

Protective effects of Butylated Hydroxytoluene (BHT) against sodium Arsenite (NaAsO₂) - Induced hepatotoxicity in Wistar Rats

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Abstract

One of the major environmental contaminants that can be found on a global scale is arsenic. Sodium arsenite (NaAsO₂) is one of the compounds of arsenic which is considered very toxic. Long term exposure to NaAsO₂ can cause chemical complications in various organs of the body such as the liver, kidney, testes and brain tissues. Butylated hydroxytoluene (BHT) is an antioxidant that can serve as an antitoxic effect. NaAsO₂. In this topic, we examined the chemo-protective effect of BHT on NaAsO₂ induced hepatotoxicity in male Wistar rats. Wistar rats (n=42, +120g) were randomly grouped into seven treatment groups: of 7 animals each Control 1 (corn oil); Control 2 (distilled water); NaAsO₂ alone (2.5mg/kg). BHT alone (25 mg/kg); BHT only (25mg/kg); BHT+ NaAsO₂ (25mg/kg + 2.5/kg), and BHT+ NaAsO₂ (2.5g/kg + 50 mg/kg) and treated for 14 days. Markers of liver functions, inflammation, oxidative stress and antioxidant profile of liver were assayed spectrophotometrically. Our results showed that exposure to NaAsO₂ resulted in dramatic increase in the levels of AST, ALT and ALP activity Furthermore, NaAsO₂ increased the concentration of oxidative stress markers such as xanthine oxidase (XO) and lipid peroxidation (MDA) and inflammatory markers such as myeloperoxidase (MPO) and nitric oxide (NO) with a concomitant decrease in antioxidant markers such as superoxide dismutase (SOD). Glutathione-S-transferase (GST), and catalase. However, the co-exposure of rats to both BHT and NaAsO₂ can result in significant decrease the parameters above. In conclusion, exposure of rats to NaAsO₂ causes increased inflammatory and oxidative stress signals, but these harmful effects can be ameliorated by administration of antioxidants such as BHT as confirmed by this research.

Keywords: Arsenic; Sodium arsenite; Oxidative stress; Antioxidants; Butylated hydroxytoluene (BHT); Reactive oxygen species (ROS)

1. Introduction

Arsenic (As), a chemical element in the nitrogen group (Group 15 [Va] of the periodic table), existing in both grey and yellow crystalline forms. A small amount exists in the native state, in 90–98% purity, generally in association with such metals as antimony and silver. Most, however, is combined in more than 150 different minerals, as sulfides, arsenides, sulfoarsenides, and arsenites. Mispickel, or arsenopyrite, FeAsS, is among the most common of arsenic-bearing minerals; others are realgar, As₄S₄; orpiment, As₂S₃; loellingite, FeAs₂; and enargite, Cu₃AsS₄. Arsenic oxide is also common. Most commercial arsenic is recovered as a by-product of the smelting of copper, lead, cobalt, and gold ores.

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Only one stable isotope of arsenic that of mass 75, occurs in nature. Among the artificial radioactive isotopes is one of mass 76, which has a half-life of 26.4 hours. Arsenic-72, -74, and -76 have been used in medical diagnostic procedures.

Arsenic is used in metallurgical applications because of its metalloid properties. About 1 % arsenic content is desirable in the manufacture of lead shot, for example, because it improves the roundness of the molten properties of copper and brass. Elemental arsenic is also used in bronzing and in pyrotechnics. Very highly purified arsenic finds applications in semiconductor technology, where it is used with silicon and germanium, as well as in the form of gallium arsenide, GaAs, for diodes, lasers, and transistors.

Because arsenic has a range of oxidation states from -3 to +5, it can form a variety of different kinds of compounds. Among the most important commercial compounds are the oxides, the principal forms of which are arsenious oxide (As_2O_3) and arsenic pentoxide (As_2O_5). Arsenious oxide, commonly known as white arsenic, is obtained as a by-product from the roasting of the ores of copper, lead, and certain other metals as well as by the roasting of arsenopyrite and arsenic sulfide ores. Arsenious oxide provides the starting material for most other arsenic compounds. It is also utilized in pesticides and serves as a decolourizer in the manufacture of glass and as a preservative for hides. Arsenic pentoxide is formed by the action of an oxidizing agent (e.g., nitric acid) on arsenious oxide. It comprises a major ingredient of insecticides, herbicides, and metal adhesives (Britannica, 2023).

