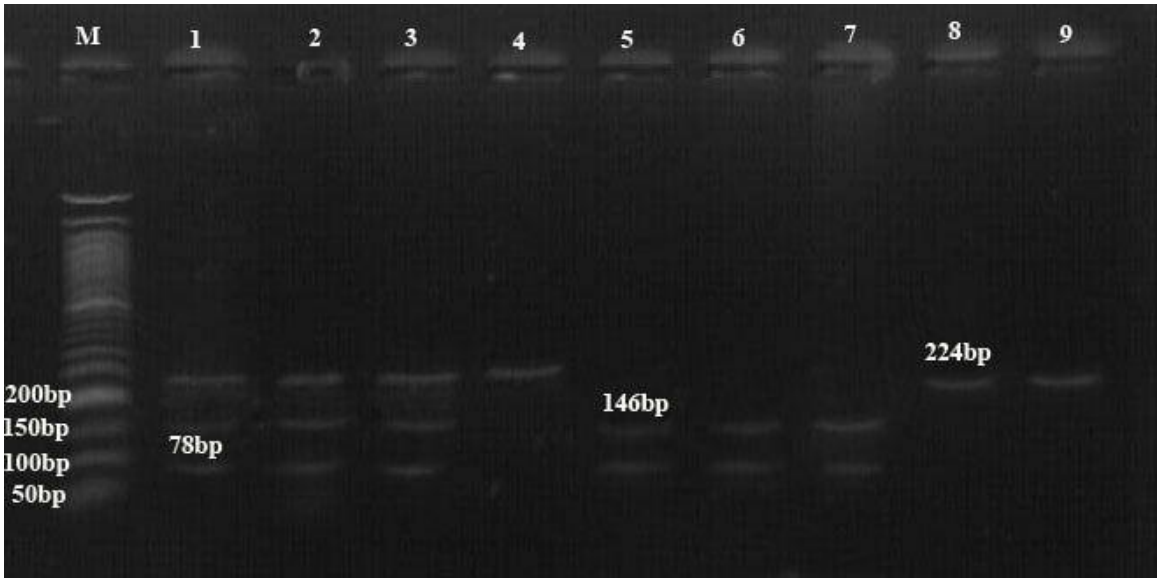



**Fig. (4-9): Electrophoresis patterns of allelotyping of GSTP1 (rs1695) gene of breast cancer patients and healthy control groups using *BsmAI* enzyme by PCR-RFLP method**

M: DNA ladder (50 bp); Lanes 1,2&3 refer to heterozygous allele (GA) had 3 bands with 224, 146 & 78 bp; Lanes 5, 6,7 refer to homozygous allele (GG) had a two band with 78,146 bp molecular size ;Lanes 4, 8&9 refer to a a homozygous allele (AA) had a single band with 224 bp molecular size. Electrophoresis conditions, 1% agarose, 75 V, 20 mA for 1h , stained with ethidium bromide**.**

### 4.2.1.1 The Genotypes Distribution of rs1695 Polymorphisms with Allele Frequency in Control and Case Groups.

the *GSTP1* (rs1695) gene polymorphism was studied in breast cancer cases and control . The distribution observed in *GSTP1* (rs1695) gene polymorphism in cases group and control group are showed in Table ( **4-2**). The highest genotype in control group was wilde homozygote AA (80%) followed by GA heterozygote genotype (16.7%) and homozygote genotype GG(3.3%)and wilde homozygote AA (80%). In breast cancer disease , the highest genotype was wilde homozygote AA (82.8%)followed by GA heterozygote genotype (8.6%)and GG homozygote genotype (8.6%).

**Table (4-2): Genotype distribution and odd ratio of *rs1695* polymorphisms between the patients vs healthy control**

| Genotype rs1695 | Patients No.(%) | Control No.(%) | Significance level | O.R | CI (95%) |
|---|---|---|---|---|---|
| AA[a] | 58 (82.8%) | 24 (80%) | | | |
| AG | 6(8.6%) | 5 (16.7%) | 0.2832 | 0.4966 | 0.1382 to 1.7835 |
| GG | 6(8.6%) | 1 (3.3%) | 0.4114 | 2.4828 | 0.2835 to 21.7410 |
| Total No. | 70 | 30 | - | - | - |
| Allele | Frequency | Frequency | - | - | - |
| A | 0.87 | 0. 88 | 0.233 | 1.1171 | 0.4404 to 2.8333 |
| G | 0.13 | 0.12 | - | - | - |

P ≤ 0.05 ; OR=(95%CI); [a] reference

In other study, in other study evaluated the polymorphic deletion of GSTP1 (rs1695), SNP in breast cancer patients treated with single-agent doxorubicin versus single-agent docetaxel as well as the expression of these genes in breast cancer tissue(Romero et al.,2012).

