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Protective Effects of Clove Extract on Oxidative and DNA Damage in liver of rats Exposed to Ethanol

A thesis

Submitted to the council of the college of veterinary medicine, university of Kerbala in partial fulfillment of the requirements for the degree of master of science in veterinary medicine/ Physiology

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هُوَ الَّذِي جَعَلَ الشَّمْسَ ضِيَاءً وَالْقَمَرَ نُورًا وَقَدَّرَهُ مَنَازِلَ لِتَعْلَمُوا عَدَدَ السِّنِينَ وَالْحِسَابَ ۚ مَا خَلَقَ اللَّهُ ذَٰلِكَ إِلَّا بِالْحَقِّ ۚ يُفَصِّلُ الْآيَاتِ لِقَوْمٍ يَعْلَمُونَ

صدق الله العلي العظيم

سورة يونس ايه رقم (5)

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Summary

The current study was conducted in the animal house of the College of Veterinary Medicine - University of Karbala to investigate the protective role of clove extract on oxidative stress and changes in gene expression and DNA resulting from exposure to ethanol alcohol،Alcohol consumption causes comprehensive liver disorders, designated as alcoholic liver disease (ALD). Cytochrome P450 2E1 (CYP2E1) in MEOS is one of the major ROS generators in liver and is recognized as a risk factor for ALD, Forty mature wister male rats weighting (140 – 190g) were used and divided randomly into four groups with ten males per group. All groups were dosed by oral intubation daily during the experiment period (four weeks), where the first group (the control group) was dosed with distilled water at an amount (1 ml per 1 kg) for a period of four weeks, while the second group (the clove group) was dosed with clove extract at a concentration (1 mg per kg) for four weeks and the third group (Ethanol group) ethanol was dosed at a concentration of (1 g per kg) and the last group was dosed with ethanol and then clove extract and at a concentration (1 g per kg) for ethanol and (1 mg per kg) for clove extract for a period of four weeks as well. Body weight of all rats (40 rats) has been monitored throughout the experiment. At the end of each treatment and control groups period, blood samples were obtained from heart for assessment of the following enzymes (catalase , glutathione peroxidase , Liver function test , TBARS and protein carbonyl) qRT-PCR technique was used for the quantification of CYP 2E1 gene expression levels relative to Housekeeping gene B actinin gene expression levels in rat liver have ethanol toxicity and treated with clove for estimation of fold changes in gene expression levels of target gene (CYP2E1) and housekeeping gene (B- actinin), Blood samples were collected from all rats, and samples were coded to avoid the possibility of bias. one ml of s blood was drawn once

from the heart of animals by heparin injection. Samples were immediately transferred on ice to the central scientific laboratory in the veterinary medicine college, Ten microliter of tissue grinding cells were mixed with 75 µl of 0.5% lower melting point agarose in order to determine DNA damage analysis for all animals in this study (experimental and control) was done using the comet assay, the results were found in all of each the experimental periods (10-d, 20-d and 30- d), there was no significant difference ($P>0.05$) in body weight between male rats, the result also showed the effect of ethanol and clove on the catalase concentration in serum rats (U/ml), the treated male rats (T2) registered low significant ($P<0.05$) level of catalase throughout the experimental groups (C,T1 and T3), the oral intubation of (1g/kg) from ethanol and (100mg/kg) clove extract (T3) group rats caused significant ($p<0.01$) decrease in serum GPx concentration as compared to ethanol group(T2), it also found a significant elevation ($P<0.05$) in the liver enzymes (AST, ALT, ALP and Total bilirubin) in the second group (T2) and third (T3) groups when compared with male rats of control and first treatment group, on the other hand treated male rats (T2) registered highest significant ($P<0.05$) level of TBARS throughout the experimental groups (C,T1 and T3) male rats, the result showed a significant ($P<0.01$) increase in the Serum protein carbonyl after four weeks in T2 as compared to control group. On the other hand, among comet assay techniques the result found significant decrease of DNA in head in ethanol groups (30.829 ±5.82) rather than other groups as (90.104± 0.628, 91.057 ±0.727and 73.17 ± 8.13) for Control, cloves, and ethanol plus cloves, respectively.

Reverse transcriptase real-time PCR results have shown that CYP450E2 mRNA expression levels increased more than 10-folds in Treatment group 2 (ethanol treatment) male rats treated compared with corresponding of control

male rats (slightly more than 2-fold) as well as in the treated male rats (around 1-fold).

In conclusion;the current study indicates that clove extract worked as antioxidant substance and the present study suggest that the ethanol exposure causes oxidative stress and liver disease and we recommended to avoiding exposure through treatment with clove extract .

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List of Abbreviations

CYP2E1	Cytochrome p450 2E1
ALP	Alkaline Phosphatase
ALT	Serum Alanine transaminase
AST	Serum Aspartate transaminase
TB	Total bilirubin
PC	Protein carponyl
TBARS	Thiobarbituri cacid reactive substance
GPX	Glutothion peroxidase
Cat	catalase
EDTA	Ethylene diamin tetraacetic acid
ROS	reactive oxygen species
ALD	Alcoholic liver disease
ARLD	Alcoholic-related liver disease
SOD	Superoxide dimutase
ADH	Alcohol dehydrogenase enzyme
MEOS	Microsomal ethanol oxidation system
ALDH	Aldehyde dehydrogenase
NAD	Nicotinamide adenine dinucleotide
DPPH	1,2hydrazinopyridinediphenyl
LDM	Lanosterol 14-α-demethylase
TBXAS1	ThromboxaneA2synthase
CPR	Cytochrome P450 reductase
RNS	Reactive nitrogen species
RCO	Reactive carbonyl
qRT-PCR	Quantitative Reverse Transcriptase Real-Time
BHT	butylated hydroxyl toluene,

Chapter One

Introduction

Introduction

Alcohol-related liver disease (ARLD) or Alcoholic liver disease (ALD), , is a term that encompasses the liver manifestations of alcohol overconsumption, including alcoholic hepatitis, fatty liver, ,liver fibrosis or cirrhosis chronic hepatitis, it is the main cause of liver disease around the world. Although anyone who drinks a lot of alcoholc beverages for a long time will develop steatosis (fatty liver disease), the process is short-lived and reversible , More than 90% of alcoholism will develop fatty liver, while about 25% will develop more severe alcoholic hepatitis, and 15% will develop cirrhosis (Foulds *et al*., 2017).

However, many patients who undoubtedly drink a lot, are already alcohol dependent and exhibit elevated serum transaminase levels, do not show a high ALT and ALP levels. This suggests that other factors cause elevated ALT and ALP levels in some patients. One of these factors may be the severity of the liver disease (Nyblom *et al*.,2004). red blood cell macrocytosis (mean corpuscular volume > 100) and elevations of alkaline phosphatase , serum gamma-glutamyl transferase (GGT), and bilirubin level as well as. Folic acid level is reduced in alcoholic patients due to decreased intestinal absorption, increased bone marrow requirement for folate in the presence of alcohol (Niemelä,2007).

Oxidative stress and reactive oxygen species (ROS) toxicity are considered to be one of the main potential mechanisms leading to alcoholic liver damage and mitochondrial changes, Previously, it was showed that acute and chronic ethanol exposure stimulated the production of reactive oxygen species in rat liver cells, presumably at the mitochondrial level (Bailey *et al*., 1999).

The main mitochondrial antioxidant enzymes used to counteract these ethanol-related increases in ROS include manganese superoxide dismutase (MnSOD) and glutathione peroxidase-1 (GSHPx-1). Mitochondria Mn-SOD catalyzes the decomposition of superoxide into hydrogen peroxide, while GSHPx-1 metabolizes hydrogen peroxide into water. Ethanol use in vivo (Polavarabo *et al.,* 1998) and cell culture models (Pereira *et al.,* 1995) have shown adaptability to ethanol-related oxidative stress reaction.

Ethanol-related protein oxidation, it was found A very mature and delicate technology the measurement is based on the observation that ROS species can attack producing amino acid residues and carbonyl functions, which can then react with 2,4-dinitrophenylhydrazone to form detectable dinitrophenylhydrazone by spectrophotometry (Reznick *et al.*,1994). They constitute a significant increase in chronic ethanol consumption the concentration of protein carbonyl in liver protein

It diverses medicinal plants that are being used in the ameliorated alcoholic liver disease due to their antioxidants capacity, clove is one of these plants,containing many high nutritional values that support the functions of the body's organs and protect them from exposure to diseases. The nutritional value of a small handful of cloves has been estimated to contain many calories, carbohydrates, dietary fiber, 30% of magnesium and 4% of Vitamin K and 3% of Vitamin C, among other vitamins,(Guan *et al*., 2007).

Due to alcohol-induced oxidative stress is linked to the metabolism of ethanol involving both microsomal and mitochondrial systems and ethanol metabolism is directly involved in the production of reactive oxygen species

(ROS) and reactive nitrogen species (RNS) there for the study was aiming at the following :

1- The antioxidant property of clove extract and ameliorating oxidative stress and reduced damage response evoked by alcoholic induced liver disease.

2-Evaluate the effect of clove extract on activty of cytochrome P450E1 (CYP2E1) gene expression and Assessment of DNA damage in blood using comet assay.

Chapter Two

Literature Review

2.Literature Review :

2.1.Alcohol ingredients:

Historically, alcohol has been used socially, in both religious and non-religious rituals, as well as as a food ingredient, and it has been used medicinally. Alcohol consumption by different cultures precedes written history. Although it was used in the past for therapeutic purposes, due to its ability to cause toxicity, so it is no longer recommended as a therapeutic treatment, alcohol produces intoxicating effects because it has the ability to move from the intestine to the bloodstream and into the brain due to its chemical composition and its solubility in water (Anderson & Baumberg, 2006).

Ethanol is an organic chemical compound that belongs to the alcohol family. Its chemical formula is (C2H5OH) and the molecular formula is (C2H6O) . It is generally called alcohol.Ethanol is a colorless, flammable substance formed by the fermentation of sugar, used in the manufacture of alcoholic beverages and perfumes, and as a fuel in ethanol-powered mechanical engines(Osunkoya & Okwudinka,2011).

2.2. Etymology:

Ethanol is the systematic name of the International Union of Pure and Applied Chemistry for double-carbon molecules and (prefix "eth-"),(Prentki & Madiraju,2008). The term 'ethyl' is An English version of the German word äthyl, coined in 1838 by Liebig. This is similar to the related term 'methyl' both as it originated from the Greek, and the participle part 'yl', which is equivalent to 'hyle' meaning substance. However, the previous part differs - 'ETH', equivalent to 'aither', meaning ether. Thus the word 'ethyl' is

an abbreviation of 'aither hyle'(Russell & Setchell,1992) Liebig used the term 'ethyl alcohol' to distinguish ethanol from other alcohols.

The term "alcohol" now refers to a broader class of substances named in chemistry, but in common parlance it is still the name ethanol. Eventually a loan from medieval Arabic Al Kuhl,(Tanaka *et al*.,1997) the use of alcohol in this sense was introduced in the mid-18th century. Before the period, Middle Latin alcohol was referring to "sublime substance; distilled spirit" The use of "wine spirit" (abbreviated for the full expression alcohol of wine) was recorded 1753, Its systematic use in chemistry dates back to 1850. Ethanol is present in nature as a by-product of yeast metabolism. As such, the ethanol will be present in the yeast habitat. Generally, ethanol can be found in ripe fruits (Nava-Ocampo *et al.*,2004).

2.3. Chemical structure of alcohol:

The molecular formula for ethanol is C2H6O, so ethanol contains two carbon atoms and one oxygen atom. However, the ethanol's structural formula, C2H5OH, provides more detail, and indicates the presence of a hydroxyl (OH-) group at the end of the 2-carbon chain (Figure 2.1).

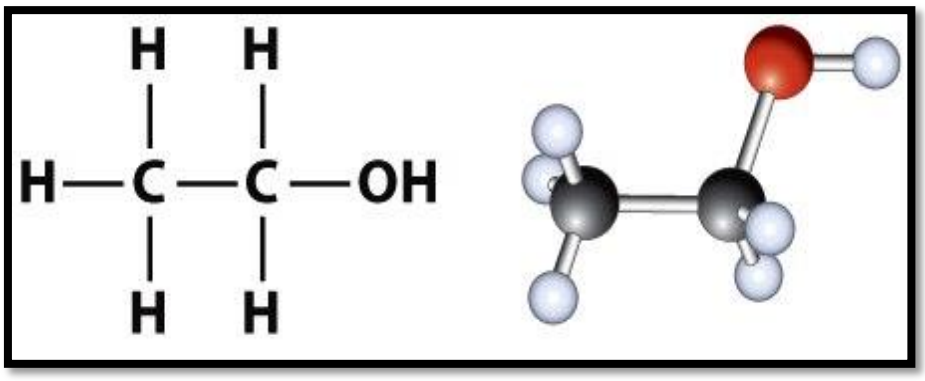


Figure 2.1 Two common ways to represent the structure of ethanol. the left is the atomic stick representation of the structural formula and the right is the ball and stick model (Guo *et al.*,2003)**.**

2.4. Physical properties:

A polar compound characterized by the formation of hydrogen bonds between its molecules, as it contains a hydroxyl group that works to increase the cohesion of bonds. A colorless, volatile liquid with a specific density of 816. It boils at 80° C. It dissolves in polar solvents such as water, as it has hydrogen bonds with water. It burns with a smokeless blue flame and is not always visible in natural light. Ethanol is slightly more refractive than water, with a refractive index between 1.36242 (λ = 589.3 in nanometers and 18.35 °C) (Harris,2009).

2.5. Properties of ethanol :

Ethanol is miscible with a number of solvents, miscible with water and with many organic solvents, including acetic acid, acetone, benzene, carbon tetrachloride, chloroform, ether, ethylene glycol, glycerol, nitromethane, pyridine, and toluene(Agarwal,2001). Miscible with light aliphatic hydrocarbons, such as pentane and hexane, and with aliphatic chlorides such as trichloroethane and tetrachloroethylene(David,2000).The miscibility of ethanol with water contrasts with the immiscibility of long-chain alcohols (five or more carbons), and the aqueous miscibility decreases sharply with increasing number of carbons (Behin & Vahed,2007).The mixing of ethanol with alkanes is limited to alkanes up to [Gerdecane] and mixtures with dodecane. The higher dodecane alkanes show a miscibility gap under a certain temperature (about 13 °C) (Behr & Roll,2005).

Ethanol is an interesting molecule. As a result of the presence of the hydroxyl terminal group, it is polar (hydrophilic), so it dissolves in water. However, it is also slightly nonpolar (hydrophobic) due to the presence of the carbon 2 chain. The electric charges between carbon atoms are not separated, thus reducing interactions between molecules in aqueous

solutions. In general, ethanol is less soluble in water, due to the presence of carbon chains (saturated with hydrogen) that give a molecule a hydrophobic character (feared water), however, in the case of ethanol, the OH-polar group dominates due to the fact that the carbon chain is short enough that Ethanol gives its polar character. In alcohols with relatively long carbon chains (4 or more) they are less soluble in water because the polar effects of the -OH group are not sufficient to overcome the hydrophobic nature of the carbon chain. One of the most important properties of ethanol is its solubility, as it has the ability to move across biological membranes and around the body. , It fits into the pores (holes) in the biological membrane(Kagan *et al*.,2014). In fact, since it is a small molecule (molecular weight = 46 g / mol) that is distributed in any area within the body where water is present. Ethanol has the ability to penetrate the binary lipid layers of biological membranes because it contains a 2-carbon chain in ethanol that makes it slightly lipophilic (Goldstein & Chin,1981) .

2.6. Metabolism of alcohole:

Ethanol is a kind of alcohol that exists in nature and alcoholic beverages. It is metabolized through complex catabolic pathways. In humans, there are several enzymes that first process ethanol into acetaldehyde, and then into acetic acid and acetyl-CoA. Once acetyl-CoA is formed, it becomes a substrate of the citric acid cycle, which ultimately produces cellular energy and releases water and carbon dioxide. Due to the existence and availability of enzymes, human adults and fetuses process ethanol in different ways. The genetic variation of these enzymes can lead to differences in catalytic efficiency between individuals. Due to the high concentration of these enzymes, the liver is the main organ for metabolizing ethanol. The average

human digestive system ferments its contents to produce approximately 3 grams of ethanol per day.(Tillonen,2000)

Therefore, the catabolism of ethanol is vital to life, not only for humans, but also for all known organisms. Certain amino acid sequences in the enzymes used to oxidize ethanol are conserved (unchanged) and can be traced back to the last common ancestor over3.5 bya. (Agarwal ,2001)This function is necessary because all organisms produce a small amount of alcohol through a variety of ways, mainly through fatty acid synthesis, (Ohlrogge & Jaworski,1997) glycerolipid metabolism (Prentki & Madiraju,2008) and bile acid biosynthesis pathways (Walters *et al*.,2009). If the body does not have a mechanism to catabolize alcohol, they will accumulate in the body and become toxic. This may be the evolutionary principle of alcohol catabolism also through sulfotransferase.

Genetic variation affects alcohol metabolism and drinking behavior. (Agarwal,2001) Gene expression and ethanol metabolism in human adults, ethanol is converted to acetaldehyde. In adults, ethanol is mainly oxidized to acetaldehyde by the liver enzyme alcohol dehydrogenase IB (Class I) and β peptides (ADH1B, EC 1.1.1.1) using NAD+. The gene encoding this enzyme is located at the locus on chromosome 4. (Getman *et al*.,1992)

The enzyme encoded by this gene is a member of the alcohol dehydrogenase family. Members of this enzyme family metabolize a variety of substrates, including ethanol, retinol, Other, fatty alcohols, hydroxysteroids, and lipid peroxidation products. This encoded protein is composed of several homodimers and heterodimers of α, β and γ subunits. It is highly active in ethanol oxidation and plays a major role in ethanol catabolism. The three genes encoding α, β, and γ subunits are organized in tandem as gene clusters in genome fragments. (Wu *et al.*,2001)

In human embryos and fetuses, ethanol is not metabolized by this mechanism, because the ADH enzyme has not been expressed in large amounts in the liver of human fetuses (the induction of ADH enzyme only starts after birth, and it takes several years to Reach adult level).(Ernst van Faassen&Onni Niemelä,2011) Therefore, the fetal liver cannot metabolize ethanol or other low molecular weight exogenous substances. In the fetus, ethanol is metabolized at a much slower rate by different enzymes from the cytochrome P-450 superfamily (CYP), especially CYP2E1.

The low rate of fetal ethanol clearance is the reason for the important observation that long after the adult ADH enzyme activity in the maternal liver clears ethanol from the maternal circulation, the fetal compartment still retains high levels of ethanol. (Nava-Ocampo *et al.*,2004) After organogenesis (approximately 50 days of pregnancy), CYP2E1 expression and activity have been detected in various human fetal tissues. (Carpenter *et al.*,1996) It is known that exposure to ethanol can promote further induction of this enzyme in fetal and adult tissues. CYP2E1 is the main contributor to the so-called microsomal ethanol oxidation system (MEOS)(Cederbaum *et al.*,2009) , and its activity in fetal tissues is believed to have a significant impact on the toxicity of maternal ethanol consumption, In the presence of ethanol and oxygen, CYP2E1 is known Release superoxide radicals and induce the oxidation of polyunsaturated fatty acids into toxic aldehyde products, such as 4-hydroxynonenal (HNE). Acetaldehyde to Acetic Acid (Parker *et al.*,2011).

The enzymes involved in the chemical conversion of acetaldehyde to acetic acid are the aldehyde dehydrogenase 2 family (ALDH2, EC 1.2.1.3). In humans, the gene encoding this enzyme is located at position q24.2 on chromosome 12 (Durocher *et al.*,1995). There are mutations in this gene,

leading to significant differences in catalytic efficiency between people. (Zhang *et al.*,2002).