Sodium arsenite is an inorganic sodium salt with formula with $NaAsO_2$. It has a role as an insecticide, an antibacterial agent, an herbicide, a rodenticide, a carcinogenic agent, an antineoplastic agent. It is an arsenic molecular entity and an inorganic sodium salt. Sodium Arsenite, aqueous solution appears as an aqueous solution of a solid. It becomes toxic in the body by ingestion, inhalation, or skin absorption. Used as an antiseptic, in insecticides and herbicides, to preserve hides and in making dyes (PubChem, 2023).

Hepatotoxicity is defined as injury or liver damage caused by exposure to drugs or other no pharmacological agents. It is an adverse drug reaction that may be uncommon but serious, and therefore, have a considerable impact on health. Hepatotoxicity generates between 1/600 and 1/3500 of all hospital admissions, 2–3 % of hospitalizations for jaundice, 10 % of acute jaundice hepatitis (being more than 40 % in people over 50 years of age) and between 15 and 30 % of cases of fulminant hepatic failure. Although hepatotoxicity is less frequent than other adverse drug effects, due to its severity and is the most common cause of drug withdrawal in the pharmaceutical market, it is assessed as a major adverse event, so, it is a frequent impediment to the development of drugs by pharmaceutical companies (Paniahqua *et al.*, 2017).

Antioxidants are used in food to protect it from deleterious effects of oxidation and are also employed as dietary supplements to neutralize the adverse effects of oxidative stress (Shahidi, 2015). Antioxidants have the ability to scavenge free radicals in the human body and have been suggested to contribute to the protective effect of plant-based foods on diseases such as cardiovascular disease (CVD), cancer, and type 2 diabetes. However, evidence from supplementation studies using various antioxidants, including vitamin C, vitamin E, carotenoids, zinc, or selenium, does not support the hypothesis that antioxidants decrease risk of these diseases (Stanner *et al.*, 2013). Antioxidants are mainly classified into two types: synthetic antioxidants and natural antioxidants. The most common synthetic antioxidants are BHA, BHT, ascorbic acid, and gallic acid esters. Synthetic antioxidants are cheap, stable, and effective and have undergone several toxicological tests. However, synthetic antioxidants are toxic, carcinogenic, non-biodegradable and pose environmental problems. Natural antioxidants are considered safer and healthier compared with their synthetic origin. Antioxidants having phenolic groups are the most widely used ones. Natural antioxidants can be produced from vegetables, fruits, spices, herbs, nuts, oilseeds, cereals, legumes, animal and microbial sources (Saibaba, 2023).

Butylated Hydroxytoluene (BHT) is a most commonly used commonly used antioxidant recognized as safe for use in foods containing fats, pharmaceuticals products, rubber and oil industries (Yehye *et al.*, 2015).

Liver is the largest organ, and it accounts for approximately 2% to 3% of the average body weight. It has two lobes (right and left lobe). The liver is a critical organ in the human body that is responsible for an array of functions that help support metabolism, immunity, digestion, detoxification, vitamin storage among other functions. It comprises around 2 % of an adult's body weight. The liver is a unique organ due to its dual blood supply from the portal vein (approximately 75 %) and the hepatic artery (approximately 25 %) (Kalra *et al.*, 2022).

Aim and objectives of the study

The present study is focused at assessing the chemo-preventive property of Butylated hydroxytoluene (BHT) in ameliorating sodium arsenite-induced toxicity in male Albino Wistar rats.

The specific objectives include:

- To evaluate the influence of BHT and NaAsO₂ on serum and liver function enzymes and liver architecture in sodium arsenite exposed rats.
- To evaluate the impact of BHT on oxidative stress biomarkers in sodium arsenite-induced toxicity in rats.
- To determine the influence of BHT on pro-inflammatory and anti-inflammatory response in sodium arsenite-exposed rats.
- To evaluate the effect of BHT on the mean body weight and Organo-Somatic Indices (OSI) in arsenite exposed rats.

2. Literature review

2.1. Sodium arsenite

Sodium arsenite refers to the inorganic compound with the formula NaAsO₂. Also, sodium arsenite is called sodium meta-arsenite, it is sodium salt of arsenous acid (Grund *et al.*, 2005).

Sodium arsenite is a poisonous substance that is obtained as a concentrated solution or dry powder by treating arsenic trioxide with sodium hydroxide, that consists in some cases of a mixture of sodium ortho-arsenite Na₃AsO₃ and sodium meta-arsenite NaAsO₂, and that is used chiefly as an insecticidal bait and weed killer (Merriam-Webster, 2023).