Some studies have linked GSTP1 polymorphisms and toxicity to taxanes (Tran et al.,2006, Mir et al.,2009) .(Marsh et al.,2007) did not find any significant association between GSTP1 genotypes and outcome or toxicity in ovarian cancer patients treated with platinum plus taxane chemotherapy. GSTP1 is particularly active in catalyzing the reactions with propenal derivatives, whereas GSTA1 are more active with 4-

hydroxyalkenals (products of lipid peroxidation) than with base propenals (Berhane et al.,1994). Some studies (Gilbert *et al*., 1993 and Colovai *et al*., 1992) also found that an increased level of GSTP activity was inversely related to hormone progesterone receptor status.

Several studies of GSTP1 expression in breast tumors have been conducted (Arun et al.,2005, Keith et al.,1990). However, the results are not conclusive and the contribution of GSTP1 to the inactivation of chemotherapy drugs and their metabolites in breast cancer tissue remains unknown as well as how much this inactivation may account in survival and treatment outcome since GSTP1 is expressed in many other tissues as liver and red blood cells. The GSTs are expressed in a tissue-specific manner (Tu et al.,1983) . GSTP1 is the major GSTs expressed in breast tissue (Forrester et al.,1990).

Some studies were investigating the association of the GSTP1 polymorphisms with breast cancer risk. For example, Samson et al., (2007) reported a non-significant elevation in the risk of breast cancer was observed among women who had the GSTP1 Val/Val genotype (Samson et al., 2007). While, Ge et al., (2013) reported a positive association between GSTP1-Ile105Val polymorphism and breast cancer risk (Ge et al., 2013). The dissimilar results from different studies may be due to environmental, geographic, race, and other factors .

Also, GSTP1 is a gene that is related to DNA repair, and keeps DNA from an impairment, controls detoxification and metabolism, so preventing tumor incidence. The GSTP1 gene methylation often shows tumors progression, including breast cancer or unfavorable prognosis (Schnekenburger et al., 2014). Consistent with the role of methylation of GSTP1 in the tumor's progression, a study showed significantly increased gene methylation of GSTP1 in breast cancer cells, which was positively associated with tumor size and TNM stage, and negatively associated

with the expression of ER/PR (Schnekenburger et al., 2014). This evidence could elucidate the main role of disrupted GSTP1 in breast susceptibility. This could explain the role of key single nucleotide polymorphisms in the pathogenesis of GSTP1. Genetic variations based on their positions in a gene could alter the gene function (Salimi et al., 2017; Nejati et al., 2018). The SNPs on the promoter of a gene may alter the gene expression however the SNPs in the intron regions could alter the production of mature mRNA by interfering with the splicing process (Mobasseri et al., 2019; Zamani-Badi et al., 2019). But, the missense mutations could alter the structure and function of proteins (Noureddini et al., 2018; Bafrani et al., 2019) ,what may be true for GSTP1-Ile105Val genetic variation. Evaluation of the impacts of genetic variations by biological experiments is a very difficult process and evaluation of these impacts could be much easier with the in silico tools (Tameh et al., 2018; Zamani-Badi et al., 2019). In other study, also employed the SNP effects bioinformatics tool to evaluate the molecular effects of Ile105Val SNP on the GSTP1 gene and found that this polymorphism decreases the amyloid propensity of GSTP1 protein and therefore, the pathogenic effect of Ile105Val may arise from this issue.

Our findings showed no association between *GSTP1* gene with susceptibility to breast cancer women ($P \geq 0.05$).

### 4.2.2. Genotyping of *GSTA1* (rs3957357) Gene Polymorphisms

For GSTA1 (rs3957357) genotyping, the genomic DNA was amplified using specific primers and accomplished by the Thermo-cycler apparatus under the optimal conditions The results revealed that the presence a one bands (400bp) of the target sequence of GSTA1

(rs3957357) gene in agarose gel (Fig. 4-10).