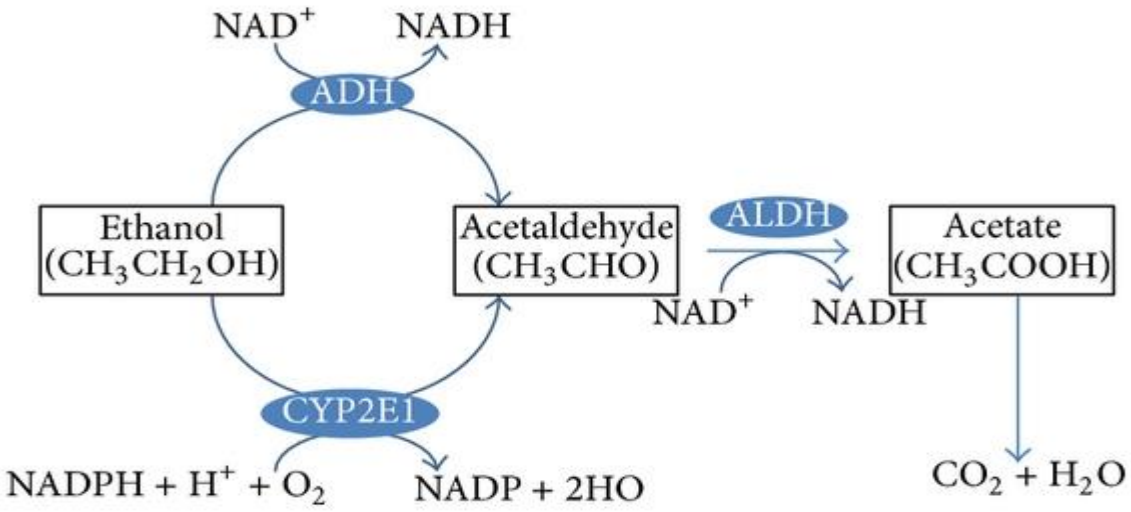


Figure 2.2:The pathway of ethanol metabolism. Ethanol is metabolized into acetaldehyde by alcohol dehydrogenase (ADH) and the microsomal enzyme cytochrome P450 2E1 (CYP2E1). The ADH enzyme reaction is the main ethanol metabolic pathway involving an intermediate carrier of electrons, namely, nicotinamide adenine dinucleotide (NAD+). Acetaldehyde is rapidly metabolized by aldehyde dehydrogenase (ALDH) in the mitochondria to acetate and NADH. And acetate is eventually metabolized in the muscle to carbon dioxide and water (Kawaratani *et al.*,2013).

2.7.The Toxic effects of ethanol alcohole:

There are four characteristics that must be taken into account when studying the toxic effects of ethanol alcohol (distribution, absorption, organ function, and filtration). Ethanol is rapidly absorbed through the intestines and pumped parallel to all organs and tissues through the blood and passes mainly to the liver. Solutions of absolute ethanol and aqueous ethanol are widely used in various pharmaceutical and cosmetic forms and are used in the manufacture of alcoholic beverages(Benjamins *et al*.,2011).

It is rapidly absorbed from the gastrointestinal tract and its vapors can pass through the lungs, mainly metabolized in the liver to acetyldehyde which in turn is later oxidized to acetate. Ethanol is a central nervous system (C.N.S) depressant, as ingestion of small to moderate amounts may lead to the appearance of symptoms of toxicity represented by incoordination of muscle work, poor vision, slurred speech. . . . etc. As for consuming large amounts (or large concentrations), it may cause inhibition of bulbar reflexes such as lethargy, forgetfulness and amnesia, hypothermia, hypoglycemia, stupor, coma, respiratory depression, and cardiovascular collapse. . The lethal concentration of alcohol in the blood is estimated at 400-500 mg/100 ml (Babu *et al.*,2003)

Although the symptoms of alcohol intoxication are usually observed in those who abuse alcoholic beverages, it is possible that these symptoms appear when taking pharmaceutical preparations containing it as a sweetener, if taken in large quantities.

2.8.Alcohole and liver:

Chronic alcoholism has many health risks, from high blood pressure to stroke. People are most familiar with the negative effects of alcohol on the liver. Heavy drinking is defined as women drinking 8 or more drinks per week and men drinking 15 or more drinks per week. Even a single binge eating can cause serious physical injury, damage or potential death(Foster& Marriott,2006) .

The liver breaks down and filters out harmful substances in the blood and makes proteins, enzymes and hormones that the body uses to fight off infections. It can also convert vitamins, nutrients and medicines into substances that our body can use. The liver is also responsible for cleaning our blood, producing bile for digestion and storing glycogen for energy. The liver processes more than 90% of alcohol. The rest is excreted through urine, sweat and breathing. The body takes about an hour to process an alcoholic beverage. This time frame increases with each drink(Hemkin, 2018).

The higher a person's blood alcohol content, the longer it takes to process alcohol. The liver can only process a certain amount of alcohol at a time. When someone drinks too much alcohol, the alcohol that has not been processed by the liver circulates in the blood(Cho *et al.*,2019).

Alcohol in the blood begins to affect the heart and brain. This is how people get drunk. Long-term alcohol abuse can damage liver cells, leading to liver scarring (cirrhosis), alcoholic hepatitis, and cell mutations that may lead to liver cancer. These conditions usually progress from fatty liver to alcoholic hepatitis to cirrhosis, although alcoholics may develop alcoholic cirrhosis without first developing hepatitis (Arulkumaran *et al.*,2009).

Mixing alcohol with other drugs is also very dangerous for liver. When certain drugs are used in combination, such as acetaminophen, such as

Tylenol, it can cause severe damage to liver. Other drugs used in combination with alcohol include antibiotics, blood thinners, antidepressants, sedatives, pain relievers, and muscle relaxants.

Compared with moderate drinkers, heavy drinkers have a higher risk of a series of liver diseases. As many as 20% of heavy drinkers develop fatty liver, although fatty liver can usually be reversed by abstaining from alcohol. Alcoholic hepatitis is an inflammation that causes liver degeneration, which can further develop into liver cirrhosis and may even be fatal. However, this can also be reversed by abstinence(Weathermon & Crabb,1999).

2.9.Clove:

Kingdom: Plantae

Division: Magnoliophyta- flowering plants.

Class: Magnoliopsida – dicotyledons plants.

Order: Myrtales

Family: Myrtaceae

Genus: Eugenia

Species:S.aramaticum

Comman name: cloves ,aryophyllus (Fringe)

Figure (2-3): clove (Dorenburg *et al*.,2003)

2.10.Effect of cloves on the health:

Clove has both volatile and non-volatile oils, such as tannins, flavonoids and steroidsand the glycoside that makes it of important medical significance.Eugenol is the effective compound of clove oil, It accounts for 70-95% of the total oil weight and has many uses, such as Used as a local anesthetic in dentistry (Curtis,1990) ،The bactericidal properties of clove can be used to treat certain viral infections. In some tropical regions of Asia, it is usually used to treat diseases such as malaria, cholera and tuberculosis.

It has a stimulating effect on the mind and body because it can stimulate memory and the body. It has been used to prepare for labor because it stimulates and enhances uterine contractions during labor. And strengthen it during the perinatal period. It also eliminates fever, cleans the body, disinfects the stomach and intestines, reduces head and nerve pain, strengthens immunity and relieves allergic inflammation, and is used with olive oil when muscle weakness and paralysis(Cortés-Rojas *et al* .,2014)،

The treatment of plant extracts has been widely used to treat different types of diseases, including arteriosclerosis, diabetes and cancer(Hemnani & Parihar,1998) He has indicated (Lee & Shibamoto,2001) The eugenol derivative is used to make stabilizers and antioxidants for plastics and rubber. Clove oil is also used as a good antibacterial, antidote, ,antifungalو antiperspirant and deodorant(Duke *et al*.,2003). As stated by the researcher Clove oil is used to treat various skin diseases such as pimples and acne. It is also used to treat severe burns and dermatitis, and to reduce skin sensitivity (Lans *et al* .,2008).

2.11.The antioxidant efficacy of cloves:

Most of the aromatic and spicy plants, especially clove buds and their oils are well known. The most important thing was has various biological activities, including its antioxidant and anti-inflammatory effects. Because of its ability to dispense with hydrogen atoms, and their interacting with 2,1 hydrazinopyridinediphenyl (DPPH), the dark purple is reduced to light yellow(Fum *et al*.,2007). They are two compounds Eugenol and Isoeugenol were extracted from cloves have an inhibitory effect on the lecithin that occurs by the system Fe^{+2} H_2O_2 (Toda *et al* ., 1994).

The antioxidant activity of some plant is related to the concentration of these substances. Cloves act first, followed by sage, then rosemary, thyme and ginger are The weakest, and it is believed that clove has an effective antioxidant effect, which can inhibit the effectiveness of free radicals and lead to the occurrence of cancer (Shahidi *et al* .,1995) .

Plants containing flavonoids can effectively prevent gastric ulcers, the main reason is that they have antioxidant properties(Harborne & Williams,2000) Also note(Abdel-Wahhab Aly,2005) The use of cloves and

cardamom as a treatment reduces the level of liver enzymes in the serum. The effectiveness of this treatment is due to the fact that both cloves and cardamom contain antioxidants, which contain phenolic compounds that can inhibit the production of free radicals.

Cloves are important in preventing stress and vascular failure caused by streptozotocin drugs that can lead to diabetes in mice, and these activities are due to the presence of oils in cloves, especially eugenol, so it prevents stress inducing pathological changes by antioxidants(Nangle *et al*.,2006) It was also found that the aqueous extract of cloves has a great effect in physiological resisting stress. The extract in a high dose can prevent the development of peptic ulcers in a group of mice stimulated by a stress-restricted cold peptic ulcer and reduce the level of biochemical markers that lead to biochemical damage to cells. Additionally, it decreased the hypoxic-inducing effect of spasticity in mice treated with clove extract(Singh *et al*.,2009) Compared with synthetic antioxidants such as butylated hydroxyl toluene(BHT), the ethanol extract of clove buds is the most effective at inhibiting free radicals (Nagababu *et al*., 2010).

2.12.Oxidative stress and Reactive oxygen species (ROS):

Is an imbalance in the oxidants and antioxidants system towards the production of more oxidants that reflects an imbalance between the systemic manifestations of reactive oxygen species and the ability of the ecosystem to easily detoxify the intermediate reaction or repair the resulting damage. It plays a major role in various diseases (Sies *et al*.,2017).

Reactive oxygen species are produced by reducing molecular oxygen to superoxide radicals O2.Or hydrogen peroxide radicals HO- then form hydrogen peroxide H2O2 (Bienert *et al*., 2006).

Superoxide radicals can disrupt important group Fe-S containing enzymatic metabolism processes or alter their catalytic activity (Van-Breusegem *et al*.,2001) .HO- radicals are present in acidic environments, they can cross biofilms and oxidize lipids by extracting protons from polyunsaturated fatty acids. O2- radicals are rapidly converted to H2O2 by the Superoxide dismutases (SOD) enzyme, and H2O2 radicals can deactivate the enzymes by oxidizing the thiol group (Halliwell,2006).

The harmful properties of (O2- and H2O2)are most pronounced when they react in the presence of metal ions to form highly reactive (HO-) through the (Haber-Weiss) reaction (Kehrer, 2000). Since (HO-) is highly reactive, cells do not possess enzymatic defense mechanisms to remove ROS and rely on mechanisms that prevent their formation. These mechanisms include the removal of (O2 and H2O2) and the isolation of metal ions that catalyze.

the (Haber-Weiss) reaction with metal-binding proteins such as Ferritins and Metallothionine (Hintze and Theil, 2006; Mittler *et al*.,2004). An important feature of ROS chemistry is its conversion to other radicals, in addition to its reaction with H2O2 and the formation of HO. , O2 can also react with the nitric oxide radical (NO) and form peroxynitrite (ONOO-),(ONOO-) quickly turns into (ONOOH) Peroxynitrous , which is a strong oxidizing agent, capable of destroying all vital molecules and leads to the formation of many harmful radicals (Halliwell,2006) .

2.13. Plant defense mechanisms against ROS toxicity:

Levels of ROS (inside the cell) are kept to a minimum by mprotective mechanisms. With An increase in ROS occurs during

certain periods of development as well as in response to certain types of stress. Plant cells possess both enzymatic and non-enzymatic defense mechanisms that can overcome oxygen toxicity and delaying the harmful effects of free radicals (Larson, 1988;Ames *et al*.,1993;).

The enzyme protective system includes SOD, Catalase (CAT) and Ascorbt peroxidase (APX).they are mechanics in removing ROS(Asada *et al*., 2006; Bowler *et al*., 1992; Wellekens *et al*., 1997),These enzymes are able to remove, neutralize ROS. The balance between SOD and APX or the activity of Catalase In cells it has a key role in removing ROS (Bowler *et al*.,1992) without these defenses, plants cannot efficiently convert light energy into chemical energy. SOD acts as a main line of defense to stimulate the conversion of superoxide radicals to molecular oxygen and hydrogen peroxide, then The enzymes CAT and APX remove hydrogen peroxide. SOD and CATs are the most important in cells as antioxidant enzymes, their combined activity converts (O2) and hydrogen peroxide into water and oxygen, thus avoiding cellular damage. Mitochondria and chloroplasts contain mechanically removed ROS R (Mittler *et al*.,2004) Other antioxidants such as ascorbic acid and glutathione are found in high concentrations in chloroplasts and other cellular components, and they have a major role as plant defense mechanisms against oxidative stress (Foyer & Noctor, 1998).

A number of investigations have shown that exposure to ethanol will reduce the content of vitamin C and E. For example, after ingestion of ethanol, a decrease in vitamin C levels was observed in the testes of rats (Bakpinar & Tugrul,1995).

In healthy volunteers, after drinking 84 grams of ethanol, pretreatment with vitamin C (1 gram per day for 3 consecutive days) can reduce alcohol toxicity (mediated by the acetaldehyde cycle) (Wickramasinghe & Hasan,1994). Long-term use of ethanol will increase the level of ascorbic acid free radicals in the bile of alcohol-treated rats (Reinke *et al*.,1997). Ascorbic acid free radicals are considered to be a marker of oxidative stress (Buettner, ,1993). As far as vitamin E is concerned, long-term use of ethanol can significantly reduce the content of this vitamin in mitochondria and microsomes in the cytochromes of rat liver, Kupffer cells (Takeyama *et al*.,1996) and blood cells (Takeyama *et al*.,1993, Koch *et al*.,1994). Low serum vitamin E levels have also been observed in the liver of alcoholics with or without liver disease (Bjorneboe & Bjorneboe,1993) and in patients with alcoholic cirrhosis (Bell *et al*.,1992).

The combination of increased influx of oxygen free radicals and subsequent loss of cellular redox homeostasis can lead to various DNA damages. These include DNA strand breaks, basic modifications (such as 8-hydroxyguanine and thymine glycol), and deoxyribose hydrolysates (Laval,1996). Recent studies have shown that ethanol may exert its cytotoxicity through DNA damage, possibly through NADPH-dependent electron transfer and the oxidation of ethanol metabolite acetaldehyde to produce ROS (Fridovich,1989). After an acute dose of ethanol, the number of single breaks in rat brain cell DNA increased significantly (Singh et al.,1995); ethanol combined with acetaldehyde-induced DNA lysis of rat hepatocytes (Rajasinghe *et al*.,1990), and was also observed in rat thymocytes Ethanol-induced DNA fragmentation and cell death (Ewald &Shao,1993). However, ethanol alone does not cause DNA strand breaks in

human lymphocytes in vitro, while acetaldehyde can cause single-strand and double-strand breaks (Singh *et al*.,1995), indicating that ethanol may cause DNA cross-strand breaks as the main metabolite. The harmful effects of acetaldehyde can be mediated through the formation of DNA cross linkers (Kuykendall & Bogdanffy,1992) and the acetaldehyde-protein pathway (Baraona *et al*.,1993); the removal and repair of DNA crosslinks may cause DNA strand breaks.

2.14.Cytochrome P450 2E1 Definition:

Cytochrome P450 2E1 (CYP2E1) has always been the focus of liver research because it is related to Biological activation of many metabolites and carcinogens (Tu *et al*., 1983; Yang *et al*., 1990). It is known that it is induced by ethanol, and long-term alcohol intake has been associated with several cases of liver disease and cancer (Koop *et al*., 1986;Tsutsumi *et al*., 1989). The latest report by(Cederbaum *et al*.,2009). It has also been shown that when overexpressed in human liver cancer cells, CYP2E1 mediated substrate metabolism is involved in the process of apoptosis (Chen *et al*., 2014) .

The previous characteristics of the CYP2E1 cDNA clone indicate that it is very conserved in various animal species (Song *et al*., 1986; Khani *et al*., 1988; Martignoni *et al*., 2006). Interestingly CYP2E2, which is different from CYP2E1 by only 16 amino acids, was also reported in rabbits. (Khani *et al*., 1988). Previous reports have also shown that CYP2E1 exists in both rat and human brains (Cheung *et al*., 2005).

The expression of CYP2E1 during the embryogenesis and fetal formation of human head tissues was also demonstrated (Choudhary *et al*., 1997).

Therefore, detailed characterization of CYP2E1 nucleotide level expression is very important for a better understanding of brain toxicology that may be mediated by multiple chemicals, including alcohol. Although the existence of CYP2E1 in the rat brain has been fully documented by PCR, RT-PCR and Western blot analysis for more than ten years (Hipolito *et al*., 2007), There is little information about its nucleotide sequence.

2.15.Location of Cytochrome P450 :

Cytochrome P450 is considered one of the enzymes involved in the manufacturing and metabolism processes of many molecules and compounds within the cell. These enzymes are found in many living organisms such as plants, animals, bacteria, and some viruses, and there are approximately 60 cytochrome P450 Genes in humans(Sharma *et al*., 2012). The CYP enzyme was first identified and characterized in mammals. the pigment combined with carbon monoxide showed a characteristic absorption band at 450 nm. Omura and Sato discovered that this pigment is a new type of cytochrome because it contains a heme group and is different from cytochrome b5 in liver microsomal preparations (Omura *et al*.,1964). After these findings, a detailed study of bacterial CYP enzymes was subsequently carried out.

In all kingdoms of life: animals, plants, fungi, protists, bacteria and archaea, as well as viruses, cytochrome (P450) enzymes have been found (Lamb *et al*.,2009) However, they are not ubiquitous. And, for example, it has not been found in Escherichia coli. As of 2018, more than 300,000 distinct CYP proteins are known. (NelsonDR,2009; Nelson, 2018).

CYPs are present in electron transport chains and are known as terminal oxidase enzymes, mainly comprising systems containing P450. The

designation “P450” came from the peak of the light spectrum at the wavelength of the enzyme's maximum absorption (450 nm) when it is in the reduced state and complexed with carbon monoxide. Most CYPs often bond with a protein partner to deliver one or more electrons to reduce iron (and ultimately molecular oxygen).(Hrycay& Bandiera *et al*.,2015).

It was known that the causes the increasing use of genome sequencing CYP enzymes are evolutionarily conserved and exist in all areas of life, bacteria, archaea and eukaryotes, and may exist in viruses (Lamb *et al*.,2020).

2.16.Cytochrome P450 Nomenclature

Enzymes CYP and The genes encoding CYP enzyme are named after the superfamily root symbol CYP, followed by a number to refer the gene family, a capital letter to indicate the subfamily, and another number to reference a single gene. Then it was use italics when referring to genes according to the convention( ).

For example, the gene that encodes the enzyme CYP2E1 is CYP2E1, which is an enzyme involved in the metabolism of acetaminophen (acetaminophen). in spite of CYP450 or CYP450 is sometimes used as a synonym. However, indicating the catalytic activity and the names of the compounds used as substrates. Examples include CYP5A1, CYP51A1, lanosterol 14-α-demethylase, sometimes based on its substrate (Lanosterol) and activity (DeMethylation) Informally abbreviated as LDM, thromboxane A2 synthase, abbreviated as TBXAS1 (ThromBoXane A2 synthase 1), (Chen *et al*.,2014). With the development of the CYP field, it is necessary to standardize the nomenclature of CYP enzymes as ensure that no duplicate gene names and newly discovered CYPs are assigned to the right family and

subfamily Current naming guidelines indicate that new CYP family members must share at least 40% amino acid identity, and subfamily members must share at least 55% amino acid identity .

A naming committee is responsible for assigning and tracking basic gene names (Cytochrome P450 homepage) and allele names (CYP Allele Nomenclature Committee),(Nelson *et al* ., 2009)(Nelson *et al* ., 2011). P450-containing systems According to the properties of electron transfer proteins, CYP can be divided into several categories { Microsomal P450 systems in which electrons are carried from NADPH via cytochrome P450 reductase (variously CPR, POR, or CYPOR).