It is primarily used as a pesticide, but has other uses such as hide preservative, antiseptic, dyeing, and soaps. A mixture of sodium meta-arsenite and sodium ortho-arsenite is produced by treating arsenic trioxide with sodium carbonate or sodium hydroxide (Grund *et al.*, 2005). Sodium Arsenite is amorphous, typically, being obtained as a powder or as a glassy mass. The compound consists of the polymer [AsO₂]_nⁿ⁻ associated with sodium cations, Na⁺. The polymer backbone has connectivity –O-As(O-)- (Eagleton, 2011)

Sodium arsenite can be inhaled or absorbed through the skin due to its carcinogenic and teratogenic effect, its contact with these substance yields symptoms like skin irritation, burns, itching, rash, thickened skin, loss of pigment, poor appetite, a metallic or garlic taste, stomach pain, nausea, vomiting, diarrhoea, convulsion, decreased blood pressure and headache.

Severe acute poisoning of sodium arsenite can lead to nervous system damage, weakness, poor coordination or pins and needles sensation or eventually paralysis and death (Jing *et al.*, 2012).

Sodium arsenite can be used primarily as pesticide but other uses include antiseptic dyeing, soap, hide, preservative (Considine, 2005). It is an appropriate chemical stressor that is induced in the production of heat shock protein (Bhagat *et al.*, 2008) and during the formation of cytoplasmic stress (Mc Ewen *et al.*, 2005).

Symptoms of exposure to sodium arsenite include:

- Weak pulse, cyanosis, and cold extremities may also be observed.
- Neurological effects such as giddiness, delirium, stupor, dizziness, and fainting. Convulsions, paralysis, and coma may also occur.
- Irritation or burning of the skin, eyes, and mucous membranes (Kegley *et al.*, 2009).

2.1.1. Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) were initially recognized as toxic by-products of aerobic metabolism. In recent years, it has become apparent that ROS plays an important signalling role in plants, controlling processes such as growth, development and especially response to biotic and abiotic environmental stimuli. The major members of the ROS family include free radicals like O^{•-2}, OH[•] and non-radicals like H₂O₂ and ¹O₂. The ROS production in plants is mainly localized in the chloroplast, mitochondria and peroxisomes. There are secondary sites as well like the endoplasmic reticulum, cell membrane, cell wall and the apoplast. The role of the ROS family is that of a double edged sword; while they act as

secondary messengers in various key physiological phenomena, they also induce oxidative damages under several environmental stress (Front. Environment Science, 2014).

2.1.2. Oxidative Stress

Oxidative stress is a phenomenon caused by an imbalance between production and accumulation of oxygen reactive species (ROS) in cells and tissues and the ability of a biological system to detoxify these reactive products. ROS can play, and in fact they do it, several physiological roles (i.e., cell signaling), and they are normally generated as by-products of oxygen metabolism; despite this, environmental stressors (i.e., UV, ionizing radiations, pollutants, and heavy metals) and xenobiotics (i.e., antiproliferative drugs) contribute to greatly increase ROS production, therefore causing the imbalance that leads to cell and tissue damage (Pizzino *et al.*, 2017).

2.1.3. Antioxidants

Antioxidants are a class of chemical substances naturally found in our food which can prevent or reduce oxidative stress of the physiological system. Antioxidants are the molecules that prevent cellular damage caused by oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from one molecule to an oxidizing agent. Oxidation reactions are known to produce free radicals. These free radicals are highly reactive species which contains one or more unpaired electrons in their outermost shell. Once they are formed, the chain reaction starts. Antioxidant reacts with these free radicals and terminates this chain reaction by removing free radical intermediates and inhibits other oxidation reactions by oxidizing themselves. Though oxidation reactions are crucial for life, they can also be damaging. Plants and animals have a complex system of multiple types of antioxidants, such as vitamin C and vitamin E, as well as enzymes, such as catalase (CAT), superoxide dismutase (SOD), and various peroxidases (Hamid *et al.*, 2010).

Oxidative stress plays a key role in causing various human diseases, such as cellular necrosis, cardiovascular disease, cancer, neurological disorder, Parkinson's dementia, Alzheimer's disease, inflammatory disease, muscular dystrophy, liver disorder, and even aging (Amik *et al.*, 2011). Besides, there are some antioxidants in the form of micronutrients which cannot be manufactured by the body itself such as vitamin E, β -carotene, and vitamin C, and hence these must be supplemented in the normal diet (Teresa *et al.*, 2011)

Antioxidants can also act as pro-oxidants when these are not present at the right place at the right concentration at the right time (Touriño *et al.*, 2008). The relative importance of the antioxidant and pro-oxidant activities is not yet explored fully and needs further research. In this chapter, authors have tried to discuss the various types, sources, synthesis, uses, and protective efficacy of antioxidant with examples.

2.1.4. Classification of antioxidants

Antioxidants can be classified into two major types based on their source, i.e., natural and synthetic antioxidants.