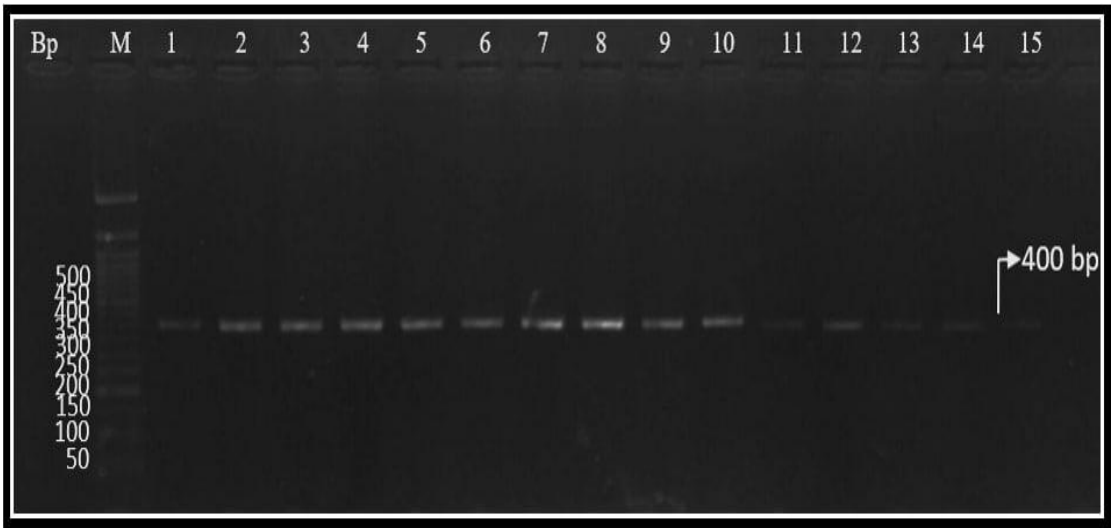
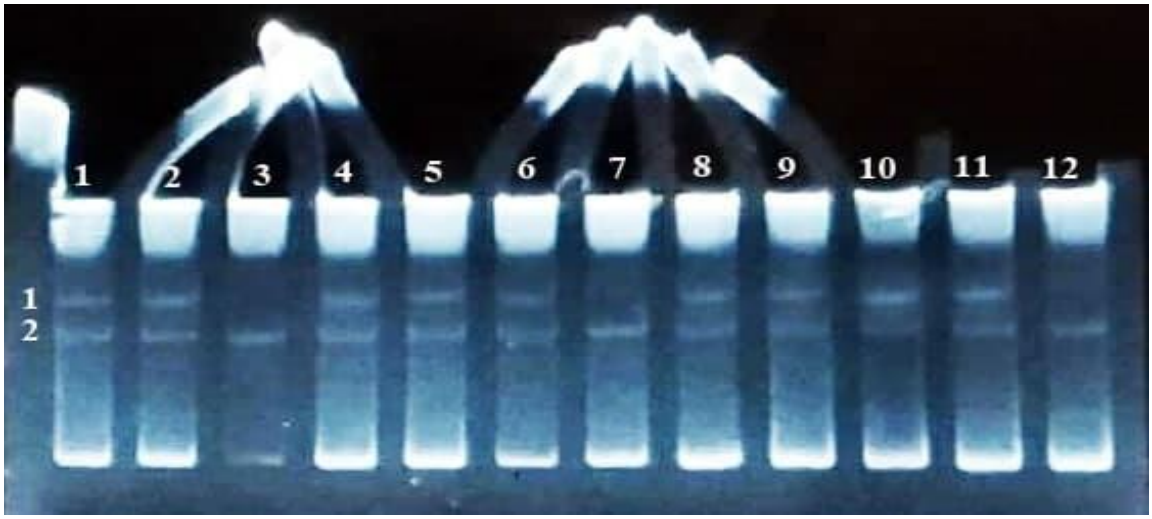



**Figure (4-10);Agarose gel electrophoresis of an amplified product patterns of Glutathion S-Transferase alpha 1 (GSTA1), (rs3957357) with specific primer.**

M: refers to DNA size marker(50bp); lanes 1 - 10 refer to PCR products of ***GSTA1*** (400bp) of breast cancer patients and lanes 11-15 healthy control groups. Electrophoresis conditions: 1% agarose concentration 1%; 75 V, 20 mA for 120 min. Staining method; ethidium bromide.