Cytochrome b5 (cyb5) can also share with in reducing power to this system after being lessen by cytochrome b5 reductase (CYB5R), Mitochondrial P450 systems which utilize adrenodoxin reductase and adrenodoxin to convey electrons from NADPH to P450, Bacterial P450 systems which use a ferredoxinX and a ferredoxin reductase to carry electrons to P450, CYB5R/cyb5/P450 systems in which both electrons wanted by the CYP come from cytochrome b5, FMN/Fd/P450 systems at first found in Rhodococcus species, in which a FMN-domain-containing reductase is Merge to the CYP, P450 only systems, which do not require external reducing power. significant ones include thromboxane synthase (CYP5), prostacyclin synthase (CYP8), and CYP74A (allene oxide synthase)}(Hanukoglu, 1996)

The final formulation of the human and mouse genome confirmed 57 CYP sequences in humans, with 58 pseudogenes and 102 CYP sequences with 88 pseudogenes in mice, calling for a naming group (Nelson *et al*.,2004) related to CYP naming for more information on the law and dozens of sequence information about Of animal and plant species.

2.17. Role of CytochromeP450 in oxidative stress :

Cytochrome P450 (CYPs) is a super family of enzymes that contain heme as a cofactor that acts as a mono-oxygen, during their reaction cycle, contribute to cellular reactive oxygen species (ROS). (Gonzalez *et al*., 1992) CYP enzymes are one of the enzymatic sources of reactive oxygen species that have a role in biology and medicine The role of reactive oxygen species in biology and medicine, They help reduce the balance of cellular (oxidative) stress and play an important role in normal cellular processes, including cell signaling and immune function (Holmstrom *et al*.,2014;Jones *et al*., 2015).

Disorder of the normal redox balance leads to oxidative stress and is involved in many disease processes, including aging and carcinogenesis, ROS is very important to biology and medicine. There are many kinds, including superoxide anion (O2-*), hydrogen peroxide (H2O2), radical hydroxyl (OH*) and even reactive carbonyl (RCO) and reactive nitrogen (RNS).

All of these may hold up the redox balance and cause oxidative stress (Sies *et al* .,2017). ROS exert biological functions and cause cell damage through modification of lipids, nucleic acids and proteins. Lipid peroxidation products, such as F2-isoprostaglandin, are produced by non-enzymatic oxidation of arachidonic acid by oxygen free radicals (Morrow *et al*.,1994) are many times used as a surrogate marker for oxidative stress and ROS levels, but it also has its own biological effects, ROS can also modify DNA, causing mutations and errors in replication, and is known to play a major role in cancer, especially pro-tumor signals, pro-survival signals and drug resistance(Moloney *et al*.,2017),

CYP enzymes, especially CYP2E1, have been shown to produce ROS and lipid peroxidation.oxidative DNA adducts are produce from interact

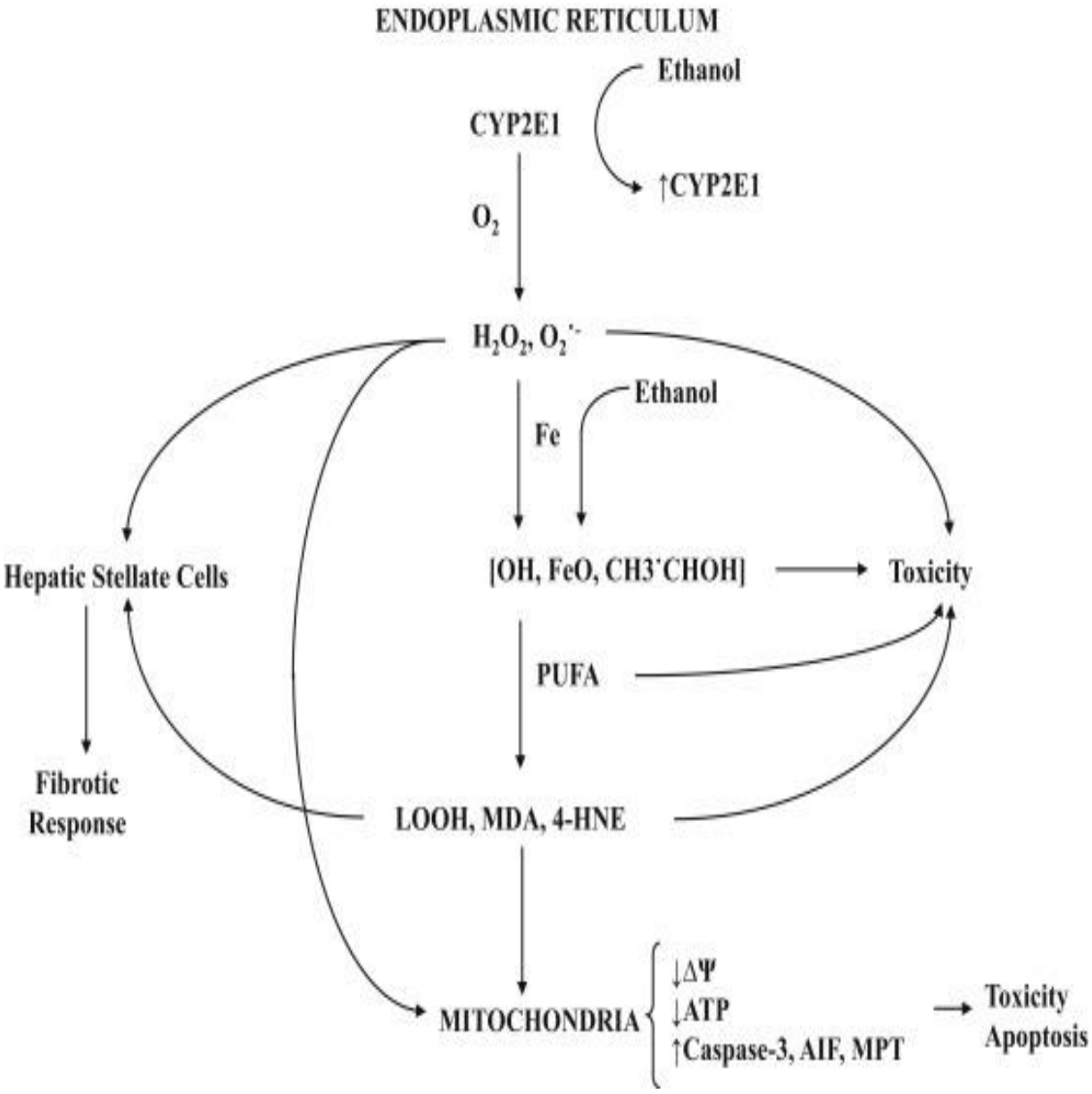
lipid peroxidation with DNA(Linhart *et al* .,2014), Excessive ROS levels and raises in oxidative stress can also modify proteins, especially the amino acid cysteine, which can damage the protein and/or cause toxicity in downstream signaling way(Schieber *et al* .,2014).

CYP enzymes play an important role in human physiology and are involved in the metabolism of biological and xenogeneous drugs as well as biosynthesis of endogenous molecules and are expressed throughout the human body ,CYP enzymes play a prominent role in phase I metabolism in approximately three quarters of drug metabolism reactions in humans, with only five isoforms being responsible for most of these reactions (Guengerich,2008) Although these enzymes can conduct a wide range of reactions, the most common is oxidation of a substrate (R)the general reaction mechanism is (Bae , *et al*.,2011)

$$RH+2H^{+}+O2^{-}+2e \longrightarrow ROH+H2O$$

In mammals, the function of these enzymes is to act on oxidation of steroids, fatty acids, and biological foreign substances. They have an important role in removing various compounds, and affect hormones by synthesizing and breaking them down, also in the biosynthesis of sterols, fatty acids, eicosanoids, vitamins, etal . These proteins have an important role in plants, where they biosynthesize defense compounds, fatty acids and hormones, However, during the catalytic CYP cycle, ROS can be established by separating the enzymatic cycle.

CYP enzymes can rise ROS through its catalytic cycle and change the redox balance, generate oxidative stress, and contribute to the development of diseases. However, some substrates can be modified by CYP enzyme to form reactive intermediates or Products can also promote disease development. It will bie concluded will now mention several physiological or disease states in which ROS or reaction products produced by CYP play a role(CYP1 family in hyperoxic lung injury, CYP enzymes in ROS-mediated hepatotoxicity, CYP2 family in metabolism of ethanol and acetaminophen, CYP3 family in drug metabolism, families in eicosanoid metabolism)(Snezhkina et al.,2019)



Figure(2.4) CytochromeP450 and oxidative stress in the rat liver(Veith and Moorthy .,2018)

Chapter Three

Materials and Method

3.Materials and Methods:

3.1.Materials:

3.1.1. Equipments and Instruments:

Table (3.1): The equipments and instruments used in this study.

No.	Equipment & instrument	Company	Country
1	Autoclave	TOMY Vertical Autoclave	Germany
2	Beaker 250 ml	HAILAO	Italy
3	Centrifuge	Hettich	Germany
4	Cold Eppendorf Centrifuge	Hermle	Germany
5	Cold rack tubes	BIO BASIC INC.	USA
6	Cylinder 100 ml	Boeco	Italy
7	Cylinder 1000 ml	Boeco	Italy
8	Digital camera	Agfa photo	China
9	ELISA reader	Biochrom	England
10	Freezer	Concord	Lebanon
11	Gel electrophoresis apparatus	Consort	Belgium
12	Hot magnetic stirrer	HYSC	Korea
13	Incubator	Memert	Germany
14	Latex gloves	Great glove	Malaysia
15	Latex gloves withoutpowder	Great glove	Malaysia
16	Liquid nitrogen can	Rockefeller	USA
17	Microcentrifuge tubes 1.5ml	Eppendorf	Germany
18	Micropestles	Geneaid	USA
19	Micropipette 1 - 50 µL	CYAN	Belgium
20	Micropipette 10 - 100 µL	CYAN	Belgium
21	Micropipette 100-1000 µL	CYAN	Belgium
23	Morter and pestle	Fisher	USA
24	Multichannal Micropipette	CYAN	Belgium
25	Nano drop	Bionner	Korea
26	PCR Thermocycler	Techne	Korea

27	Pipette tips and Pipette filter tips 10, 200, 1000 ul	Axy Gen	USA
28	Real Time PCR	Bioneer	Korea
30	Refrigerator	KIRIAZI	Egypt
31	Sensitive Balance	Sartorius	Germany
32	Spin down mixer centrifuge	Bioneer	Korea
33	Sterile syringes 1, 5, 10 ml	PROTON	Malaysia
34	Sterile test tubes	PROTON	Malaysia
35	Surgical blade	Fisher	USA
36	Syringe filte 0.20 and 0.40Micron	ISO-Disk	USA
37	Tissue forceps	Fisher	USA
38	Vortex	CYAN	Belgium
39	Water bath	Memert	Germany

3.1.2.ELISA Kits :

Table (3.2): ELISA kits which used in this study with companies and countries of origin.

No.	Type of kit	Company	Country
1	Catalase ELISA KIT	Sigma-Aldrich	USA
2	Glutathione peroxidase ELISA KIT	MyBioSoure	USA
3	Alanine aminotransferase(ALT) ELISA Kit	MyBioSoure	USA
4	Aspartate transaminase (AST) ELISA Kit	MyBioSoure	USA
5	Alkaline phosphatase (ALP) ELISA Kit	Sigma-Aldrich	USA
6	Total bilirubin(TB) ELISA Kit	MyBioSoure	USA
7	TBARS ELISA Kit	MyBioSoure	USA
8	Protein Carbonyle ELISA Kit	MyBioSoure	USA

3.1.3.Quantitative Reverse Transcriptase Real-Time PCR Kits:

Table (3.3): All kits which used in quantification of gene expression levels by qRTPCR with their companies and countries of origin:

No.	Kit	Company	Country
1	**AccuZol™ Total RNA Extraction Kit**	**Bioneer**	**Korea**
	Trizol 100ml		
2	**AccuPower® RocktScript RT PreMix**	**Bioneer**	**Korea**
	- RocketScript Reverse Transcriptase (200 u)		
	- 5× Reaction Buffer (1×)		
	- DTT (0.25 mM)		
	- dNTP (250 µM each)		
	- RNase Inhibitor (1 u)		
3	**AccuPower® Greenstar™ qPCR PreMix**	**Bioneer**	**Korea**
	- PreMix -Ethidium bromide fluorescence -Exicycler™ 20 µL reaction -8 Well strips × 12 each -DEPC – D.W. 1.8 ml × 4 tubes		

3.1.4. Primers:

Two primers were used in this study , thefirst primer used for **B-actinin** gene as House keeping gene and the second primer used for **CYP2E1** gene as target gene. These primers were designed by using NCBI- Gene Bank data base and Primer 3 design online, the primers used in quantification of gene expression using qRT-PCR techniques based Ethidium bromide DNA binding dye, and supported from (Bioneer, Korea) company.

Table (3-4): The Primers and their sequences, gene bank accession number, and references.

Primer	sequences		Reference
CYP2E1	F	5-ATGGCGGTTCTTGGATCAC-3	(Shin et al., 2005)
	R	5-GGGGATATCCTTCAAATCCAGCTG-3	
B-actinin	F	5-ACTCTGTGTGGATTGGTGGC-3	(Zhang et al., 2018)
	R	5-CGCAGCTCAGTAACAGTCCG-3	

3.1.5.Chemicals :

Table (3.5): The chemicals are used in this study and their sources:

No.	Chemicals	Company	Country
1	Agarose	Promega	USA
2	Chloroform	Labort	India
3	DEPC water	Bioneer	Korea
4	EDTA	Sigma-Aldrich	USA
5	Ethanol 100%	Labort	India
6	Ethanol 80%	Labort	India
7	Ethidium Bromide	Promega	USA
8	Formalin 10%	Labort	India
9	Isopropanol	Labort	India
10	Normal saline	Labort	India
11	RNase free water	Bioneer	Korea
12	Tris- Borat-EDTA(TBE) buffer	BIO BASIC INC.	USA

3.2.Experimental animals:

Fourty Male Wister rats (average weight 140 to 190. g), were born in the animal house of the College of Veterinary Medicine, University of Kufa. Rats were raised under controlled ambient temperature (20–25 °C) and fed a standard laboratory diet (19% protein and 3000 kcal energy) and drinking normal water.

3.3.Experimental design:

Forty mature Wister male rats (105 to 120 days old, weight 140 to 190 g) were randomly selected into four equal groups (10 control take distal water orally, 10 positive clove intake, 10 ethanol intake , and 10 mixed Ethanol

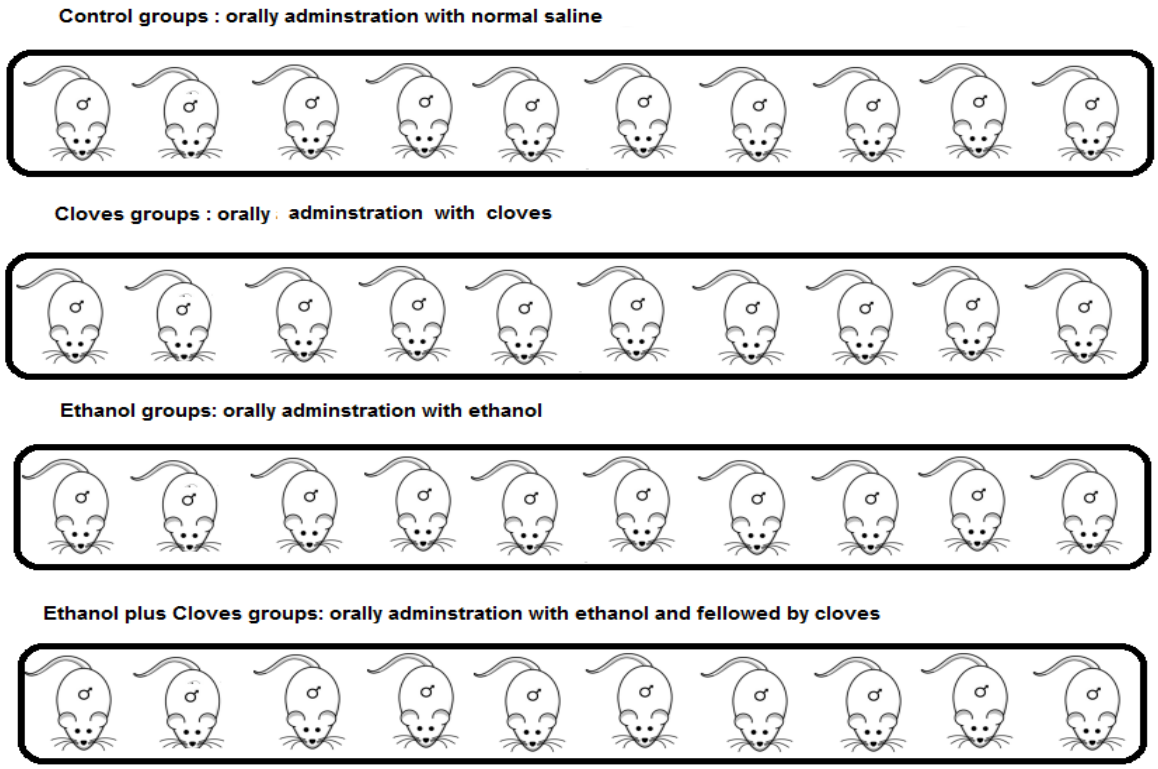
and clove intake. Each animal was identified by tail painting. The animals were treated as follows Figure (3-1):

1- The first: is the (control negative) will be administrate Distal water(1ml/kg body weight) for 4 weeks

2- The second: is the (clove positive) will be administrate clove extract 100 mg/ Kg body weight by Intragastric oral gavage for 4 weeks(Osman et al.,2012).

3 -The third group: will be administrate Ethanol (1 g/Kg body weight of 20% (V/V) ET by Intragastric oral gavage for 4 weeks to induce liver alcoholic disease(Bertola et al.,2013).

4- The fourth group: will be administrate ET + Clove together for 4 weeks.



Figure(3-1): Schematic diagram of experimental study .

Body weight of all rats (40 rats) has been monitored throughout the experiment. At the end of each treatment and control groups period blood samples were obtained from heart for the assessment of the following enzymes (protein carbonyl, and glutathione peroxidase, Liver function test, TBARS, catalase). Each animal was obtained and reserved in formalin 10% for about 6 hours and then kept by paraffin impeding method for estimation of fold changes in gene expression levels of target gene (CYP 2E1) and housekeeping gene (B- actinin) by using quantitative Real-Time PCR technique based on Ethidium bromide dye beyond scarified.

3.4. Methods:

3.4.1. Chemicals preparation:

3.4.1.1. Ethanol 70% Preparation:

Calculation of the volume of 95% ethanol for preparation of 70% ethanol (100 ml) according to the following equation:

$70 \times 100 = 95 \times V2$, where V2~ 74 ml (of 95% ethanol) plus 26 ml of DW 3A.

Total volume is 5000 µl enough to administrate 25 male rat double dose (Barreras-Urbina et al.,2018) .

3.4.1.2. Preparation of clove extract:

The extraction was conducted based on the previous method as described by Cortés-Rojas et al. (D. F. Cortés-Rojas et al.,2014**)**. The clove leaves were macerated using 70% ethanol at a ratio of 1 : 5 (sample : solvent). The sample was soaked for 24 hours and stirred at each 12 hours. Following the harvest of the macerated substance, the process was repeated twice by using the same volume of solvent. Substance was then collected and concentrated by using the rotary evaporator (45°C) within one hour. The ethanol-derived extract was obtained in paste with dark brown color **.**

3.4.1.3. preparation of agarose gel (50 ml):

1. fifty ml. of 1X TBE buffer was taken in a beaker.
2. Half gram of agarose (1%) was added to the buffer.
3. The solution was heated until boiling (using hot magnetic stirrer) and all the gel particles were dissolved.
4. The agarose was stirred in order to mix and to avoid making bubbles.
5. The solution was allowed to cool down at 50-60C°(Surzycki & Belknap,2000).