Natural Antioxidants

Natural antioxidants either are synthesized in human body through metabolic process or are supplemented from other natural sources, and their activity very much depends upon their physical and chemical properties and mechanism of action. This can be further divided into two categories, i.e., enzymatic antioxidants and non-enzymatic antioxidants.

Enzymatic Antioxidants

Enzymatic antioxidants are uniquely produced in the human body and can be subdivided into primary and secondary antioxidant.

Primary Antioxidants

Primary antioxidants mainly include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) as described below.

- Superoxide Dismutase

Superoxide dismutase (SOD) enzyme is found in both the dermis and the epidermis. It removes the superoxide radical (O_2^-) and repairs the body cells damaged by free radical. SOD catalyzes the reduction of superoxide anions to hydrogen peroxide. SOD is also known to compete with nitric oxide (NO) for superoxide anion, which inactivates NO to form peroxynitrite. Therefore, by scavenging superoxide anions, it promotes the activity of NO (Chakraborty *et al.*, 2009).

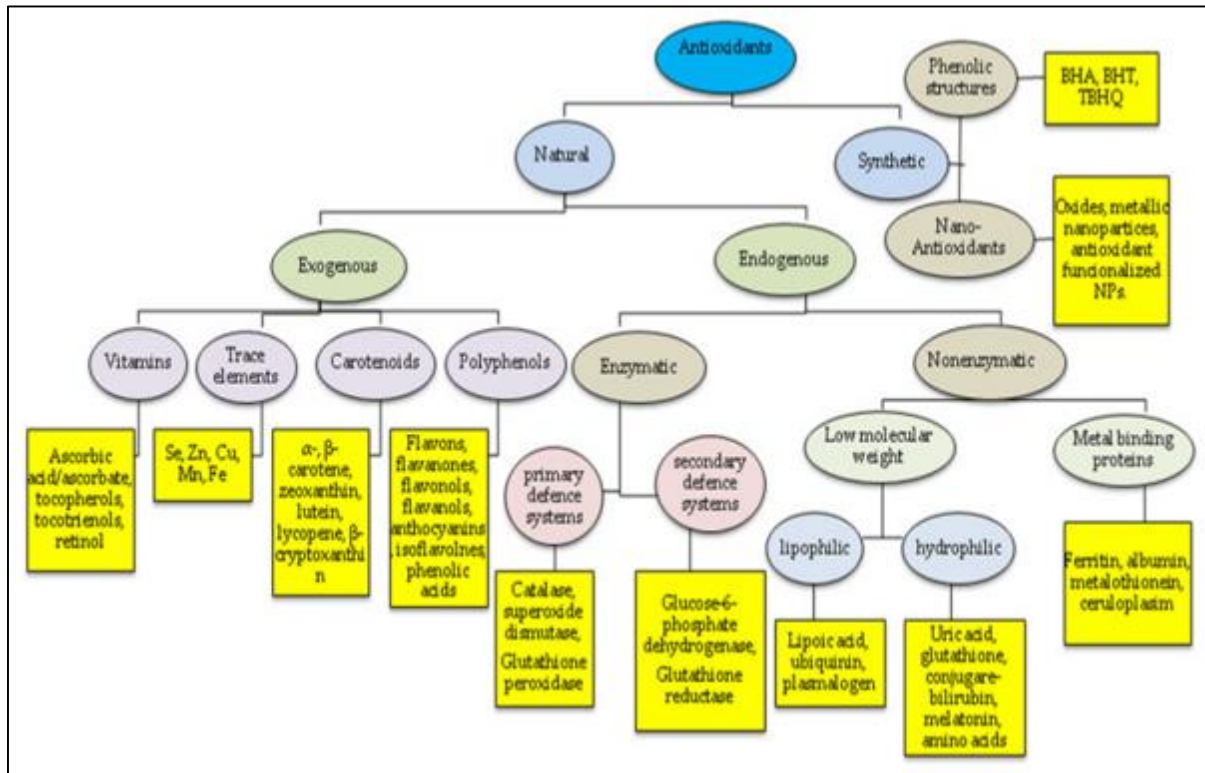
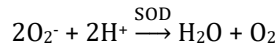
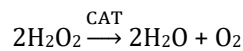


Figure 1 Antioxidants Classification (Jolanta *et al.*, 2021)

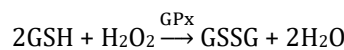
- Catalase enzyme

Catalase enzyme is found in the blood and most of the living cells and decomposes H₂O₂ into water and oxygen. Catalase along with glucose peroxidase is also used commercially for the preservation of the fruit juices, cream consisting of egg yolk, and salad by removing the oxygen (Chakraborty *et al.*, 2009).