After that, the PCR products of the (GSTA1), (rs3957357) target sequences Polymorphisms were detected by PCR-SSCP method. In the present study, variations in the GSTA1 gene were detected, As shown in Fig(4-11); of PCR-SSCP gel electrophoresis, the existence of one conformational DNA polymorphisms was found according to the number of bands:2-bands(group A) and 1band (group B) as in figure (4.11) .




**Figure( 4-11): *GSTA1* gene polymorphisms of Breast cancer patients and healthy control subjects according to the number of the bands using PCR-SSCP method .**

lanes 1 – 6 refer to *GSTA1* (400bp) polymorphism of breast cancer patients and lanes 7 - 12 healthy control groups, lanes 1,2,4,5,6,8,9,10,11 refer to A group (two bands)GSTA1 polymorphism ,and lanes 3,7,12 refer to B group (one band) GSTA1 polymorphism

### 4.2.3. The Haplotype distribution of GSTA1 (rs3957357) gene by the number of bands and their association with Breast cancer patients and control groups.

the *GSTA1* (**rs3957357**) gene polymorphism was studied in breast cancer cases and control . The distribution observed in *GSTA1* (**rs3957357**) gene polymorphism in cases group and control group are showed in Table ( **4-3**). The results also showed that conformational polymorphism distributions among the haplotypes were 1-bands (92.85%) and 2-band (7.15 % ) respectively in patient group . The results demonstrate that there is an significant association between DNA polymorphisms according to the number of bands with patients as compared with the control groups (OR 26 ;95% CI 7.95-85.01) as shown in table (4-3).

**Table (4-3): PCR-SSCP haplotype distribution of *GSTA1* (rs3957357) gene by the number of bands and their association with Breast cancer patient and control groups**

| Conformational haplotypes | Patient group No. (%) | Control group No. (%) | *P*-value | OR | 95% CI |
|---|---|---|---|---|---|
| 1-bands | 65 (92.85%) | 10(33.33%) | 0.0001* | 26 | 7.95 - 85.01 |
| 2-bands | 5 (7.15%) | 20 (66.66%) | | | |
| Total number | 70 | 30 | | | |

***P ≤ 0.05; OR: Odd ratio ; CI : confidence interval**

In this study observed association between GSTA1 gene polymorphism and breast cancer risk.

Inherited polymorphisms in enzymes that activate or detoxify chemotherapy drugs are thought to account for some of the variability in toxicity and efficacy of cancer treatment.( Evans et al.,1999) The GST enzymes catalyze the glutathione-dependent detoxification of several chemotherapeutic drugs or their metabolites.( Hayes et al.,1995, Dirven et al.,1996), Polymorphisms that result in reduced (e.g., GSTP1 single nucleotide polymorphisms (SNP) or no (e.g., GSTM1 and GSTT1 deletion polymorphisms) activity of certain GST enzymes are recognized. These polymorphisms may alter the metabolism of chemotherapeutic drugs and modify the effectiveness of therapy, as suggested by reports that GST polymorphisms predict differences in outcomes of treatment for cancers including breast cancer( Kelsey et al.,1996 , Ambrosone et al.,2001), leukemias(Anderer et al.,2000 , Stanulla et al.,2000)and colorectal cancer(Stoehlmacher et al.,2002).

GSTA1 and other GSTs of the class are the predominant GSTs in human liver(van Ommen *et al*.,1990, Rowe *et al*.,1997), the major site of