3.4.1.4. Casting of the horizontal agarose gel:

1.The agarose solution was purified into a gel block, and allowed for 30 minutes to become a gel at room temperature. The comb was carefully removed and the gel was placed in the gel tray.

2. The tray was filled with 1×TBE -electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel(Surzycki& Belkna,2000).

3.4.2. collection of the samples:

At the end of the experiments, food was withheld for 16-18 hr and all animals were sacrificed (anesthetized by chloroform inhalation). The chest and abdomen were opened by thoracotomy and laparotomy. Blood was withdrawn from the heart. LIVER was removed by craniotomy before following investigations were performed**.**

From each rat, 5 ml of blood were collected from the heart using disposable syringe. The blood samples were placed in serum tube and left for 30 minutes. The serum was prepared via centerfugation at 3000 rpm for 10 minutes and kept frozen at -20 °C,For the assessment of the following enzymes concentrations in serum (protein carbonyl, glutathione peroxidase, Liver function test, TBARS and catalase)**.**

3.5.Estmation of levels enzymatic antioxidants:

3.5.1. Estmation of catalase (CAT) levels (U/ml):

Serum rat **catalize enzyme** level(U/ml) was measured using ELISA kit (Table 3.2)(Weydert and Cullen.,2010). in appendix (I)(Weydert and Cullen.,2010)

3.5.2. Estmation glutathione peroxidase enzyme(GPX) levels(ng/ml):

The ELISA was used to examine the glutathione peroxidase enzymes, and the method for examining the enzymes was carried out according to the instructions of the mybiosource company as shown appendix (II)(Weydert and Cullen.,2010) .

3.5.3.Liver function markers:

3.5.3.1. Estmation Serum Alanine Aminotransferase (ALT)(U/ml), Aspartate Aminotransferase (AST)(U/ml)and Alkaline phosphatase(ALP)(mg/dl):

The mindray apparatus was used to examine the enzymes, and the method for examining the enzymes was carried out according to the instructions of the manufacturer producing the device , as shown in appendix (III)(Akter *et al*.,2021).

3.5.3.2. Estmation of Serum Total bilirubin (µg/ml):

The ELISA was used to examine the enzymes, and the method for examining the enzymes was carried out according to the instructions of the manufacturer mybiosource company as shown in appendix (IV)(Akter *et al*.,2021).

3.5.4. Lipid peroxidation:

3.5.4.1. Estmation of Serum thiobarbituric acid(nmol/L):

The ELISA was used to examine the enzymes, and the method for examining the enzymes was carried out according to the instructions of the manufacturer mybiosource company as shown in appendix (V)(Monserrat *et al*.,2017).

3.5.4.2.Estmation of Serum Protein carbonyl (nmol/g):

The ELISA was used to examine the enzymes, and the method for examining the enzymes was carried out according to the instructions of the manufacturer mybiosource company as shown in appendix (VI) (Pirinccioglu *et al* .,2010)

3.6. Molecular analysis:

3.6.1.Quantitative Reverse Transcriptase Real-Time PCR:

qRT-PCR technique was used for quantification of Cyp450 2E1 gene expression levels relative to Housekeeping gene B actin gene expression levels in rat liver have ethanol toxicity and treated with clove. This technique was done according to method described by (Saelens et al., 2018) as follow:

3.6.1.1.Collection of rat liver:

The tissues were cut with a disposable scissor into 3×15 µm section and placed in eppendrof tubes, Tissue were digested by the following process:

3.6.1.1.1. Total RNA extraction:

Total RNA were extracted from rat liver by using (TRIzol® reagent kit) and done according to the company following instructions :

1- Liver tissue was homogenized by grinding in liquid nitrogen, and the tissue powder was transferred into DEPC – treated Eppendorff tube contains 1 ml of TRIzol® reagent. The tubes were shaken vigorously for 30 seconds.

2- Chloroform (200 µl) was added to each eppendorff tube and shaken vigorously for 15 seconds.

3- The mixture was incubated on ice for 5 minutes.

4- The tubes were spined at 12,000 rpm , 4C° , for 15 minutes.

5- Supernatant was transferred to a new eppendorff tube, and isopropanol (500 µl) was added.

6- Mixed mixture by inverting the tube 4-5 times and incubated at 4C° for 10 minutes .

7- Spined at 12,000 rpm , 4C° for 10 minutes.

8- The Supernatant was discarded.

9- Adding 80% Ethanol (1 ml) and Vortex again.

10- Spined at 12,000 rpm , 4C° for 5 minutes.

11- The Supernatant was discarded and the pellet dried.

12- RNase free water (30µl) was added to the sample with vortexing until dissolving.

13- The extracted RNA sample was kept at -20(Lee et al.,2011).

3.6.1.1.2. DNase inactivation (DNase I) Treatment:

The extracted total RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme) and done according to the method described by promega company, USA instructions are follow:

1- RNA (1µl) and 10 × reaction buffer with Mgcl2 (1µl) and DNase I, RNase-free (1µl) and DEPC treated water (7µl) were added to Eppendorff tube.

2- The mixture was incubated at 37C° for 30 minutes.

3- 1µl EDTA was added and incubated at 65C° for 10 minutes.

4- A volume of DNase Inactivation reagent equal to 20% of RNA sample was added to each RNA sample. The tubes vortexed to mix the DNase Inactivation Reagent with RNA sample.

5- All RNA samples left at room temperature for 2 minutes with flicking the tubes once or twice during this period to resuspend the DNase inactivation regent.

6- The tubes were centrifuged at (12,000 rpm) for 1 minute to allow the DNase inactivation reagent separated from RNA sample solution, then, the RNA solutions transferred to new eppendorff tube(Crawford et al.,2006).

3.6.1.1.3. Assessing of RNA yield and quality:

Three quality controls were performed on isolated RNA.the First one is to determine the quantity of RNA (ng/µL) that has been isolated by used Nano drop UV/VIS spectrophotometer (OPTIZEN POP. MECASYS Korea), the second is to purify RNA by reading the absorbance in spectrophotometer at 260 nm and 280 nm in same Nanodrop machine, and the third is the integrity of the RNA by prepared gel electrophoresis, as follow:

1- After opening up the Nano drop software, chosen the appropriate application (Nucleic acid, RNA).

2- A dry Chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 1µl of ddH_2O onto the surface of the lower measurement pedestal.

3- The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of the appropriate blanking solution was added as black solution which is same elution buffer of RNA samples.

4- After that, the pedestals are cleaned and pipet 1µl of RNA sample for measurement.

5- The purity of RNA, also determined by reading the absorbance in Nanodrop spectrophotometer at 260 nm and 280 nm, so the RNA has its absorption maximum at 260 nm and the ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is

generally accepted as “pure” for DNA; a ratio of ~2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

6- After that, the integrity of the RNA was determined by prepared gel electrophoresis as following:

a- 1% agarose gel was prepared in 0.5X TBE buffer and heating by using (hot magnetic stirrer) for 2 minutes until disappear all crystals in agarose solution.

b- After cooling, (3 µL) of Ethidium bromide was added to the solution, then the gel was poured in the tray and left until solidifying. Then transferred into electrophoresis machine which containing same 0.5X TBE buffer.

c- The RNA samples were prepared by mixed 5µl of RNA sample with 1µl of loading dye**.**

d- Then, all amount was transferred into agarose gel wells, then running the electrophoresis power at 100 Volt for 1 hours, then the RNA bands are seen by U.V light (Fleige & Pfaffl,2006).

3.5.1.1.4. cDNA synthesis

DNase-I treatment total RNA samples were used in cDNA synthesis step by using AccuPower® RocktScript RT PreMix kit that provided from Bioneer company, Korea and done according to company instructions as follow:

1- All DNase-I treatment total RNA samples were adjusted at same concentrations that measured by nanodrop by DEPC water.

2- RNA was converted to cDNA by prepare of Reverse transcription PreMix reaction as following table:

Table (3-6): Reverse Transcription PreMix Reaction for converting RNA to cDNA:

RT PreMix	Volume
Total RNA (100ng/µL)	10 µL
Oligo(dT) 15 primer 10 pmole	2 µL
DEPC water	8 µL
Total	**20 µL**

3- 20 µL of RT PreMix was added into AccuPower RocketScript RT PreMix tubes that contains Reverse transcription enzyme at lyophilized form.

4- The lyophilized pellet were dissolved completely by vortex and briefly spinning down.

5- The RNA converted into cDNA under the following thermo cycler conditions:

Step	Temperature	Time
cDNA synthesis	50°C	1 hour
Heat inactivation	95°C	5 minutes

6- Finally, the samples were stored at - 20C° until performed qRT-PCR(Deprez et al.,2002) .

3.6.1.1.5. qRT-PCR based Ethidium bromide I Dye Detection:

qRT-PCR was performed using AccuPower® Greenstar™ qPCR PreMix reagent kit (Bioneer, Korea) and Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer, Korea). According to method described by (Cheon *et al.*,1999). The Ethidium bromide based qRT-PCR PreMix reagent kit is designed for PCR amplification of cDNA for (Cyp450 2E1) target gene by using, Cyp450 2E1 primer and (B-actin) Housekeeping gene. The Ethidium bromide dye that used in this kit is DNA binding dye which reacted with new copies of amplification specific segment in target and housekeeping gene, then the fluorescent signals recorded in Real Time PCR thermocycler.

A genomic DNA standard curve was generated from B-actin gene of Wistar rat (27.9Mbp) were taken from NCBI-Gene Bank information is approximately (~1×10^7) copies(Jozefczuk *et al*.,2011).

3.5.1.1.6. Experimental design of qRT- PCR:

For quantification of (CYP2E1) gene expression in treatment and control samples at duplicate, internal control gene as housekeeping gene (B actin) was used to normalize gene expression levels, therefore, CYP2E1- target gene, and B actin housekeeping gene are showing in the following tables:

A) qRT-PCR Master Mix for Cyp450 2E1 target gene

qPCR PreMix		Volume
cDNA template		10 µL
Primers	Cyp450 2E1 -F	2 µL
	Cyp450 2E1 -R	2 µL
DEPC water		6 µL
Total		20 L

B) qRT-PCR Master Mix for B-actin housekeeping gene

qPCR PreMix		Volume
cDNA template		10 µL
Primers	B actin-F	2 µL
	B actin -R	2 µL
DEPC water		6 µL
Total		20 µL

After that, qPCR PreMix were added into *AccuPower* GreenStar qPCR PreMix tube. Then, real-time PCR tubes sealed by the optical adhesive film and mixed by vigorous vortexing for resuspension of PreMix pellet. The tubes centrifuge at 3,000 rpm, for 2 min, then start Exicycler™ 96 Real-Time Quantitative Thermal Block instrument and loaded the following Program according to kit instruction(Zhang *et al*.,2018):

Table (3-7):After reaction is completed, perform data analysis.

Step	Temp.	Time	Cycle
Pre-Denaturation	95 °C	5 min	1
Denaturation	95 °C	20 sec	45
Annealing/Extension	60 °C	45 sec	
Detection(Scan)			
Melting	-		1

3.6.1.1.7. Data analysis of qRT-PCR:

The data results of q RT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) Livak method that is described by (Livak and Schmittgen, 2001). The relative quantification method, quantities obtained from q RT-PCR experiment must be normalized in such a way that the data become biologically meaningful. In this method, one of the experimental samples is the calibrator such as (Control samples) each of the normalized target values (CT values) is divided by the calibrator normalized target value to generate the relative expression levels. After that, the ΔCT Method with a Reference Gene was used as following equations:

Table (3.8): C_T values required for relative quantification with reference gene as the normalizer.

	Test	Calibrator (cal)
Target gene	CT(target, test)	CT(target, cal)
Reference gene	CT(ref, test)	CT(ref, cal)

First, normalize the CT of the reference (ref) gene to that of the target gene, for calibrator sample:

ΔCT (calibrator) = CT (ref, calibrator) – CT (target, calibrator)

Second, normalize the CT of the reference (ref) gene to that of the target gene, for the test sample:

ΔCT (Test) = CT (ref, test) – CT (target, test)

ΔΔCT= ΔCT (test) – ΔCT (calibrator)

Fold change = $2^{-\Delta\Delta CT}$

Ratio (reference/target) = 2CT (reference) – CT (target)

So, the relative expression was divided by the expression value of a chosen calibrator for each expression ratio of test sample(Zhang et al.,2013).

3.6.2. Comet assay:

Blood samples were collected from all rats, and samples were coded to avoid the possibility of bias. 1 ml of s blood was drawn once from the heart of animals by heparin injection. Samples were immediately transferred on ice to the central scientific laboratory in the veterinary medicine college (Olmedo et al., 2003)

DNA damage analysis for all animal in this study (experimental and control) was the comet assay according to (Bell et al.,2013). Comet slides were prepared in duplicate per subject. Ten mictoleter of tissue grinding cells were mixed with 75 µl of 0.5% lowmelting point agarose . The mixture was cast into previously coated frosted slide with0.5% normal melting point agarose and allowed to gel to solidify. Then it wereplaced in cold lysis buffer (2.5 M NaCl , 10 mMTris-base , 100 mM disodium-EDTA, 1% Na-sarcocinat, 10% DMSO, 1% Triton X- 100, pH 8) for 2 hour at 4°Cbefore

DNA was treated with alkaline solution (0.3 M mNaOH, 1 mM EDTA-NA2,pH 13.2) for 20 minutes to allow for unwinding of the DNA strands. Following , alkaline electrophoresis was run for 30 min at 300 mA and 24 volts , the DNA strands migrate toward the anode according to size.

The extent of migration depends on the number of strand breaks in the nucleoid. The electrophoresis slides were neutralized by washing twice for 5 min in neutralizing buffer (0.4 M Tris, pH 7.5) and once in water before dehydration in 100% ethanol for 20 min, and then it dried in 50°C for 30min to be stored until use. For examination , stored slides rehydrated with chilled water for 30 min and it stained with 85 µl of ethidium bromide for 5 min and the slides washed to remove the excess of stain , covered with coverslip. The migration of damaged DNA was visualized under a florescence microscope at 400x magnification, with a 450-490 nm emission filter and 515 excitation filter . The comet parameters(tail length ,Head DNA%,tailarea) are then evaluated by a software .In this study a total of 40experimental rats were screened per subject (50 cells / eachslide). Undamaged cells resemble an intact nucleus without a tail, while damaged cell has the appearance of a comet, its parameter resemble estimating the degree of DNA damage (14) figure (4-2).

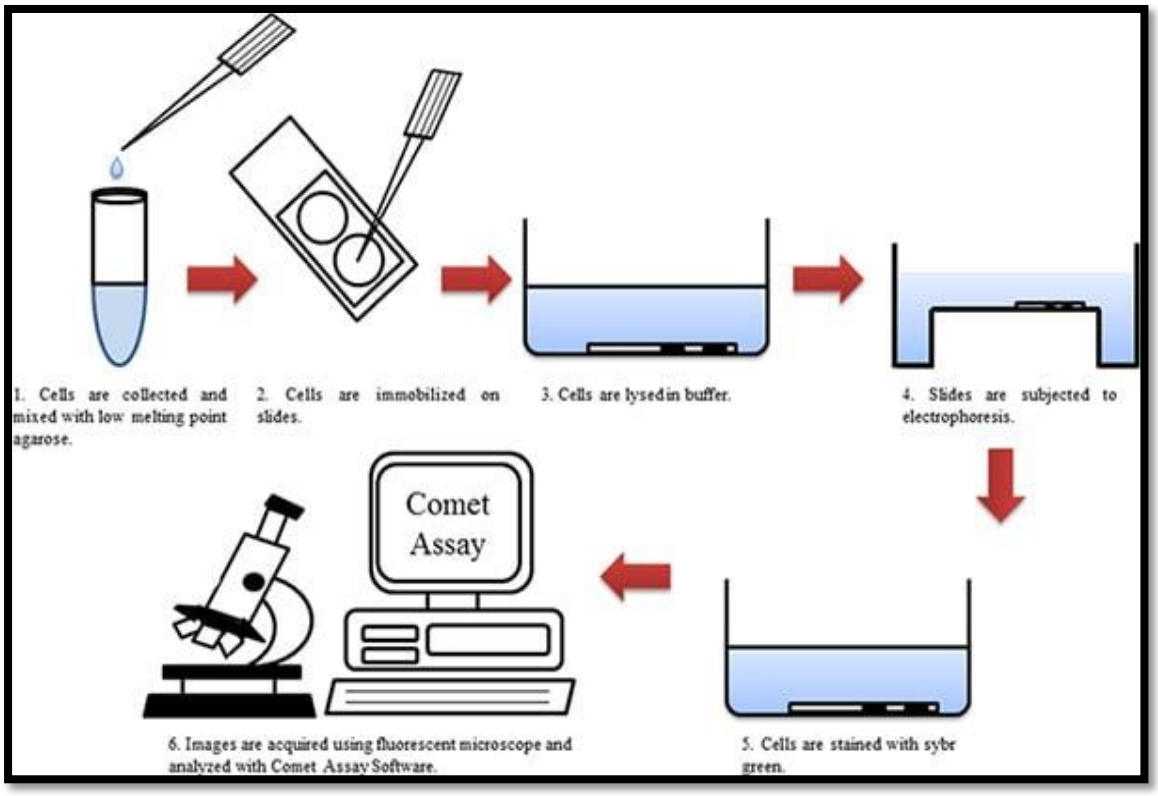


Figure (4-2) showed DNA comet assay procedure(Collins;2004)

3.7. Statistical analysis:

All the values are expressed as mean ± SE. Data of each of 23d, 30d and 50d periods of the experiment were analyzed using Anova Tukey HSD test to estimate the significant differences between groups. P value less than 0.05 was considered significant (Abdi, & Williams, (2010).

The data results of q RT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) Livak method that described by (Livak and Schmittgen, 2001). The relative quantification method, quantities obtained from q RT-PCR experiment must be normalized in such a way that the data become biologically meaningful. In this method, one of the experimental samples is the calibrator such as (Control samples) each of the normalized target values (CT values) is divided by the calibrator normalized target value to generate the relative expression levels (Livak and Schmittgen, 2001).

Chapter Four

Result

4. Result:

4.1.Body wight:

The body weight of the rats at the beginning of the study was similar in studied groups. At each the experimental periods (10-d, 20-d and 30- d), there was no significant difference (P>0.05) in body weight gain between male rats (figure 4-1).

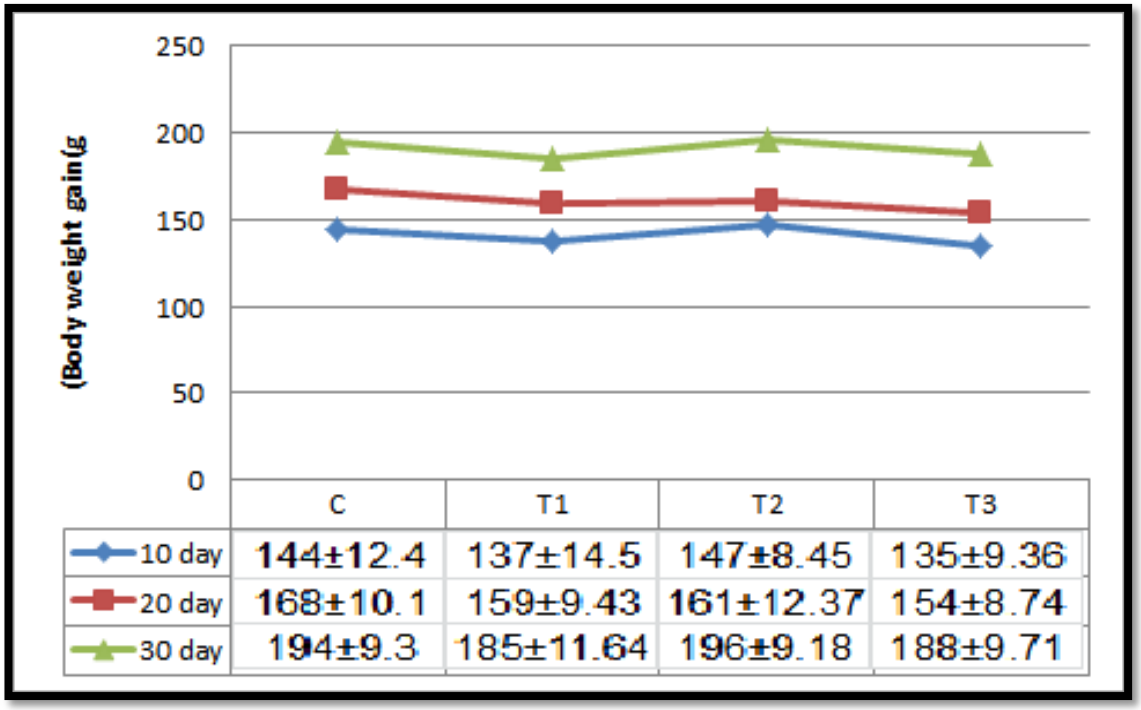


Figure (4-1): Effect of ethanol and clove against body weight gain (g) of 10d, 20d, and 30d old male rats.