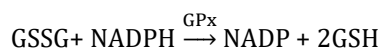
- Glutathione Peroxidase

Glutathione peroxidase (GPx) is a group of selenium dependent enzymes, and it consists of cytosolic, plasma, phospholipid hydroperoxide, and gastrointestinal glutathione peroxidase (Chakraborty *et al.*, 2009). GPx (cellular and plasma) catalyzes the reaction of H₂O₂ by reduced glutathione (GSH); as a result, oxidized glutathione (GSSG) is produced and it is again recycled to its reduced form by glutathione reductase (GR) and reduced nicotinamide adenine dinucleotide phosphate (NADPH).



Secondary Antioxidants

Secondary antioxidant includes glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH). G6PDH generates NADPH. GR is required to recycle the reduced glutathione (GSH) using secondary enzyme GR and NADPH



Glutathione is a cysteine containing peptide-type antioxidant and is synthesized in the body cells. The thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced. A high level of glutathione is found in the cells (~3,100 µg/g of tissue) (Hissin *et al.*, 1976), maintained in the reduced form (GSH) by the enzyme GR, and in

turn reduces other metabolites and enzyme systems, such as ascorbate. Due to its high concentration and its role in maintaining redox state in the cells, it is considered one of the most important cellular antioxidants.

Non-enzymatic Antioxidants

They are a class of the antioxidants which are not found in the body naturally but are required to be supplemented for the proper metabolism (Raygani *et al.*, 2007). Some of the known non-enzymatic antioxidants are minerals, vitamins, carotenoids, polyphenols, and other antioxidants as listed below;

- Minerals

Minerals are required in the body cells for the proper functioning of the enzymes. Their absence is known to affect the metabolism of many macromolecules. They include selenium, copper, iron, zinc, and manganese. They act as cofactors for the enzymatic antioxidants.

- Iron (Fe)

Iron is the most abundant trace metal found to bound with protein in the biological system. Normally the concentration of free iron is very low and the low concentrations of iron-binding proteins promote ROS production, lipid peroxidation, and oxidative stress (Dabbagh, 1984). Hence iron supplementation helps in reducing the oxidative stress.

- Magnesium (Mg)

Magnesium is a cofactor for glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) involved in pentose cycle which catalyzes the production of NADPH from NADP during the glucose metabolism and hence maintains the normal ratio of GSH to GSSG and a normal redox state in cells. Deficiency of magnesium reduces GR activity and GSSG does not reduce to GSH, hence causing oxidative damage to the cells (Fang, 2002).

- Selenium (Se)

Selenium is also a very important component of enzymatic antioxidant. In the presence of selenium (Se), glutathione peroxidase (GPx) plays a protective role against oxidation of lipid and protects the cell membrane and takes part in H₂O₂ and lipids' hydroxyperoxide metabolism. Hence, Se behaves like vitamin E and can be substituted in place of vitamin E and is used to prevent the risk of cancer and cardiovascular diseases (Sikora *et al.*, 2008).

- Copper (Cu), Zinc (Zn), and Manganese (Mn)

SOD is a class of enzyme that consists of different types of SODs, depending upon their metal cofactor such as Cu–Zn and Mn. Cu–Zn SOD is found in the cytosol having Cu and Zn at their active sites which helps in proton conduction, whereas Mn-SOD is found in mitochondria and has Mn at its active site. These metals are responsible for SOD's antioxidant activities.

- Vitamins

Vitamins form the class of micronutrients required for the proper functioning of the body's antioxidant enzyme system, such as vitamin A, vitamin C, vitamin E, and vitamin B. They cannot be synthesized in our body and hence need to be supplemented in the diet.

- Vitamin A

Vitamin A is helpful in night vision and in maintenance of epithelial cells in mucus membranes and skin. Because of its antioxidant properties, it assists immune system also and is found in three main forms: retinol, 3, 4-didehydroretinol, and 3-hydroxyretinol. The main sources of this include sweet potatoes, carrots, milk, egg yolks, and mozzarella cheese.

- VitaminC

Vitamin C is water soluble and is also called as ascorbic acid. It is found in fruits (mainly citrus), vegetables, cereals, beef, poultry, fish, etc. It is helpful in preventing some of the DNA damage caused by free radicals, which may contribute to the aging process and the development of diseases, such as cancer, heart disease, and arthritis.

- Vitamin E

Vitamin E is a lipid-soluble vitamin. This consists of eight different forms such as α -, β -, γ -, and δ -tocopherol and α -, β -, γ -, and δ -tocotrienol.