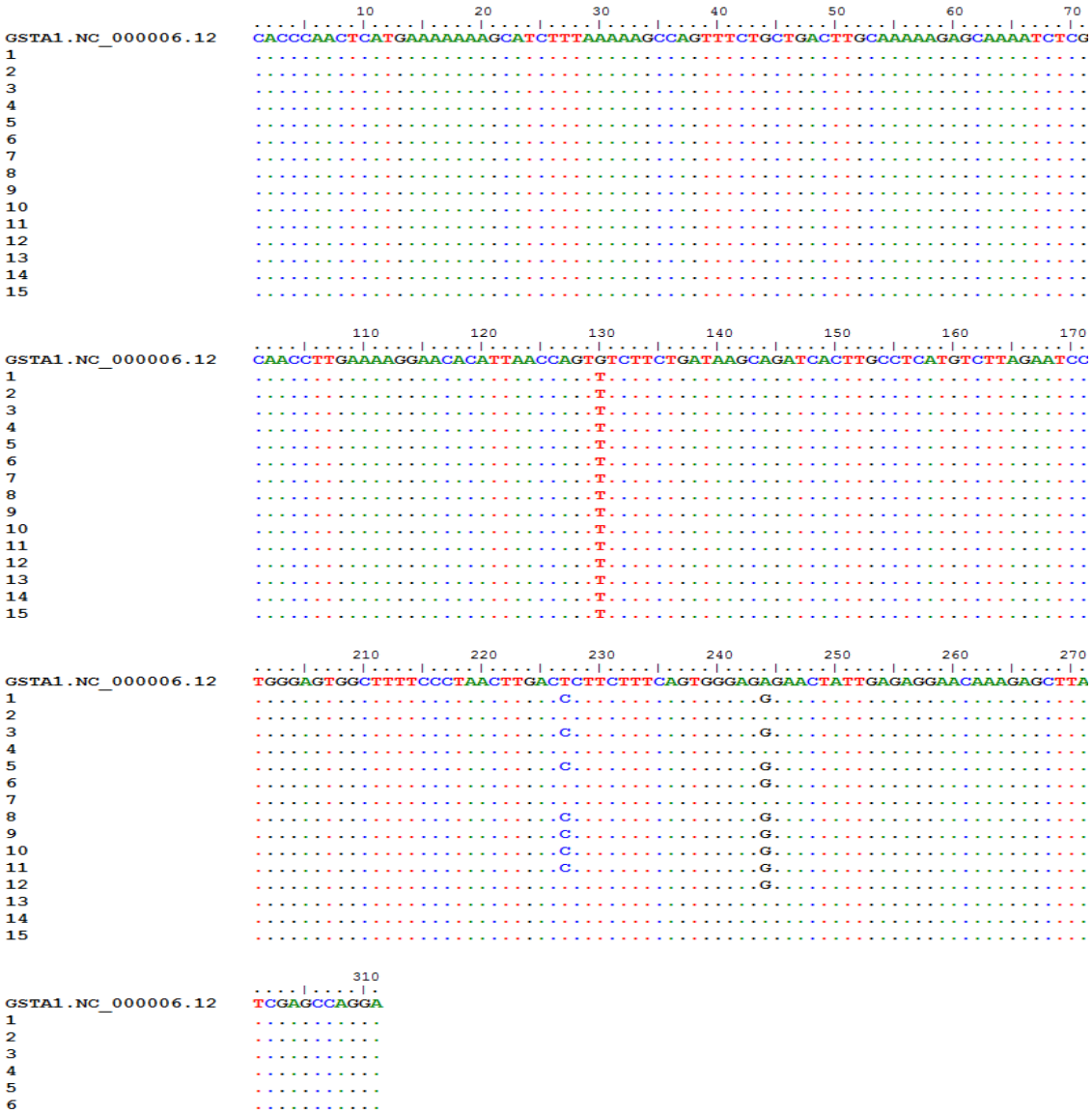
drug metabolism, and are also expressed in other tissues.( Howie *et al*.,1990, Morel *et al*.,2002). In vitro studies have shown that among human GSTs, *GSTA1* has the highest catalytic activity for glutathione conjugation of nitrogen mustard chemotherapy agents,( Dirven *et al*.,1996) including metabolites of cyclophosphamide (CP), ,( Dirven *et al*.,1994) which is used in combination chemotherapy for breast cancer. A polymorphism that influences the hepatic expression of GSTA1 has recently been described.( Coles *et al*.,2001,Morel *et al*.,2002) Liver cytosols from individuals who carried the variant GSTA1*B allele, which consists of several linked SNPs in the proximal promoter region of the *GSTA1* gene, had reduced levels of *GSTA1* enzyme.( Coles *et al*.,2001) Because of the importance of the *GSTA1* enzyme in metabolism of chemotherapeutic drugs, it can be hypothesized that individuals carrying the low expression GSTA1*B allele may have altered responses to chemotherapy.

(Board et al.,2002)To our knowledge, no studies have considered genotypic variants affecting _ class GST enzymes in relation to outcomes of cancer treatment. Other study determined genotypes at the GSTA1 proximal promoter polymorphism in normal DNA from women who received CP as part of combination chemotherapy for breast cancer and evaluated overall survival in relation to GSTA1 genotype.