- **Values represents Mean ± SE.**
- **C: male rats orally intubation with distal water (1ml/kg).**
- **T1: male rats orally intubation with Clove (100 mg/kg of body weight).**
- **T2: male rats orally intubation with ethanol (1 g per kg).**
- **T3: male rats orally intubation with ethanol and Clove (1 g/kg ethanol and 100mg/kg body weight).**
- **N=10 rats per each group.**

4.2.Levels of enzymatic antioxidants:

4.2.1. Levels of catalase:

Figure (4-2) clarified the effect of ethanol and clove on the catalase concentration in serum rats (U/ml). Treated male rats (T2) registered low significant ($P<0.05$) level of catalase throughout the three experimental groups(T1,T2 and T3) compared with control male rats. The concentration of T2 was found (472.17± 2.548), T3 groups (507.33± 2.641), otherwise the result found non significant differences between control group and treatment groups (T1), it was recorded as (524.94±0.931) and (533.15±2.14) respectively. Appendix(VII)

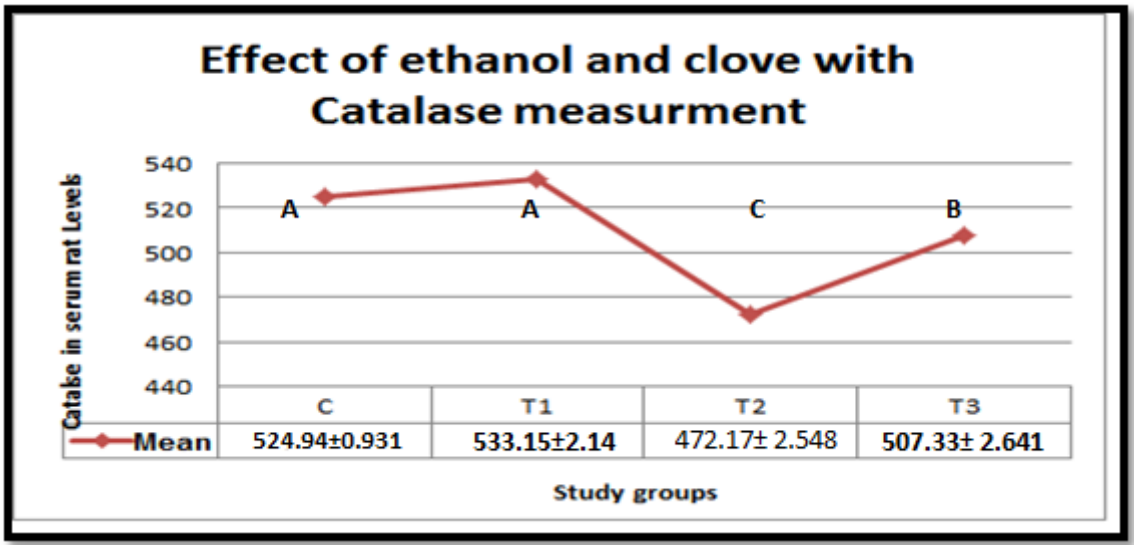


Figure (4-2): Effect of ethanol and clove with Catalase concentrations (U/ ml) of male rats.

- **Values represents Mean ± SE.**
- **C: male rats orally intubation with distal water (1ml/kg).**
- **T1: male rats orally intubation with Clove (100 mg/kg of body weight).**
- **T2: male rats orally intubation with ethanol (1 g per kg).**
- **T3: male rats orally intubation with ethanol and Clove (1 g/kg ethanol and 100mg/kg body weight).**
- **N=10 rats per each group.**

4.2.2.Serum Glutathione peroxidase (Gpx) Concentration:

Table (4-3) shows the concentration of GPx in serum in the control group and three treated groups along the length of the experiment, and the table showed a significant ($P < 0.01$) increase after four weeks in T2 compared to the control group, and the result also showed that the oral intubation caused by (1 g/kg body weight) ethanol and (100 mg/kg) clove extract (T3) in rats resulted in a significant ($p < 0.01$) decrease in serum GPx concentration compared to the ethanol group (T2). , the result did not find any significant association between the control group and the clove group, with respect to cloves.Appendix(VII)

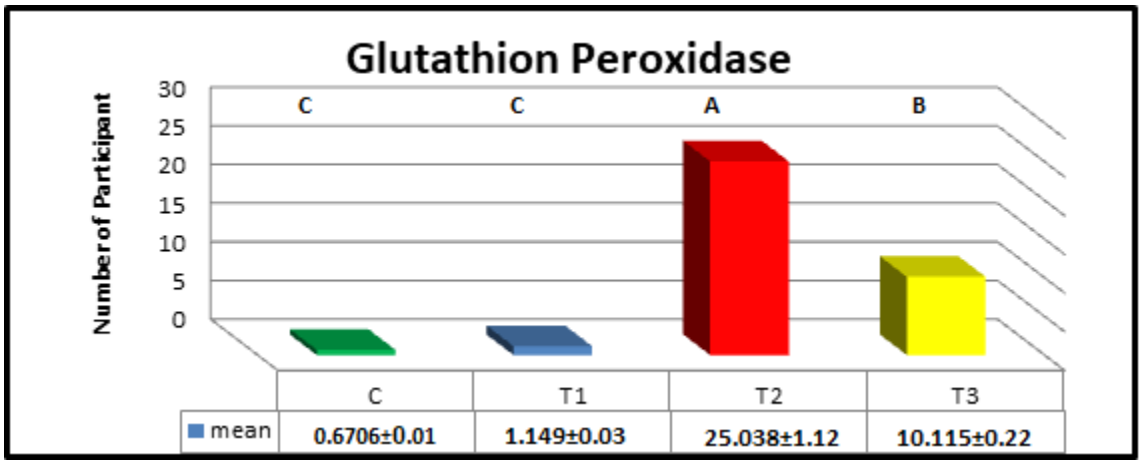


Figure (4-3): Effect of ethanol and clove against Glutathion peroxidase old male rats.

- **Values represents Mean ± SE.**
- **C: male rats orally intubation with distal water (1ml/kg).**
- **T1: male rats orally intubation with Clove (100 mg/kg of body weight).**
- **T2: male rats orally intubation with ethanol (1 g per kg).**
- **T3: male rats orally intubation with ethanol and Clove (1 g/kg ethanol and 100mg/kg body weight).**
- **N=10 rats per each group.**

4.3.Liver function test:

4.3.1.Alanine aminotransferase Test(ALT):

Results of ALT concentration in the serum treated male rats(U/ml), illustrated in figure (4-4), revealed significant elevation ($P<0.05$) in the second group (T2) and third (T3) groups when compared with male rats of control and group (T1). It was recorded as (40.46 ± 1.198) and (31.88 ± 0.393) respectively, However, in the, ALT levels in serum male rats of the two group (control and T1 group) registered values near each other as (24.683±0.336) and (26.75±1.268), respectively. Appendix(VII)

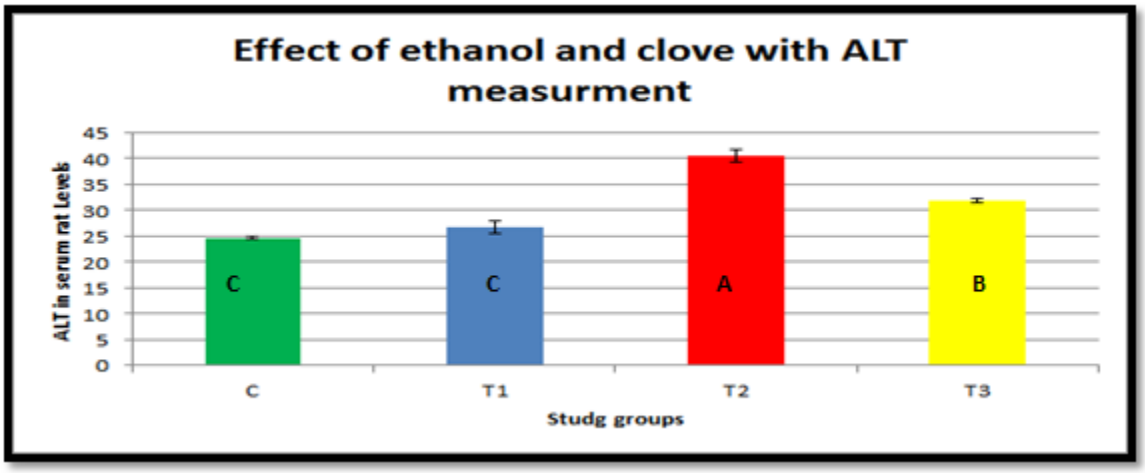


Figure (4-4): Effect of ethanol and clove against Alanine aminotransferase(U/ml) old male rats.

- **Values represents Mean ± SE.**
- **C: male rats orally intubation with distal water (1ml/kg).**
- **T1: male rats orally intubation with Clove (100 mg/kg of body weight).**
- **T2: male rats orally intubation with ethanol (1 g per kg).**
- **T3: male rats orally intubation with ethanol and Clove (1 g/kg ethanol and 100mg/kg body weight).**
- **N=10 rats per each group.**

4.3.2.Aspartate transaminase (AST):

Figure (4-5) clarified the effect of ethanol and clove on the AST concentration in serum rats (U/ml). Treated male rats (T2) registered the highest significant ($P<0.05$) level of AST throughout the three experimental groups (T1,T2 and T3) compared with control male rats. The concentration of T2 was found (150.94± 0.987), T3 groups (139.93± 0.617), otherwise the result found non-significant differences between control group and treatment groups(T1), it was recorded as (131.33±0.605) and (133.9±0.77) respectively. Appendix(VII)

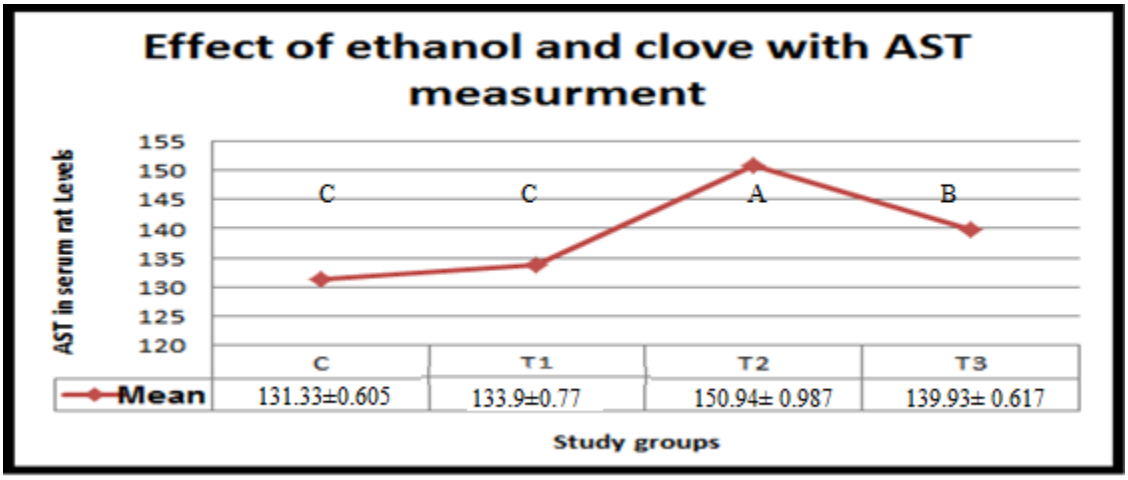


Figure (4-5): Effect of ethanol and clove with AST concentrations (U/ml) of male rats.

- **Values represents Mean ± SE.**
- **C: male rats orally intubation with distal water (1ml/kg).**
- **T1: male rats orally intubation with Clove (100 mg/kg of body weight).**
- **T2: male rats orally intubation with ethanol (1 g per kg).**
- **T3: male rats orally intubation with ethanol and Clove (1 g/kg ethanol and 100mg/kg body weight).**
- **N=10 rats per each group.**

4.3.3.Alkaline Phosphatase (ALP):

Figure (4-6) depict serum concentrations of ALP (mg/dl) in male rats, showing insignificant differences (P>0.05) between experimental groups throughout the three experimental groups (T1, T2, and T3) compared with control groups, the result was (183.84±0.17), (141.73±0.739), (143.71±0.704) and (136.15±2.72) respectively. Appendix(VII)

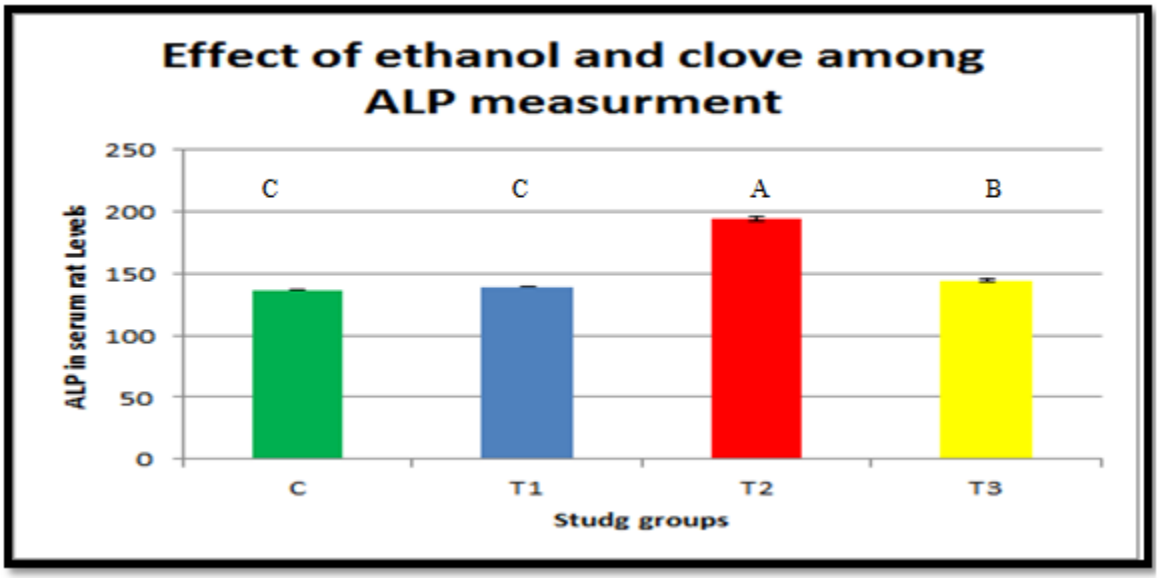


Figure (4-6): Effect of ethanol and clove with ALP concentrations (mg/ dl) of male rats.

- **Values represents Mean ± SE.**
- **C: male rats orally intubation with distal water (1ml/kg).**
- **T1: male rats orally intubation with Clove (100 mg/kg of body weight).**
- **T2: male rats orally intubation with ethanol (1 g per kg).**
- **T3: male rats orally intubation with ethanol and Clove (1 g/kg ethanol and 100mg/kg body weight).**
- **N=10 rats per each group.**

4.3.4.Total bilirubin (TB):

Figure (4-7) clarified the effect of ethanol and clove on the TB concentration in serum rats (µg/ml). Treated male rats (T2) registered highest significant (P<0.05) level of TB throughout the three experimental groups (T1,T2 and T3) compared with control male rats. The concentration of T2 was found (11.55± 0.58), T3 groups (10.29± 0.17), otherwise the result found non significant differences between control group and T1 treatment groups, it was recorded as (9.57± 0.35) and (9.94± 0.17) respectively. Appendix(VII)

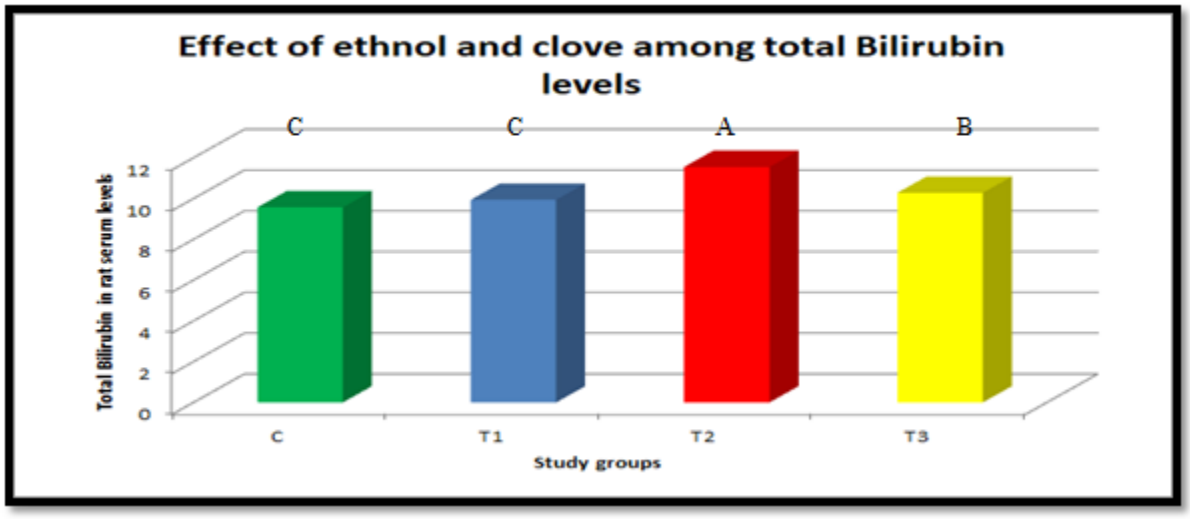


Figure (4-7): Effect of ethanol and clove with Total bilirubin concentrations (µg/ml) of male rats.

- **Values represents Mean ± SE.**
- **C: male rats orally intubation with distal water (1ml/kg).**
- **T1: male rats orally intubation with Clove (100 mg/kg of body weight).**
- **T2: male rats orally intubation with ethanol (1 g per kg).**
- **T3: male rats orally intubation with ethanol and Clove (1 g/kg ethanol and 100mg/kg body weight).**
- **N=10 rats per each group.**

4.4.Lipid peroxidation :

4.4.1.Thiobarbituric acid reactive substance TBARS assay:

Figure (4-8) clarified the effect of ethanol and clove on the TBARS concentration in serum rats (nmol/L). Treated male rats (T2) registered the highest significant (P<0.05) level of TB throughout the three experimental groups (T1,T2 and T3) compared with control male rats. The concentration of T2 was found (3.2± 0.15), T3 groups (2.44± 0.2), otherwise the result found non-significant differences between control group and T1 treatment groups, it was recorded as (2.19± 0.13) and (2.29± 0.13) respectively. Appendix(VII)

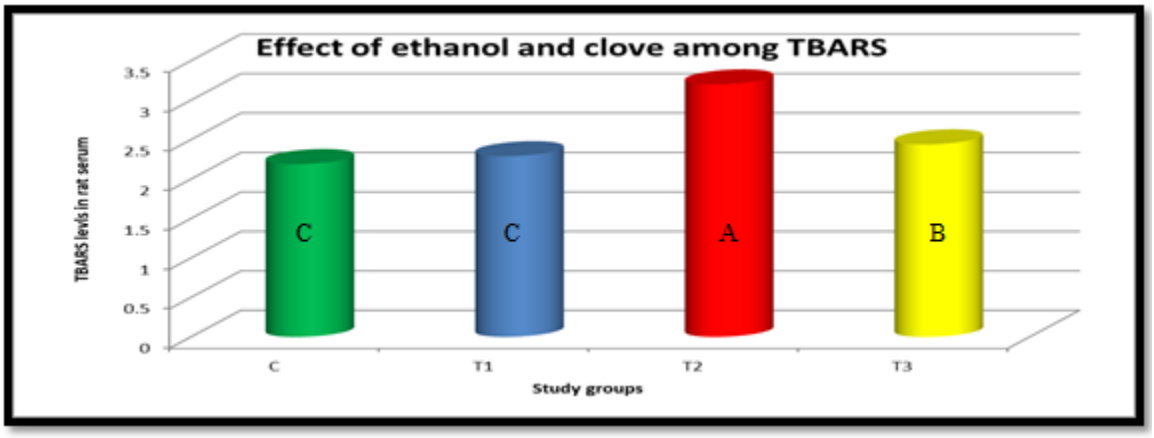


Figure (4-8): Effect of ethanol and clove with TBARS concentrations (nmol/L) of male rats.