- Carotenoid

Carotenoid consists of β -carotene, lycopene, lutein, and zeaxanthin. They are fat-soluble colour compounds found in fruits and vegetables. β -Carotene is found mostly in radish-orange-green colour food items including carrots, sweet potatoes, apricots, pumpkin, mangoes, and cantaloupe along with some green and leafy vegetables, including collard greens, spinach, and kale. Lutein is abundant in green leafy vegetables such as collard greens, spinach, and kale (Hamid *et al.*, 2010). Lutein is best known for its role in protection of retina against harmful action of free radicals and also prevents atherosclerosis (Sikora *et al.*, 2008).

2.2. Butylated hydroxytoluene (BHT)

Butylated hydroxytoluene (BHT), also known as **dibutylhydroxytoluene**, is a lipophilic organic compound, chemically a derivative of phenol that is useful for its antioxidant properties. (Yehye *et al.*, 2015). BHT is widely used to prevent free radical-mediated oxidation in fluids (e.g. fuels, oils) and other materials.

2.2.1. Structure of BHT

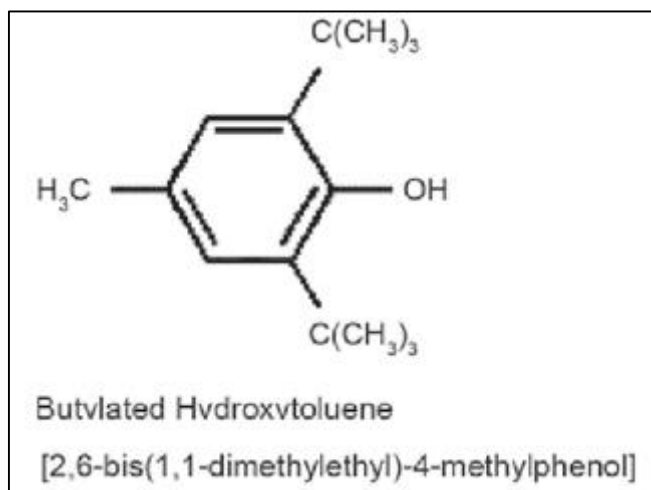


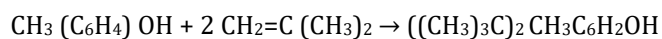
Figure 2 Structure of BHT (Bhupendrasinh *et al.*, 2011)

2.2.2. Natural sources of BHT

Three distinct cyanobacteria and the green alga *Botryococcus braunii* are among the phytoplankton species that can naturally make BHT (*Cylindrospermopsis raciborskii*, *Microcystis aeruginosa*, and *Oscillatoria sp.* (Babu *et al.*, 2008). The lychee fruit's pericarp also contains BHT (Jiang *et al.*, 2013). BHT is produced by a variety of fungi, including *Aspergillus conicus*, which lives in olives.

2.2.3. Biosynthesis of BHT

The chemical synthesis of BHT in industry has involved the reaction of p-cresol (4-methylphenol) with isobutylene (2-methylpropene), catalyzed by sulphuric acid (Fiege, 2002).



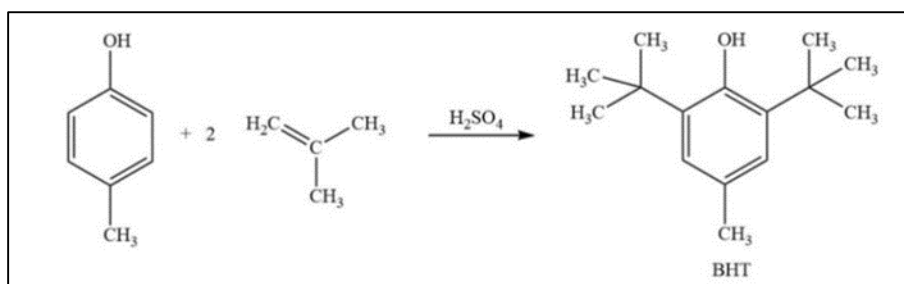


Figure 3 Synthesis Of BHT (Fiege, 2002)

2.2.4. Mechanism Of Action Of BHT

The molecular structure of BHT is what gives it its antioxidant qualities because, like other synthetic phenolic antioxidants, it has a labile hydrogen atom in the hydroxy group that can be donated and diminish free radicals produced during the first stage of lipid oxidation. BHT is oxidized as a result, and the radical that is produced as a result is stabilized by electronic delocalization in the benzene ring. BHT can prevent the spread of radical oxidation in this way, delaying the oxidation of lipids and extending the shelf life of foods (the method of quenching responses may vary depending on the radical, BHT has been shown to effectively react against strong oxidizing radicals such as singlet oxygen, hydroxyl radicals (OH), and peroxy radicals (OOR). The reaction pathways previously proposed for BHT antioxidant activity under experimental condition are summarized in Figure below.