### 4.3.Result Of Sequencing Technique

When translating the DNA sequence using by Bio Edit program version 7.2.5 according to the reference sequence alignment of the human *GSTA1* gene ID: NG_008731.1. observed the result Significant differences in allelic and genotypic frequencies of *GSTA1* rs3957357 were present between breast cancer patients and control groups (*GSTA1*

rs3957357A/G/T (52803891) ,revealed the substitutions of Adenine to Guanine A>G in sample (1,3,5,6,8,9,10,11,12)in the site (244) of the sequence, and Guanine to Thymine G>T in sample all the site (130) of the sequence ,and Thymine to Cytosine o T/C in sample (1,3,5,8,9,10,11) in the site(227) of the sequence.




**Figure(4-12):Sequences alignment result for GSTA1 gene fragment by Bio Edit Program version**

Glutathione S-transferases (GSTs) detoxify toxic molecules by conjugation with reduced glutathione and regulate cell signaling.In other

study , Single nucleotide polymorphisms (SNPs) of GST genes have been suggested to affect GST functions and thus to increase the risk of human hepatocellular carcinoma (HCC). As *GSTA1* is expressed in hepatocytes and the rs3957357C>T (TT) SNP is known to downregulate GSTA1 mRNA expression, study were:- (i) to explore the relationship between the TT SNP in *GSTA1* and the occurrence of HCC:- (ii) to measure *GSTA1* mRNA expression in HCCs (Akhdar *et al* .,2016).Glutathione S-transferases (GSTs) are phase II enzymes that are involved in the detoxification of a wide range of carcinogens, Another study showed the novel GSTA1*A and GSTA1*B genetic polymorphism results in differential expression, with lower transcriptional activation of GSTA1*B (variant) than that of GSTA1*A (common) allele. Considering that cruciferous vegetables induce GSTs, which metabolize tobacco smoke carcinogens, hypothesized that the variant GSTA1*B genotype may predispose women to breast cancer. Particularly among low cruciferous vegetable consumers and among smokers. Thus, we evaluated potential relationships between GSTA1 polymorphisms and breast cancer risk, in relation to vegetable consumption and smoking status in the Long Island Breast Cancer Study Project (1996-1997), a population-based case-control study.

In other 15 study were enrolled, and the results indicated that GSTA1 BB genotype was associated with elevated cancer risk, especially in colorectal cancer. Further stratifications showed that GSTA1 BB genotype was associated with increased cancer risk in Caucasian populations and in the study with population-based controls ( Deng *et al*.,2015), and glutathione transferase A1 (GSTA1) and the extent of oxidative damage in patients with transitional cell carcinoma (TCC) of the urinary bladder(Savic-Radojevic *et al*.,2013).

In present study, Where we have observed changes in the nitrogenous bases, and this thus leads to a change in the amino acid that forms the nucleic acid, and thus will affect the function of the gene in the defense against breast cancer, The GST enzymes catalyze the glutathione-dependent detoxification of several chemotherapeutic drugs or their metabolites, This function will stop due to the variations we have seen in the structure of the studied gene (Townsend et al.,2003).

# Conclusion and Recommendation

**Conclusions:-**

1-The study demonstrated that the frequency of Significant association was reported between decrease in antioxidant when using chemotherapy and breast cancer risk.

2-The study demonstrated Chemotherapy caused a decrease in the level of glutathione activity(GSH

3- Significant effects were found between lipids profiles (TG, TC, HDL LDL,V-LDL) and breast cancer patients.

4-Association GSTA1 genes with breast cancer risk,and No association was reported between polymorphisms of GSTP1 and breast cancer risk.

**Recommendations:-**

1-Further larger studies using large number of mutations of other genes are recommended to shed more light on the molecular diagnosis of breast carcinogenesis.

2- Detection of other biochemical marker(GPX, Selenium, vitamin C ,vitamin D,vitamin E).

3-Study of the immune system of breast cancer patients.