- **Values represents Mean ± SE.**
- **C: male rats orally intubation with distal water (1ml/kg).**
- **T1: male rats orally intubation with Clove (100 mg/kg of body weight).**
- **T2: male rats orally intubation with ethanol (1 g per kg).**
- **T3: male rats orally intubation with ethanol and Clove (1 g/kg ethanol and 100mg/kg body weight).**
- **N=10 rats per each group.**

4.4.2. Serum protein carbonyl Concentration(PC):

Figure (4-9) illustrated the serum Protein carbonyl (nmol/g) in the control and three treated groups along the experimental period , the table showed a significantly (P<0.01) increase after four weeks in T2 (2.78± 0.11) as compared to control group (2.09± 0.05) ,the result also showed that oral intubation of (6g/kg B.W) from ethanol and (100mg/kg) clove extract (T3) (2.27± 0.07) in rats caused significant (p<0.01) decrease in serum protein carbonyle concentration as compared to ethanol group (T2).on the other hand , the result did not find a significant association between control and clove group (2.18± 0.05), respectively. Appendix(VII)

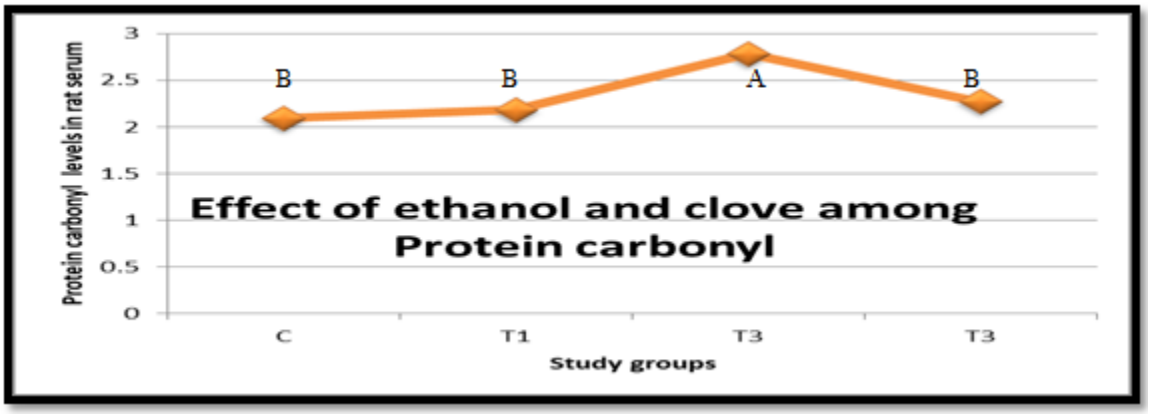


Figure (4-9): Effect of ethanol and clove with PC concentrations (nmol/g) of male rats.

- **Values represents Mean ± SE.**
- **C: male rats orally intubation with distal water (1ml/kg).**
- **T1: male rats orally intubation with Clove (100 mg/kg of body weight).**
- **T2: male rats orally intubation with ethanol (1 g per kg).**
- **T3: male rats orally intubation with ethanol and Clove (1 g/kg ethanol and 100mg/kg body weight).**
- **N=10 rats per each group.**

4.5.Molecular analysis

4.5.1.Determination of DNA damage by commet assay in liver of male rats.

The results of comet assay of four groups were listed in table (4-1). In this study, a significant increase in comet assay in rats that DNA damage (Comet Area) was observed in rats given ethanol it found significant increase of ethanol groups (1555.667 ±88.895) rather than other groups as (1528 ±135.011, 1523±133.08 and 1543.51± 109.12) for Control, Cloves, and ethanol plus cloves, respectively, On the other hand it found significant decrease of DNA in head in ethanol groups (30.829 ±5.82) rather than other groups as (90.104± 0.628, 91.057 ±0.727and 73.17 ± 8.13) for Control, cloves, and ethanol plus cloves, respectively. Regarding the last position (tail area), the results were significant differences our result was found to be more effect on the final portion of target DNA which was determine DNA damage, it found 69.171 ±5.82 in the ethanol group rather than other groups as (8.943 ±0.727, 8.780±0.720 and 26.8±38.13), for control group, cloves, ethanol plus cloves, respectively.

Table (4-1) : DNA damage assessment of male rats treated with alcohol and clove extract

Treatment parameter	**Control group** N=10 **Mean±SD**	**Cloves group** N=10 mean±**SD**	**Ethanol group** N=10 mean±**SD**	**Ethanolplus cloves** N=10 mean±**SD**	**P.Value**
Comet Area (px) **Mean±SD**	**1528±135.011 C**	**1523±133.08 C**	**1555.66±88.89 A**	**1543.51±11.12 B**	**0.047**
%DNA in Head **Mean±SD**	**90.104±0.628 A**	**91.057 ±0.727 A**	**30.829 ±5.82 C**	**73.17± 8.13 B**	**0.036**
Tail Area (px) **Mean±SD**	**207.67±31.09 C**	**201.54±29.97 C**	**1051 ±238.434 A**	**450.51±22.39 B**	**0.038**
%DNA in Tail **Mean±SD**	**9.343 ±0.727 C**	**8.780±0.720 C**	**69.171 ±5.82 A**	**26.8±38.13 B**	**0.027**

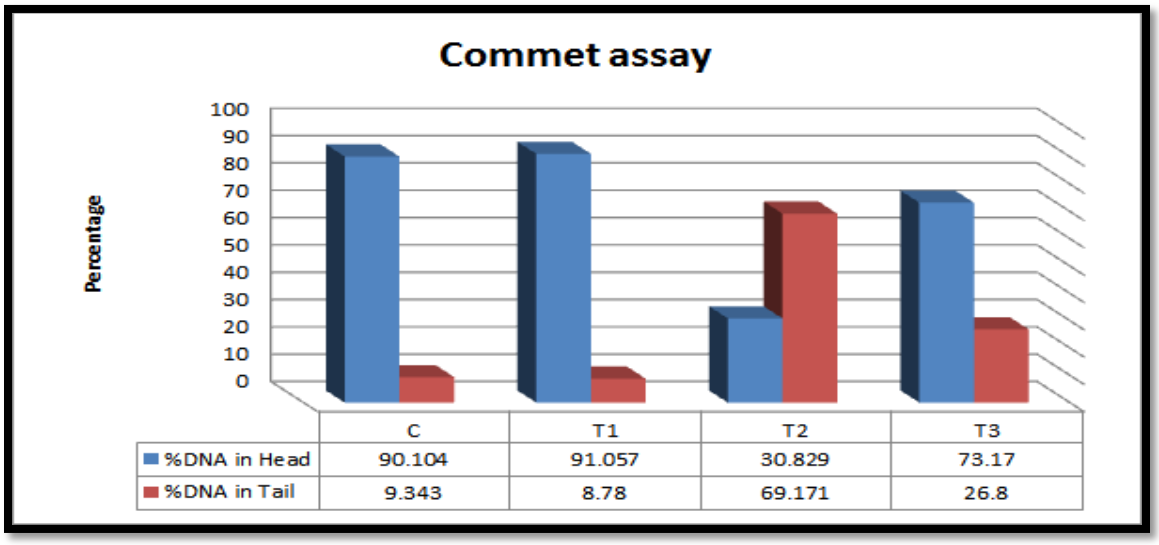


Figure (4-10): Determination of DNA damage assessment by using commet assay

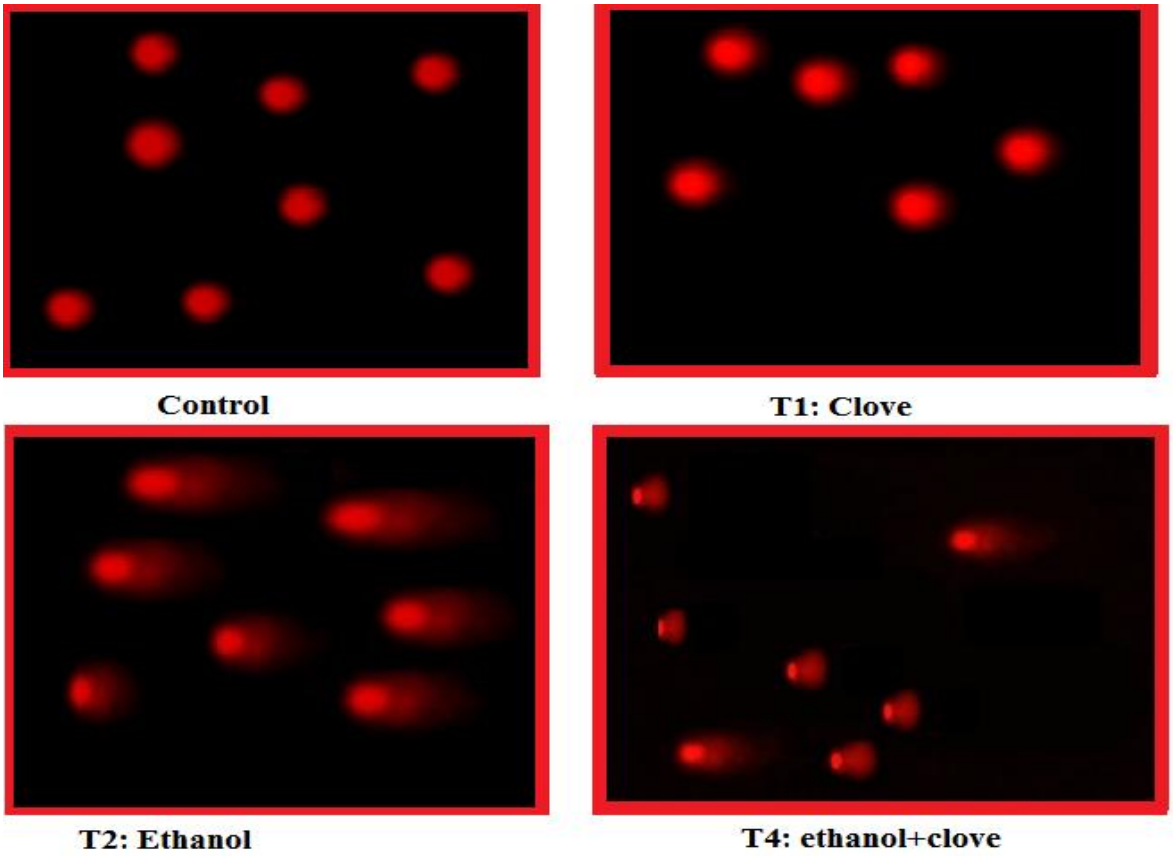


Figure (4-11):Determination of DNA damage assessment by using commet assay

4.5.2.The Concentrations and Purity of Total RNA:

Total RNA concentrations and purity were assessed using Nano drop spectrophotometer in absorbance readings (260/280 nm). All liver tissue samples used in the present study during the four experimental groups gave high concentrations of total RNA and appeared quantitatively enough to proceed in quantitative reverse transcriptase real- time PCR. The purity of total RNA samples (also assessed using agarose gel electrophoresis) of liver tissues obtained from experimental male rats recorded different band thickness figure (4-12). The figure shows clear 18s and 28s band.

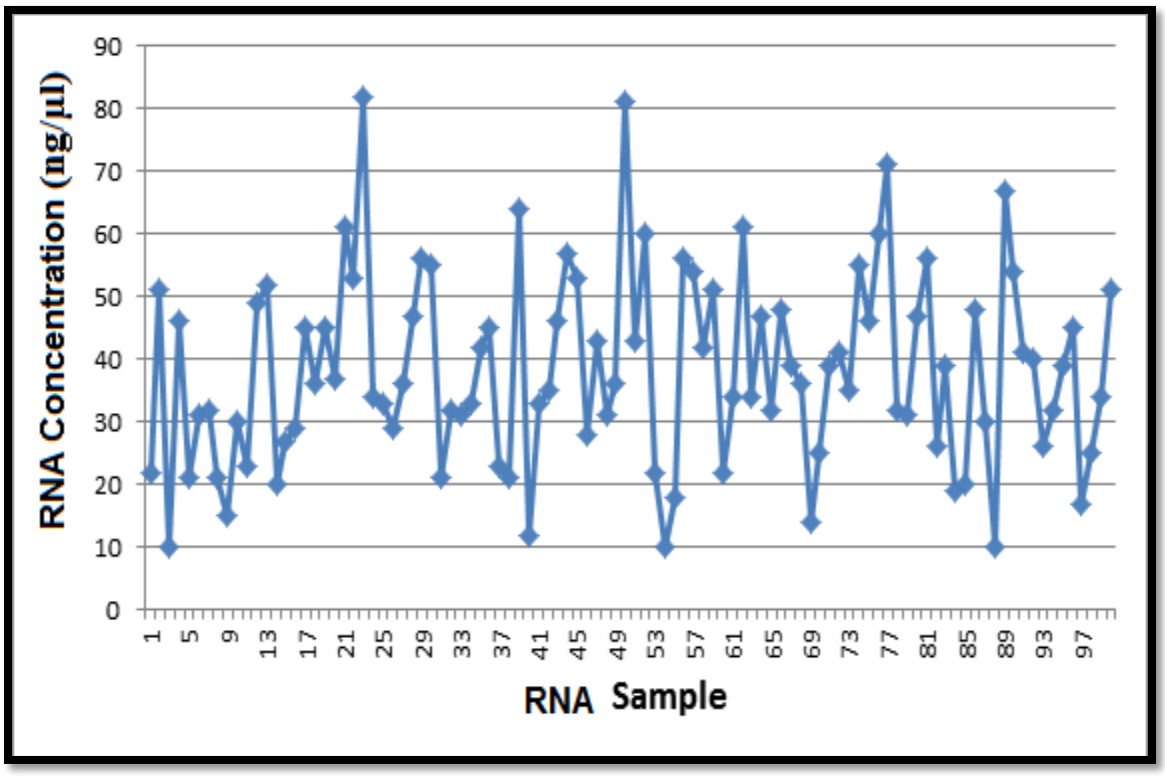


Figure (4-12): : Concentration of RNA extracted from liver tissue in all study groups.

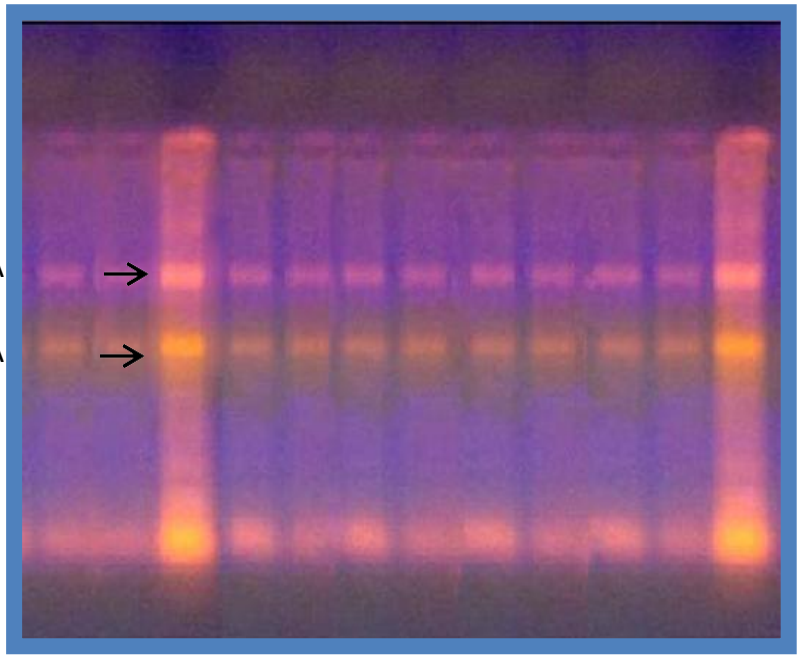


Figure (4-13): agarose gel electrophoresis analysis of total RNA in liver tissues of male rats .

4.5.3.Quantitative Reverse Transcriptase Real- Time PCR:

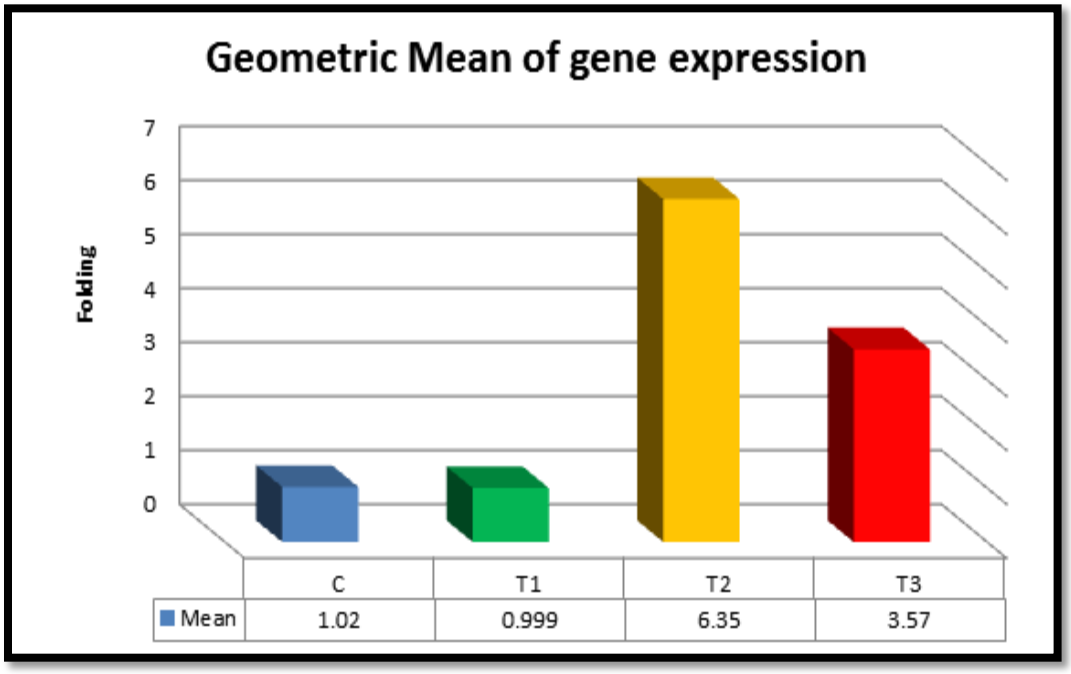
Data analysis of Ethidium Bromide based reverse transcriptase real-time PCR assay were divided into relative quantification of CYP2E1 gene expression level normalized by housekeeping gene expression (Actinin- B).

4.5.4.Relative quantification of target gene expression:

To calculate the relative expression of target gene (CYP2E1) in rat liver tissue, the 2^ΔCt (livak) method used by normalizing the gene expression of target gene with expression of housekeeping gene (B-actinin) as reference gene. At first, the threshold cycle number of target gene normalized to that of reference gene in all treatment groups and calibrator

(control groups). Second,the ΔCt of treatment groups and the ΔCt of control group is normalized, and finally the expression ratio (fold change) was calculated (2^ ΔCt).

4.5.5.Relative quantification of CYP2E1 gene expression:



Reverse transcriptase real-time PCR results have shown that CYP2E1 mRNA expression level increased more than 10-folds in Treatment group 2 (ethanol treatment) male rats treated compared with corresponding of control male rats (slightly more than 2-fold) as well as in the treated male rats (around 1-fold) (figure 4-14) Geometric mean of gene expression.