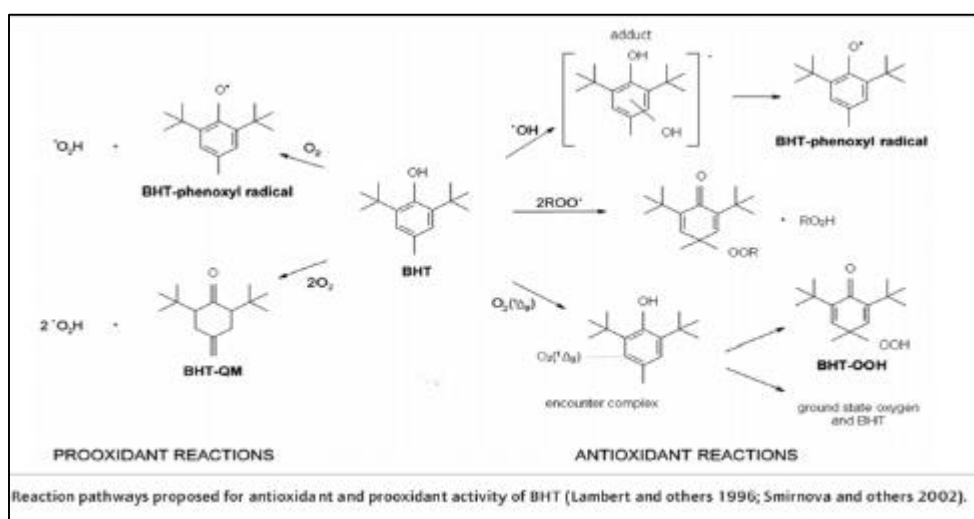


Figure 4 Mechanism of action of BHT (Lambert *et al.*, 2002)

2.3. The Liver

The liver is the body's largest solid organ. It performs hundreds of other crucial tasks, such as clearing contaminants from the blood supply, regulating blood clotting, and preserving healthy blood sugar levels. It is in the right upper abdomen, below the rib cage (Centre for Liver Disease and Transplantation, 2020).

The body's metabolic processes are the liver's main areas of function. They consist of transforming or breaking down food components like fats and proteins to release energy, vitamins, and minerals while also reducing the danger that toxins pose to the body and eliminating them from the bloodstream.

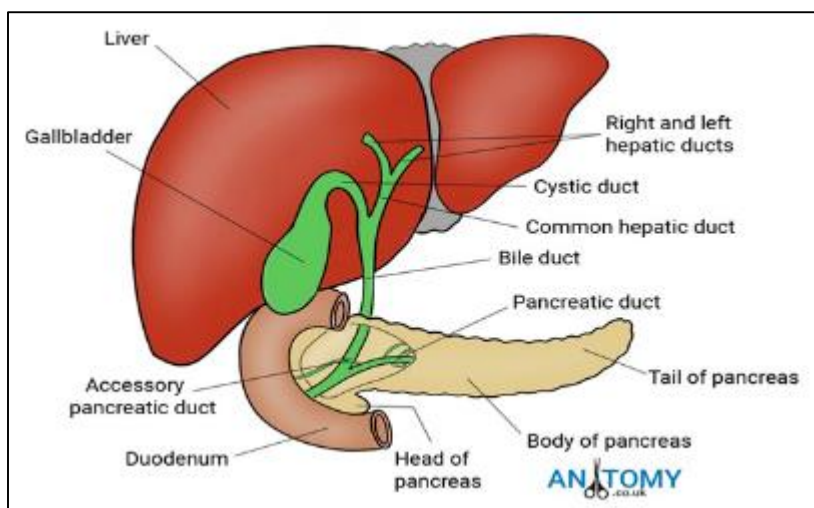


Figure 5 Structure of Liver (Anatomy, 2023)

2.3.1. Biotransformation of toxicants in the liver

Biotransformation, sometimes referred to as metabolism, is the structural modification of a chemical by enzymes in the body. Chemicals are biotransformed in several organs, including the liver, kidneys, lungs, skin, intestines, and placenta, with the liver being the most important. Chemicals absorbed in the gastrointestinal tract must pass through the liver, where they can be biotransformed and thus eliminated before being distributed to other parts of the body. This phenomenon is known as the first-pass effect. As a result, smaller amounts of certain chemicals are distributed throughout the body after oral administration than after other exposure routes, such as intravenous or intramuscular injections. Biotransformation of a chemical primarily facilitates its excretion into urine or bile; however, certain chemicals are biotransformed into more toxic forms and, as a result, biotransformation of chemicals is not always beneficial (Bergmeyer, 1974).