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## الخلاصة

سرطان الثدي هو أكثر أنواع السرطانات شيوعًا التي تصيب النساء في جميع أنحاء العالم. بحثت الدراسة الحالية في العلاقة بين تعدد الأشكال الجينية لجينات Glutathione S-Transferase ( (GSTP1)pi1 و Glutathione S-Transferase Alpha 1 (GSTA1)) وانخفاض إنزيم مضادات الأكسدة بين مرضى سرطان الثدي وتأثير العلاج الكيميائي له. تم إجراء هذا التحقيق على 70 مريضًا (جميعهم من الإناث) ممن تأكدت إصابتهم بسرطان الثدي عن طريق الفحوصات التشريحية المرضية التي حضرها مركز الأورام في مدينة مرجان الطبية في محافظة بابل ، وتم استخدام 30 من النساء اللائي يبدو أنهن يتمتعن بصحة جيدة كعنصر تحكم.

كانت فترة الدراسة ما بين سبتمبر 2020 إلى مايو 2021 في كلية العلوم بجامعة بابل ، مختبر التكنولوجيا الحيوية ، كان هذا البحث دراسة حالة ضابطة ، تم جمع عينات دم من 70 مريضة بسرطان الثدي ، وتم جمع 30 عينة كمجموعة ضابطة.

جمعت عينات الدم من 70 امرأة مصابة بسرطان الثدي و 30 امرأة شاهدة لتحديد مستوى الدهون ومستوى بيروكسيد الدهون. أظهرت النتائج زيادة ملحوظة في مستويات Malondialdehyde (MDA) في مرضى سرطان الثدي ، بينما أظهرت ملامح الدهون ،الدهون الثلاثية (TG) ، والكوليسترول الكلي (TC) ، والدهون عالية الكثافة (HDL) ، والبروتين الدهني منخفض الكثافة (LDL) و كثافة البروتين الدهني المنخفض جدا (VLDL)، أظهرت انخفاضًا في الارتباط بسرطان الثدي.

في هذه الدراسة تم تحديد محددات مضادات الأكسدة مثل نشاط سوبروكسيد ديسموتاز (SOD) ، نشاط كاتاليز (CAT) ، بيروكسيد الهيدروجين (H2O2) ، سعة إنزيمات مضادات الأكسدة الكلية (TAC) وملف الدهون وعلامة بيروكسيد الدهون مثل Malondialdehyde (MDA). كشفت الدراسة الحالية عن تعدد أشكال الجينات GSTPI (rs1695) و GSTAI (rs3957357) في مرضى سرطان الثدي مع السيطرة.

تم تقسيم الخصائص الديموغرافية لمرضى سرطان الثدي إلى ثلاث مجموعات حسب عدد جرعات العلاج الكيميائي مع مجموعة السيطرة.

أظهرت نتائج الدراسة الحالية وجود فروق بين مجموعة السيطرة والحالة المصابة بسرطان الثدي في بعض الواسمات البيوكيميائية ، حيث كان نشاط Superoxide dismutase (SOD) في مجموعة السيطرة 64.40 ± 23.76 U / ml بينما انخفض نشاطها في مجموعات

الحالة بشكل ملحوظ. كانت المجموعة الأولى (1-5 جرعات) 40.89 ± 28.55 وحدة / مل ، المجموعة الثانية (6-10 جرعات) 40.51 ± 27.55 وحدة / مل والمجموعة الثالثة (> 10 جرعات) وصلت إلى 43.65 ± 28.75 وحدة / مل. . كان نشاط الكاتلاز (CAT) المسيطر 16.08 ± 8.38 وحدة / مل بينما انخفض نشاطه في مجموعات الحالة بشكل ملحوظ. كانت المجموعة الأولى 9.51 ± 6.59 وحدة / مل ، أما المجموعة الثانية فبلغت 9.90 ± 4.66 وحدة / مل والمجموعة الثالثة 9.25 ± 4.62 وحدة / مل. كان تركيز GSH في السيطرة 17.57 ± 9.09 ميكرو مول / مل بينما انخفض نشاطه في مجموعات الحالة بشكل ملحوظ ليصل إلى 11.01 ± 5.55 ميكرو مول / مل في المجموعة الأولى ، أما المجموعة الثانية فكانت 13.03 ± 8.29 ميكرو مول / مل والمجموعة الثالثة وصلت إلى 11.07 ± 5.55 ميكرو مول / مل. بينما كان تركيز H2O2 في مجموعة السيطرة 1.22 ± 1.18 نانومول / مل بينما انخفض تركيزه في مجموعات الحالة بشكل ملحوظ ليصل إلى 2.36 ± 1.27 نانومول / مل في المجموعة الأولى ، أما المجموعة الثانية فكانت 1.94 ± 0.79 نانومول / مل وزاد في المجموعة الثالثة لتصل إلى 2.39 ± 1.18 نانومول / مل.