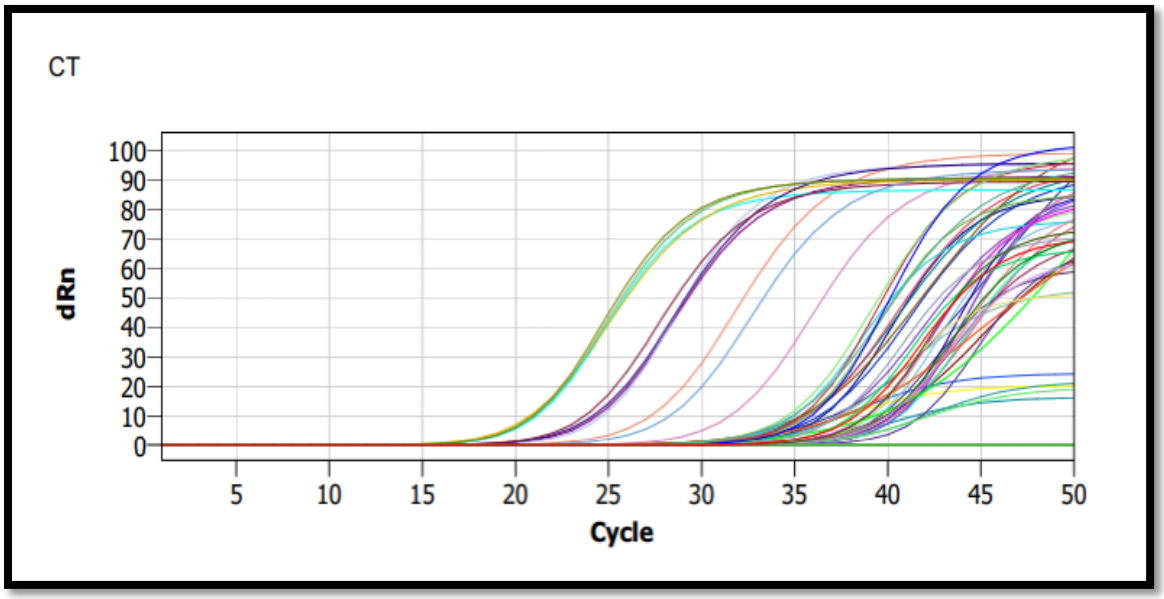


Figure (4-15): Effect of ethanol on *CYP450E2* gene expression (fold change) in liver tissues of male rats.

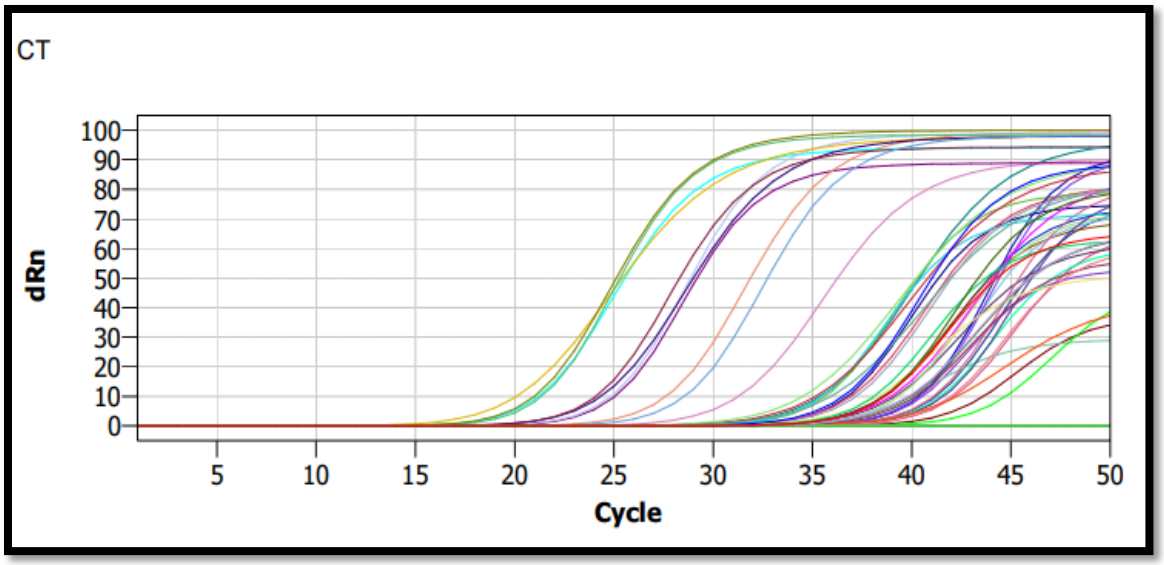


Figure (4-16): Effect of ethanol on B-actinin gene expression (fold change) in liver tissues of male rats.

Chapter Five

Discussion

5. Discussion:

5.1.body weight:

Figure (4-1) shows the relationship of the weight of the rats with the substances that the Rats were dosed with during the experiment period, The body weight of all the Rat at beginning of the study was similar in the study groups. At each of the experimental periods (10-d, 20-d and 30- d), there was no significant difference (P>0.05) in body weight gain between male rats, during Alcohol consumption, this result was agreement with (Kołota *et al*.,2019) who found no significant differences between his groups and he decided that Alcohol consumption may influence hepatic antioxidant defense in adolescent male rats, but without influence on body weight gain., Other study showed a positive relationship between Alcohol consumption without antioxidant , and Body weight, they typically refer to the calories in alcohol as the leading cause of weight gain ; alcohol provides 7 calories per gram(French *et al*.,2010).

5.2. Levels of enzymatic antioxidants:

5.2.1. Catalase enzyme :

The Figure (4-2) clarified the effect of ethanol and clove on the catalase levels in serum rats (U/ml). the treated male rats (T2) registered low significant (P<0.05) concentration of catalase throughout the three experimental groups (T1 and T3) compared with control male rats. The concentration of T2 was recorded as (472.17± 2.548), and T3 groups (507.33± 2.641).

Several studies reported the increase in catalase activity after chronic ethanol feeding (Orellana *et al*., 1998), this result was in agreement with (Das & Vasudevan, 2007) the catalase activity relies on the cellular level of

H2O2, the ability of catalase to metabolize ethanol may be increased under oxidative stress with the increase in cellular H2O2 production (Wu & Cederbaum, 2003). This was observed with perfused rat liver, when fatty acids were added and the process of peroxisomal β oxidation lead to the generation of H2O2 and oxidation of ethanol. This raises the possibility that under conditions of oxidant stress (and H2O2 production) catalase-mediated ethanol oxidation may be increased in T2 Treatment.

5.2.2. Glutathione peroxidase(GPX):

Figure (4-3) illustrated the serum GPx concentration in the control and three treated groups along the experimental period , the table showed a significant ($P<0.01$) increase after four weeks in T2 as compared to other groups, it was found that the concentration of GPx 25.038 ng/ml, Glutathione reductase activities were increased in both intracellular compartments and catalase activity was increased as a consequence of ethanol exposure This study demonstrates that chronic ethanol-induced alterations in the glutathione/GSHPx-1 antioxidant system might promote oxidative modification of liver proteins, namely those of the mitochondrion, which could contribute to the adverse effects of ethanol on the liver(Bailey *et al*.,2001). Through our observation on Figure (4-3), there was a clear decrease in the fourth group (T3) that was dosed with clove extract and alcohol compared with the third group (T2).

The most important thing about the plant clove was that it has various biological activities, including its antioxidant and anti-inflammatory effects. Because of its ability to dispense with hydrogen atoms, and their interaction with 2,1 hydrazinopyridinediphenyl (DPPH), the dark purple is reduced to light yellow(Fum *et al*.,2007). They are two compounds Eugenol and

Isoeugenol were extracted from cloves which have an inhibitory effect on the lecithin that occurs in the system Fe^{+2} H_2O_2(Toda *et al* ., 1994).

5.3. Liver function test:

5.3.1.Alanin aminotransferase(ALT):

The Results of ALT concentration in the serum treated male rats, illustrated in figure (4-4), revealed significant elevation ($P<0.05$) in the second (T2) and third (T3) groups when compared with male rats of control and first (T1) group. It was recorded as (40.46 ± 1.198) and (31.88 ± 0.393) respectively, However, in the, ALT levels in serum male rats of the two group (first and control group) registered values near each other as (24.683±0.336) and (26.75±1.268), respectively, the result was in accordance with (Morales *et al*., 2018) who found that the groups with weekend alcohol consumption presented a significant increase of ALT liver enzymes that was six times greater than control groups of rats,. ALT catalyzes the transfer of an amino group from L-alanine to α-ketoglutarate, the products of this reversible transamination reaction being pyruvate and L-glutamate (Cascella *et al*., 2020).

ALT is commonly measured clinically as part of liver function tests and is a component of the AST/ALT ratio(Botros *et al*.,2013)When used in diagnostics, it is almost always measured in international units/liter IU/L or µkat(Alkhawaja *et al*.,2017).

5.3.2.Aspartate aminotransferase(AST):

Figure (4-5) clarified the effect of ethanol and clove on the AST concentration in serum rats (U/ml). Our result were found treated male rats (T2) registered highest significant (P<0.05) level of AST throughout the three experimental groups (T1,T2 and T3) compared with control male rats. The concentration of T2 was found (150.94± 0.987), T3 groups (139.93± 0.617), otherwise the result found non-significant differences between control group and treatment groups (T1), it was recorded as (131.33±0.605) and (133.9±0.77) respectively. This result was in agreement with (Wang *et al*., 2020) Some studies showed the effect of ethanol on the liver body of rat by using antioxidant camazolin the effect of camazolin on AST and liver injury serum biomarker enzymes (such as ALP and ALT),compared with the normal control group, the AST level of the ethanol-induced model group was significantly increased (p <0.05) and the dose of Chamazoline is 25 mg/kg body weight and 50 mg/kg body weight were decrease Compared with the ethanol-induced group.

5.3.3.Alkaline Phosphatase(ALP):

Figure (4-6) depicts serum concentrations of ALP (mg/dl) in male rats, showing non-significant differences (P>0.05) between experimental groups throughout the three experimental groups (T1, T2, and T3) compared with control groups, the result found as (183.84±0.17), (141.73±0.739), (143.71±0.704) and (136.15±2.72) respectively.

Some studies found the serum ALP level increased moderately (5.73 +- 0.33 U/ml is p <0.050. chronic ethanol feed also causes an increase in the ALP activity of the liver homogenate, but a decrease in the activity of the isolated hepatocytes, Raised serum ALP levels have been reported in

alcoholics (Alves *et al*., 1982) and in rats given ethanol (Masanobu, & Teschke, 1982). In addition, (Teschke *et al*.,1983) have reported that alcoholic fatty liver in humans is associated with increased hepatic ALP activity. However, the mechanism for increased ALP activity in response to chronic ethanol feeding remains unclear. Although it has been shown quite convincingly that serum and hepatic levels of ALP will increase in response to biliary obstruction.

5.3.4.Total bilirubin(TB):

Figure (4-7) showed the effect of ethanol and clove on the Total Bilirubin concentration in serum rats (µg/ml). the treated male rats (T2) registered highest significant ($P<0.05$) level of Total Bilirubin throughout the three experimental groups (T1 and T3) compared with control male rats. The concentration of T2 was found (11.55± 0.58), T3 groups (10.29± 0.17), Otherwise the result found non-significant differences between control group and T1 treatment groups, it was recorded as (9.57± 0.35) and (9.94± 0.17) respectively.

The result was in accordance with (O'Malley *et al*., 2015)who found that total serum bilirubin (sum of indirect and direct) concentration increased significantly after drinking from baseline to 24 hours in non-smokers.

5.4.Lipid peroxidation:

5.4.1.Thiobarbituric acid reactive substance(TBARS);

Figure (4-8) clarified the effect of ethanol and clove on the TBARS concentration in serum rats (nmol/L). Treated male rats (T2) registered highest significant ($P<0.05$) level of TB throughout the three experimental groups (T1,T2 and T3) compared with control male rats,

Thiobarbituric acid reactive substances (TBARS) are formed as a by-product of lipid oxidative damage (i.e., as degradation products of fats) and can be detected by the TBARS assay using thiobarbituric acid (TBA) as a reagent. This is an indirect measure of ROS (Irwin *et al*.,2004). So, it is occur naturally and is a marker for oxidative stress (the TBARS determination of lipid peroxidation, Through plasma thiobarbituric acid reactants (TBARS) and the level of some antioxidant enzymes such as superoxide dismutase (SOD), catalase, non-enzymatic antioxidants, vitamin C and vitamin E, significantly increased plasma TBARS concentrations, decreased SOD levels and CAT, Vit C, and Vit E have been observed in patients and this may be due to their increased use to scavenge lipid peroxides as well as their sequestration by cancer cells.

Increased levels of lipid peroxidation may be due to excessive oxidative stress(Nelson *et al*.,2006), this result was in accordance with (Harlina *et al*.,2018) who was indicated that application of clove extracts significantly reduced 2-thiobarbituric acid reactive substances and anisidine values as well as the conjugated dienes levels during curing, when he using the effect of clove extract on lipid oxidation, and fatty acid composition of salted duck eggs.

5.4.2.Protein carbonyl (PC):

Figure (4-9) illustrated the serum Protein carbonyl nmol/g in the control and three treated groups along the experimental period , the table showed a significant (P<0.01) increase after three weeks in T2 (2.78± 0.11) compared to the control group (2.09± 0.05) ,the result also showed that oral intubation of (1g/kg B.W) from ethanol and (Costa *et al*.,2018), on the other hand the result was in agreement with (Kapaki *et al*.,2007) who found protein carbonyl concentration at normal levels in human nonsmoking, otherwise , it was increased in concentration in the case of human smoking.

5.5.Molecular analysis:

5.5.1.Comet assay:

Alcohol, especially ethanol, is known to be a carcinogen and has many health risks, in the case of increased oxidative capacity (Kapaki *et al*.,2007), in this study the toxic effects on the DNA of ethanol were evaluated in rats administrated with doses of ethanol. The investigation was carried out by implementing a comet test. The level of DNA damage was determined as the percentage of cells with comets.

The results of comet assay of four groups were listed in table (4-1), The rats that were administrated with ethanol had a significant effect in the comet test, as it statistically showed (P <0.05) an increase in DNA damage compared to the control group figure (4-10) .

While the rats treated within the control group showed a significant increase (P <0.05) in the treatments compared to control group, which indicates DNA damage in rat have orally administration ethanol (Kido *et al*., 2006) The DNA damage which was initially occurred as the result of 20%

ethanol (Percentage tail DNA 69.171% and Head DNA 30.829%) was inhibited due to the ethanol extract of clove at the doses tested (percentage tail DNA 26.8% and Head DNA 73.17%) figure (4-10), These data indicate that ethanol induces DNA damage, which might be related to ethanol toxicity, these results were in agreement with (Razavi *et al*., 2014) who was found an ethanol extracts of rosemary on H2O2-induced oxidative DNA damage in human lymphocytes was inhibited.

5.5.2.Gene expression:

Figure (4-14): shows Geometric mean of relative quantification and data relevant to gene expression in different groups of study, after 30 days of treatment. Compared to the control rats, the Treatment group (T2) content in liver tissue was higher in ethanol rats ($p < 0.05$), the geometric mean of gene expression was recorded as 6.35.

while the clove extract administration treatment (T3) along with ethanol, reduced the geometric mean content of T2 significantly in the liver tissue compared to the treatment by ethanol alone ($p < 0.05$) it was recorded as 3.57, but no significant differences were found between the treatment group (T1) and control groups. Similarly,

Reverse transcriptase real-time PCR results shown that CYP2E1 mRNA expression level were increased more than 10-folds in Treatment group 2 (ethanol treatment) male rats treated compared with corresponding of control male rats (slightly more than 2-fold) as well as in the treated male rats (around 1-fold), these result was agreement with others study (Shirpoor *et al*., 2017) who was indicated that ethanol-induced heart abnormalities may in part be associated with MHC isoforms changes mediated by oxidative stress,

and that these effects can be alleviated by using ginger extract as an antioxidant molecule in wistar rats.

Furthermore, there were no significant differences in CYP4502E1 levels between the Treatment group (T1) and control groups. The geometric mean was recorded as 0.999 and 1.02 For T1 and control respectively. Ethanol administration significantly increased the expression of CYP4502E1 mRNA in the Liver tissue of the ethanol group when compared with the control group. Alcohol reduces the levels of antioxidants that can eliminate ROS, Following ethanol intoxication, the balance between prooxidants and antioxidants is disturbed to such an extent that it results in the oxidative damage of biomolecules, such as fats, proteins, or DNA, and finally leading to cell injury.

ROS production and oxidative stress in liver cells play a central role in the development of alcoholic liver disease, Cytochrome P450 enzymes (CYP) constitute the major components of MEOS. Cytochrome P450 2E1 (CYP2E1) in microsomal ethanol oxidizing system (MEOS) is one of the major ROS generators in liver and is recognized as a risk factor for alcoholic liver disease (ALD) (Chen *et al*., 2019).

Alcohol metabolism is achieved by both oxidative pathways, which either add oxygen or remove hydrogen (through pathways involving ADH, Oxidative pathways of alcohol metabolism. The enzymes alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1), and catalase all contribute to oxidative metabolism of alcohol. ADH, present in the fluid of the cell (i.e., cytosol), converts alcohol (i.e., ethanol) to acetaldehyde. This reaction involves an intermediate carrier of electrons, nicotinamide adenine dinucleotide (NAD+), which is reduced by two electrons to form NADH. Catalase, located in cell bodies called peroxisomes, requires hydrogen

peroxide (H2O2) to oxidize alcohol. CYP2E1, present predominantly in the cell's microsomes, assumes an important role in metabolizing ethanol to acetaldehyde at elevated ethanol concentrations cytochrome P450, and catalase enzymes (Lu & Cederbaum,2018). Ginger extract administration along with ethanol, significantly reduced of CYP2E1 mRNA because the clove decrease the alcoholic oxidation system.

Chapter six

Conclusions and recommendations

chapter six …….…….…….…

Conclusions:

1. Alcohol cause adverse effect in (catalase , GPX,ALT,AST,ALP,TB,TBARS,and PC) while administration clove extract improve all of above parameter .
2. Clove extract treatment caused improvement of CYP2E1gene expression and DNA damage induced by alcohol .
3. Clove may contains compounds that have anti-oxidant activity, may lower liver damage and prevent development of some liver diseases.
4. Clove extract showed has higher radical scavenging activity.
5. Our results suggest that the up regulation of CYP2E1 signaling via expression in liver cells is reduced for the control of ethanol-induced liver disease by using Cloves extract.
6. Fragmented DNA migrates from the "head" of the comet was reduced compare to the "tail" which increased based on the size of the fragment in the treated group ethanol.

Recommendations:

1- We recommended the use of Garlic as an oxidant to reduce the oxidative stress caused by alcohol or other agents
2- Study the effect of some drugs on cytochrome P4502E1 activity such as macrolides antimicrobial agent.
3- We suggest using clove extract for patient with liver disease

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Appendix

Appendix

Appendix (I)

Catalase

Procedure

Catalase activity was assessed by incubating the enzymes ample in 1.0 ml substrate (65 mmol/ml hydrogen peroxide in 60 mmol/l sodium–potassium phosphatebuffer,pH7.4)at37 °C for three minutes. There action was stopped with ammonium molybdate. Absorbance of the yellow complex of molybdate and hydrogen peroxide is measured at374nm against the blank.

Reagents

1. Sodium, potassium phosphate buffer (50mM,pH7.4): this buffer isprepared by dissolving 1.1g of Na2HPO4 and 0.27g of KH2PO4 in 100ml distilled water.

2. H2O2 (20 mM) in 50mmol/l sodium, potassium phosphate buffer: this solution is freshly diluted and standardized daily using a molar extinction coefficient of 43.6M_1 cm_1 at 240nm.

3. Ammonium molybdate (32.4mmol/l).

Table 1

Reagents	Test	Control-test*	Standard	Blank
Serum	100 µl	100 µl	-	-
D.W.	-	1000 µl	100 µl	1100 µl
Hydrogen peroxide	1000 µl	-	1000 µl	-
Mix with vortex and incubate at 37 °C for 3 min, after that, add:				
Ammonium molybdate	4000 µl	4000 µl	4000 µl	4000 µl
After that, the tubes were kept at room temperature. Changes in absorbance were recorded at 374 nm against the reagent blank.				

4. Calculation The rate constant of a first-order reaction (k) equation is used to determine catalase activity:

t: time.

S°: absorbance of standard tube

S: absorbance of test tube.

M: absorbance of control test (correction factor).

Vt: total volume of reagents in test tube. Vs: volume of serum.

3.5.4 Determination of serum concentration of superoxide dismutase (SOD) activity determination:

Preparation

1. Tris buffer (pH 8.0): was prepared by dissolving 0.258 gm of tris and 0.111 gm of Ethylenediaminetetraacetic acid (EDTA) in dH2O 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 dH2O.

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. Single 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 (Ma et al., 2009) :

SOD activity (u/ml)= (Vp-Vs)/(Vp*0.5)*(Vt/Vs)*n

Vp=Auto oxidation rate of pyrogallol rate of pyrogallol (control)

Vs= Auto oxidation rate of sample (with enzyme)

Vt=Total reaction volume (ml)

Vs= volume of enzyme used for the assay (ml)

n= dilution fold of the SOD sample

0.5= factor for 50% inhibition.

Appendix (II)

Glutathione peroxidase(GPX)

Glutathione Reductase Activity Assay:

1. Sample Preparations:

Homogenize 0.1 g tissues, 106 cells, or 0.2 ml erythrocytes on ice in 0.2 ml cold assay buffer; Centrifuge at 10,000 x g for 15 min at 4 °C; Collect the supernatant for assay and store on ice. Serum can be tested directly. Keep samples at -80 °C for storage. Add 2 - 50 µl of the samples into a 96-well plate; bring the volume to 50 µl with Assay Buffer. We suggest testing several doses of your sample to make sure the readings are within the standard curve range.

2. NADPH Standard Curve:

Dilute 25 µl of the 40 mM NADPH solution into 975 µl dH2O to generate 1 mM NADPH standard. Add 0, 20, 40, 60, 80, 100 µl of the 1 mM NADPH Standard into 96-well plate in duplicate to generate 0, 20, 40, 60, 80,100 nmol/well standard. Bring the final volume to 100 µlwith Assay Buffer. Measure O.D. 340 nm to plot the NADPH Standard Curve.

3. Positive Control (optional) and Reagent Blank:

For Positive Control use 5 - 10 µl of the GPx Positive Control into the desired well(s) and adjust to 50 µl with Assay Buffer. Add 50 µl of Assay Buffer into a well (s) as a Reagent Control (RC).

4. Reaction Mix: For each well, prepare 40 µl Reaction Mix:

33 µl Assay Buffer

3 µl 40 mM NADPH solution

2 µl GR solution2 µl GSH solution

Add 40 µl of the Reaction Mix to each test samples, Positive Control (s) and RC(s) mix well, and incubate for 15 minutes to deplete all GSSG in your sample. Add 10 µl Cumene Hydroperoxide Solution to start GPx reaction. Mix well. Measure OD 340 nm at T1 to read A1, measure OD 340 nm again at T2 after incubating the reaction at 25 °C for 5 min (or longer if the GPx activity is low) to read A2, protect from light. ΔA340 nm = [(Sample_A1-Sample_A2) – (RC_A1 – RC_A2)] .

Notes:

A. Measure the OD 340 nm before adding Cumene Hydroperoxide. Add more NADPH if the Sample OD at 340 nm is lower than 1.0 to ensure there is enough NADPH in the reaction system. 1 µl of 40 mM NADPH will give □ 0.5 OD at 340 nm.

B. If A1 reading is too low (< 0.7), it means either too much GPx or too much GSSG presence in the sample. You may need to dilute the samples, or remove GSSG from your sample using methods, such as dialyzing the sample or using spin filters (Cat.# MBS848290) to remove GSSG.

C. It is essential to read A1 and A2 in the reaction linear range. It will be more accurate if you read the reaction kinetics. Then choose A1, A2, in the reaction linear range.

5. Calculation: Plot NADPH standard Curve. Apply the ΔA340nm to the NADPH standard curve to get NADPH amount B.

GPx Activity = __ B ___ X Sample dilution = nmol/min/ml = mU/mL (T2 - T1) x V Where: B is the NADPH amount that was decreased between T1 and T2 (in nmol). T1 is the time of first reading (A1) (in min). T2 is the time of second reading (A2) (in min). V is the pretreated sample volume added into the reaction well (in ml). Unit Definition: One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 µmol of NADPH to NADP+ under the assay kit condition per minute at 25°C.

Appendix (III)

Serum determination of Alanine Aminotransferase (ALT) , Aspartate Aminotransferase (AST) and Alkaline phosphatase (ALP)

The mindray apparatus was used to examine the enzymes, and the method for examining the enzymes was carried out according to the instructions of the manufacturer producing the device.

According to mindray method for ALT and AST by UV-assay according to International Federation of clinical chemistry and Laboratory Medicine (IFCC) without pyridoxal phosphate activation .

And for ALP by International Federation of clinical chemistry and Laboratory Medicine (IFCC) modified method.

[ASSAY PROCEDURE]

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1. Determine wells for diluted standard, blank and sample. Prepare 5 wells for standard points, 1 well for blank. Add 50µL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells, respectively. And then add 50µL of Detection Reagent A to each well immediately. Shake the plate gently (using a microplate shaker is recommended). Cover with a Plate sealer. Incubate for 1 hour at 37oC. Detection Reagent A may appear cloudy. Warm to room temperature and mix gently until solution appears uniform.

2. Aspirate the solution and wash with 350µL of 1X Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Repeat 3

times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

3. Add 100µL of Detection Reagent B working solution to each well. Incubate for 30 minutes at 37oC after covering it with the Plate sealer.

4. Repeat the aspiration/wash process for total 5 times as conducted in step 2.

5. Add 90µL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 10 - 20 minutes at 37oC (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.

6. Add 50µL of Stop Solution to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.

7. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450nm immediately.

Note:

1. Assay preparation: Keep appropriate numbers of wells for each experiment and remove extra wells from microplate. Rest wells should be resealed and stored at -20oC.

2. Samples or reagents addition : Please use the freshly prepared Standard. Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of standards, samples, and reagents. Also, use separated reservoirs for each reagent.

3. Incubation: To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents are added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be controlled.

4. Washing: The wash procedure is critical. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and false elevated absorbance reading.

5. Controlling of reaction time: Observe the change of color after adding TMB Substrate (e.g. observation once every 10 minutes), if the color is too deep, add Stop Solution in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.

6. TMB Substrate is easily contaminated. Please protect it from light. FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES. MyBioSource.com

7. The environment humidity which is less than 60% might have some effects on the final performance, therefore, a humidifier is recommended to be used at that condition.

Appendix (IV)

Total bilirubin (TB)

[ASSAY PROCEDURE]

1. Determine wells for diluted standard, blank and sample. Prepare 5 wells for standard points, 1 well for blank. Add 50µL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells, respectively. And then add 50µL of Detection Reagent A to each well immediately. Shake the plate gently (using a microplate shaker is recommended). Cover with a Plate sealer. Incubate for 1 hour at 37oC. Detection Reagent A may appear cloudy. Warm to room temperature and mix gently until solution appears uniform.
2. Aspirate the solution and wash with 350µL of 1X Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Repeat 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
3. Add 100µL of Detection Reagent B working solution to each well. Incubate for 30 minutes at 37oC after covering it with the Plate sealer.
4. Repeat the aspiration/wash process for total 5 times as conducted in step 2
5. Add 90µL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 10 - 20 minutes at 37oC (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.
6. Add 50µL of Stop Solution to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
7. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450nm immediately.

Note:

1. **Assay preparation:** Keep appropriate numbers of wells for each experiment and remove extra wells from microplate. Rest wells should be resealed and stored at -20oC.

2. **Samples or reagents addition**：Please use the freshly prepared Standard. Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of standards, samples, and reagents. Also, use separated reservoirs for each reagent.

3. **Incubation:** To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents are added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be controlled.

4. **Washing:** The wash procedure is critical. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and false elevated absorbance reading.

5. **Controlling of reaction time:** Observe the change of color after adding TMB Substrate (e.g. observation once every 10 minutes), if the color is too deep, add Stop Solution in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.

6. **TMB Substrate** is easily contaminated. Please protect it from light.

7. The environment humidity which is less than 60% might have some effects on the final performance, therefore, a humidifier is recommended to be used at that condition.

[TEST PRINCIPLE]

This assay employs the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific to bilirubin has been pre-coated onto a microplate. A competitive inhibition reaction is launched between biotin labeled bilirubin and unlabeled bilirubin (Standards or samples) with the pre-coated antibody specific to bilirubin. After incubation the unbound conjugate is washed off. Next, avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. The amount of bound HRP conjugate is reverse proportional to the concentration of bilirubin in the sample. After addition of the substrate solution, the intensity of color developed is reverse proportional to the concentration of bilirubin in the sample.

[CALCULATION OF RESULTS]

This assay employs the competitive inhibition enzyme immunoassay technique, so there is an inverse correlation between bilirubin concentration in the sample and the assay signal intensity. Average the duplicate readings for each standard, control, and samples. Create a standard curve on log-log or semi-log graph paper, with the log of bilirubin concentration on the y-axis and absorbance on the x-axis. Draw the best fit straight line through the standard points and it can be determined by regression analysis. Using some plot software, for instance, curve expert 1.30, is also recommended. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Appendix (V)

thiobarbituric acid

TBARS assay protocol

A simplified protocol for the TBARS assay, and tips for success, is as follows.

1. Reconstitute TBA in glacial acetic acid (use only glacial acetic acid, as regular acetic acid affects TBA stability due to its high-water content). The reconstituted solution is typically stable for 1 week at +4°C or -20°C. Sonicate in a RT water bath if required to dissolve precipitates.

2. Use BHT in your lysis buffer during sample preparation to stop further sample peroxidation while processing. For plasma/serum samples, use phosphotungstic acid to precipitate the lipids for analysis.

3. To assay the samples, add TBA solution to your prepared samples. Incubate at 95°C for 60 minutes. Cool to room temperature in an ice bath for 10 minutes.

4. The sensitivity of the TBARS assay can be improved by using n-butanol to precipitate the MDA-TBA adduct after step 3, then dissolving the precipitate in water after removal of n-butanol.

5. Measure the output immediately after processing the samples, on a microplate reader at OD 532 nm for a colorimetric assay, and at Ex/Em = 532/553 nm for a fluorometric assay.

Appendix (VI)

Protein carbonyl

procedures

1. Preheat the Spectrophotometer or Microplate Reader for 30 min, then adjust the wavelength at 370 nm.
2. Operation table

	Control tube /well	Sample tube /well
Sample (μL)	60	60
Reagent 2 (μL)		120
Reagent 3 (μL)	120	
Mix fully, incubate at 37℃ in the dark for 1 hour		
Reagent 4 (μL)	150	150
Stand for 5 min, then centrifuge at 12000g at 4℃ for 15 min. Discard the supernatant and collect the sediment.		
Reagent 5 (μL)	500	500
Mix fully with the vortex mixer, then centrifuge at 12000g at 4℃ for 15 min. Discard the supernatant and collect the sediment		
Reagent 5 (μL)	300	300
Mix fully with the vortex mixer, then centrifuge at 12000g at 4℃ for 15 min. Discard the supernatant and collect the sediment.		
Reagent 5 (μL)	300	300
Mix fully with the vortex mixer, then centrifuge at 12000g at 4℃ for 15 min. Discard the supernatant and collect the sediment		
Reagent 6 (μL)	300	300
Mix fully with the vortex mixer, incubate at 37℃for 15 min. Then centrifuge at 12000g at 4℃ for 15 min after the sediment dissolved fully. Take 200 μL of the supernatant into the quartz cuvette or Microplate, measure the absorbance at 370 nm. △A= ASample-AControl.		

Calculation of results
Calculation formula for detection with quartz cuvette:

1. **Calculate according to the protein concentration of sample:**
   Protein carbonyl content (μmol/mg prot)
   = (△A× V total ε×d) ÷(Cpr × V sample)

=0.227×△A÷Cpr

V total: total volume of reaction system, 0.3 mL;
ε : molar extinction coefficient of carbonyl, 22 L /mmol/cm;
d: optical path of the quartz cuvette, 1 cm;
V sample: volume of sample added into the reaction system, 0.06 mL;

Calculation formula for detection with Microplate:

1. Calculate according to the protein concentration of sample: Protein carbonyl content (μmol/mg prot)
   = (△A× V total ε×d) ÷(Cpr × V sample)
   =0.454×△A÷Cpr

   V total: total volume of reaction system, 0.3 mL;
   ε : molar extinction coefficient of carbonyl, 22 L /mmol/cm;
   d: optical path of the quartz cuvette, 0.5 cm;
   V sample: volume of sample added into the reaction system, 0.06 mL

Table showing results of oxidative enzymes and liver function

Appendix (VII)

Table showing results of oxidative enzymes and liver function.

group / parameter	C	T1	T2	T3
CAT(U/ml) Mean ±SD	524.94±0.931 A	533.15±2.14 A	472.17±2.548 C	507.33±2.641 B
GPX (ng/ml) Mean ±SD	0.6706±0.01 C	1.149±0.03 C	25.038±1.12 A	10.115±0.22 B
ALT (U/ml) Mean ±SD	24.683±0.336 C	26.75±1.268 C	40.46±1.198 A	31.88±0.393 B
AST (U/ml) Mean ±SD	131.33±0.605 C	133.9±0.77 C	150.94±0.987 A	139.93±0.617 B
ALP (mg/dl) Mean ±SD	136.15±2.72 C	141.73±0.739 C	183.84±0.17 A	143.71±0.704 B
TB (µg/ml) Mean ±SD	9.57±0.35 C	9.94±0.17 C	11.55±0.58 A	10.29±0.17 B
TBARS(nmol/L) Mean ±SD	2.19±0.13 C	2.29±0.13 C	3.2±0.15 A	2.44±0.2 B
PC(nmol/g) Mean ±SD	2.09±0.05 C	2.18±0.05 C	2.78±0.11 A	2.27±0.07 B

الخلاصة :

أجريت الدراسة الحالية في بيت الحيوانات بكلية الطب البيطري - جامعة كربلاء لبحث الدور الوقائي لمستخلص القرنفل في الإجهاد التأكسدي والتغيرات في التعبير الجيني والحمض النووي الناتج عن التعرض لكحول الإيثانول ، يؤدي تناول الكحول إلى اضطرابات كبدية شاملة. ، المصنف على أنه مرض الكبد الكحولي (ALD). السيتوكروم CYP2E1 في MEOS هو أحد مولدات ROS الرئيسية في الكبد ومعترف به كعامل خطر لـ ALD. تم استخدام أربعين من ذكور الجرذان الناضجة بوزن (140 - 190) وقسمت عشوائياً إلى أربع مجموعات كل منها عشرة ذكورمجموعة. جرعت جميع المجموعات عن طريق التنبيب الفموي ، حيث تم حقن المجموعة الأولى (المجموعة السيطرة) بالماء المقطر بكمية (1 مل لكل 1 كجم) لمدة أربعة أسابيع ، في حين تم تجريع المجموعة الثانية (مجموعة القرنفل) مع مستخلص القرنفل بتركيز (1 مجم لكل كجم) لمدة أربعة أسابيع والمجموعة الثالثة (مجموعة الإيثانول) تم إعطاء جرعة من الإيثانول بتركيز (1 جم لكل كجم) والمجموعة الأخيرة تم جرعها بالإيثانول ثم مستخلص القرنفل وفي تركيز (1 جم لكل كجم) للإيثانول و (1 مجم لكل كجم) لمستخلص القرنفل لمدة أربعة أسابيع أيضًا، تمت مراقبة وزن الجسم لجميع الفئران (40 فأرًا) طوال التجربة. في نهاية كل فترة علاج ومجموعات المراقبة ،تم الحصول على عينات الدم من القلب لتقييم الإنزيمات التالية (بروتين كاربونيل وغلوتاثيون بيروكسديز ، اختبار وظائف الكبد ، TBARS، الكاتلاز) ، تم استخدام تقنية qRT-PCR لتقدير مستويات التعبير الجيني Cyp450 2E1 بالنسبة لمستويات التعبير الجيني للجين Housekeeping B actinin في كبد الفئران المتسممة بالايثانول ومعالجتها بالقرنفل لتقدير تغيرات الطيات في مستويات التعبير الجيني للجين المستهدف (Cyp450 2E1) وجين Housekeeping (بيتا اكتين) ، تم جمع عينات الدم من جميع الجرذان ، وتم ترميز العينات لتجنب احتمال التحيز. تم سحب 1 مل من الدم مرة واحدة من قلب الحيوانات عن طريق حقن الهيبارين. تم نقل العينات على الفور على الجليد إلى المختبر العلمي المركزي في كلية الطب البيطري ، وتم خلط عشرة ميكرولتر من خلايا طحن الأنسجة مع 75 ميكرولتر من درجة انصهار أقل بنسبة 0.5 ٪ من الاغاروز من أجل تحديد تحليل تلف الحمض النووي لجميع الحيوانات في هذه الدراسة (تجريبية و السيطرة) باستخدام اختبار المذنب ، ووجدت النتائج في كل الفترات التجريبية (10 - يوم ، 20 - يوم ، 30 - يوم) ، ولم يكن هناك فرق معنوي (P> 0.05) في زيادة وزن الجسم بين الذكور. أظهرت النتائج أيضًا

تأثير الإيثانول والقرنفل على تركيز الكاتلاز في فئران المصل (U / ml) ، وسجلت ذكور الجرذان المعالجة (T2) مستوى معنويًا منخفضًا (P <0.05) من الكاتلاز في جميع المجموعات التجريبية (C، T1 و T3) ، تسبب التنبيب الفموي لـ (1 جم / كجم) من الإيثانول و (100 مجم / كجم) من مجموعة القرنفل (T3) في انخفاض معنوي (P <0.01) في تركيز GPx في الدم مقارنة بمجموعة الإيثانول (T2) ، كما أنه وجدت ارتفاعًا كبيرًا (P <0.05) في إنزيمات الكبد (AST و ALT و ALP و Total bilirubin) في المجموعة الثانية (T2) والثالثة (T3) عند مقارنتها مع ذكور الجرذان الضابطة ومجموعة العلاج الأولى ، على سجلت ذكور الجرذان المعالجة من ناحية أخرى (T2) أعلى مستوى معنوي (P <0.05) من TBARS في ذكور الجرذان (C، T1، T3) ، وأظهرت النتيجة زيادة معنوية (P <0.01) في بروتين مصل الكربونيل بعد أربعة أسابيع في T2 مقارنة بمجموعة التحكم. من ناحية أخرى ، أظهرت تقنية فحص المذنب انخفاضًا ملحوظًا في الحمض النووي في الرأس في مجموعات الإيثانول (30.829 ± 5.82) بدلاً من المجموعات الأخرى مثل (90.104 ± 0.628 ، 91.057 ± 0.727 و 73.17 ± 8.13) لمجموعة التحكم والقرنفل والقرنفل. الإيثانول بالإضافة إلى القرنفل ، على التوالي.

أظهرت نتائج PCR في الوقت الحقيقي للنسخة العكسية أن مستوى تعبير CYP2E1 mRNA قد زاد بأكثر من 10 أضعاف في المجموعة العلاجية 2 (معالجة الإيثانول) ذكور الجرذان التي عولجت مقارنةً مع ذكور الجرذان اللسيطرة (أكثر بقليل من ضعفين) وكذلك في ذكور الجرذان المعالجة (حوالي ضعف واحد).

. في الختام ، تشير الدراسة الحالية إلى أن مستخلص القرنفل يعمل كمضاد للأكسدة. تشير الدراسة الحالية إلى أن التعرض للإيثانول يسبب الإجهاد التأكسدي وأمراض الكبد. نوصي بتجنب التعرض من خلال العلاج بمستخلص القرنفل.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

كلية الطب البيطري/جامعة كربلاء

فرع الفسلجة والكيمياء الحياتية والأدوية

التأثيرات الوقائية لمستخلص القرنفل على الأضرار المؤكسدة والحمض النووي في كبد الفئران المعرضة للإيثانول

رسالة مقدمة الى

جامعة كربلاء - مجلس كلية الطب البيطري

وهي جزء من متطلبات نيل درجة الماجستير في علم الفسلجة/ الطب البيطري

من قبل

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