كان مؤشر بيروكسيد الدهون مثل تركيز malondialdehyde (MDA) المسيطر 2.06 ± 1.39 ميكرومول / مل بينما انخفض نشاطه في الحالة بشكل ملحوظ ليصل إلى 2.07 ± 0.88 ميكرو مول / مل في المجموعة الأولى ، أما المجموعة الثانية فكانت 2.34 ± 1.65 ميكرو مول / مل. / مل وفي المجموعة الثالثة تصل إلى 1.28 ± 0.18 ميكرو مول / مل. كانت السعة الكلية لمضادات الأكسدة (TAC) المسيطرة 83.17 ± 50.77 وحدة/مل بينما تمت زيادة TAC في الحالة بشكل ملحوظ لتصل إلى 74.55 ± 42.83 وحدة/مل في المجموعة الأولى ، والمجموعة الثانية كانت 67.24 ± 22.73 وحدة/مل والمجموعة الثالثة كانت المجموعة 96.78 ± 61.01 وحدة/مل. كان مستوى الدهون للكوليسترول الكلي المسيطر 507.86 ± 178.62 ملغم / ديسيلتر وكانت مجموعات الحالة 260.21 ± 153.72 ملغم / ديسيلتر والدهون عالية الكثافة (HDL) في السيطرة كانت 1083.96 ± 326.02 ملغم / ديسيلتر والحالة 423.99 ± 109.54 ملغم / ديسيلتر، في حين أن الدهون الثلاثية الملحوظة (TG) في السيطرة كانت 1065.12 ± 588.85 ملغم / ديسيلتر والحالة 1045.93 ± 851.62 ملغم / ديسيلتر ، والبروتين الدهني منخفض الكثافة (LDL) في الحالة كان 412.62 ± 237.24 ملغم / ديسيلتر والسيطرة كان 789.12 ± 414.32 ملغم/ديسيلتر و كان VLDL في مجموعة الحالة 209.19 ± 170.32 ملغم / ديسيلتر ومجموعة السيطرة 213.03 ± 117.77 ملغم / ديسيلتر.

تم إجراء تعدد الأشكال الجينية لجين Glutathione S-Transferase pi1 (GSTP1) (rs1695) باستخدام تقنية PCR RFLP technique. تم إجراء تعدد الأشكال لجين Glutathione S-Transferase Alpha1 (GSTA1) (rs3957357) باستخدام تعدد الأشكال التوافقي أحادي الخيط (SSCP -PCR).

كان النمط الجيني لـ GSTP1 (rs1695) متماثل الزيجوت (80) AA٪) يليه النمط الوراثي GA متغاير الزيجوت (16.7٪) والنمط الوراثي متماثل الزيجوت (3.3) GG٪ في المجموعة الضابطة. كما كان الزيجوت البري المتماثل 82.8 AA٪ يليه النمط الوراثي GA متغاير الزيجوت (8.6٪) والنمط الوراثي GG متماثل الزيجوت (8.6٪) في مجموعة الحالة. تشير النتائج إلى عدم وجود ارتباط بين (1695) وخطر الإصابة بسرطان الثدي.

وجدت الدراسة الحالية أن الجين GSTA1 (rs3957357) له نطاقان مختلفان من التغايرات تم اكتشافهما بواسطة PCR-SSCP بما في ذلك باندة واحدة (المجموعة أ) وباندتين (المجموعة ب) ، وبعد ذلك ظهرت تقنية تسلسل الحمض النووي طفرتين منفردتين من G> A و G <T الذي لاحظ ارتباطه بسرطان الثدي.

جمهورية العراق

وزارة التعليم العالي

والبحث العلمي

جامعة بابل

كلية العلوم

قسم علوم الحياة

# فعالية الأنزيمات المضادة للأكسدة ومستوى الدهون المؤكسدة في النساء المصابة بسرطان الثدي

**رسالة مقدمة الى**

مجلس كلية العلوم / جامعة بابل

وهي جزء من متطلبات نيل درجة الماجستير في العلوم / علوم الحياة

من قبل

رغد عبيد عبد العباس عبدالله

بكالوريوس علوم الحياة / جامعة بابل (2017-2018)

**اشراف**

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