2.4. Reactive oxygen species

Reactive oxygen species (ROS), superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and singlet oxygen are formed in normal oxygen metabolism. Free radicals can initiate free radical sequence reactions that shape various free radicals (Arslan, 2014). Reactive oxygen species have been associated with many disease categories, including cancer. In addition, ROSs have been reported to increase tumour cell migration and increase the risk of metastasis and metastasis. It is known that the harmful effects of ROS are controlled by various cellular defence systems consisting of enzymatic components (catalase, glutathione peroxidase and superoxide dismutase, etc.). Epidemiological literature studies have found a relationship between low levels of antioxidants and an increased risk of cancer (Gecit *et al.*, 2012).

Although many definitions are made for free radicals, in the general definition, free radical is a chemical product that is in molecular or atomic orbit and is generally highly reactive and contains unpaired electrons. The electrons in atoms move in spaces called orbit. Each orbit has up to two electrons moving in opposite directions. Free radicals can be positively charged, negatively charged, or neutral. In biological systems, they occur mostly by electron transfer. Although free radical reactions are necessary for the defence mechanism of cells such as neutrophils and macrophages from immune system cells, overproduction of free radicals results in tissue damage and cell death (Altan *et al.*, 2006).

Electrons are orbital in atoms and are present in pairs in the spatial region. Bonds are formed as a result of the interaction between atoms and molecular structure is formed due to these bonds. Free oxygen radicals, atomic or molecular structures in the single electron, are the name given to uncommon parts. These molecules that easily exchange electrons with other molecules are also called free oxygen radicals (FOR) or reactive oxygen radicals (ROR) (Halliwell, 1991)

The form of lipid peroxidation with the result of molecular oxygen conversion to reactive oxygen species (ROS) with various environmental factors, particularly cigarette smoke, alcohol, UV rays, and other oxidants, leads to oxidative stress. As a result of this, a multi-stage carcinogenesis process is favoured by ROS in the body.

2.5. Oxidative stress

Oxidative stress is an important component in binding environmental toxicity to a multi-stage carcinogenic process. In addition, oxidative stress is characterized by the cumulative effect of more than one activity, such as a multi-stage process (three stages in a single cell; onset, elevation, and progression), such as cancer development. Reactive oxygen species (ROS) are produced in response to endogenous and exogenous stimulation. ROS can affect all these stages of carcinogenesis (Varshney *et al.*, 2013). For this reason, the term oxidative stress is used to describe the imbalance between cellular levels of oxidants and antioxidants (Hristova *et al.*, 2014).

2.6. Hepatotoxicity

Hepatotoxicity is the injury or liver damage caused by exposure to drugs; it is an adverse drug reaction that may be uncommon but serious. The hepatic injury can be classified into hepatocellular, cholestasis and mixed, caused by increase in alanine aminotransferase and alkaline phosphatase than upper limit of normal (Bergmeyer, 1974).

The risk factors include idiosyncrasy, age, gender, alcohol consumption, concomitant use of other drugs, previous or underlying liver disease, genetic and environmental factors. Liver toxicity manifestations are generally accompanied by non-specific symptoms such as abdominal pain, jaundice, fever, nausea, vomiting, diarrhoea, pruritus and rash. Identification of hepatotoxicity is a complex process to perform; therefore, clinical scales have been developed, such as the Roussel Uclaf Causality Assessment Method (CIOMS/RUCAM) and the Clinical Diagnostic Scale (M & V CDS). Additionally, there is no specific treatment for hepatotoxicity, which is based on suspending the suspected drug and treating symptoms. The most commonly associated pharmacological groups are antibiotics, nonsteroidal anti-inflammatory analgesics (NSAIDs), antidepressants and anticonvulsants. Drug-induced liver injury has been an adverse event, hard to identify, prevent and treat; thereby, the pharmacist intervention can contribute to the diminution of the deleterious effects in patient health (Bergmeyer, 1974).

2.7. Inflammation

Chronic liver inflammation is characterized by the formation of nodules and fibrous connective tissue within the liver, which hinder this organ's function. Usually, liver inflammation is associated with a more advanced stage of a hepatic condition, such as hepatitis or fatty liver disease. In addition to these problems, inflammation can also occur as a result of excessive alcohol consumption, extensive medication use, and even viral infections (Bergmeyer, 1974).

Chronic liver inflammation, or cirrhosis, is not curable therefore treatment is normally aimed at managing some of the symptoms and preventing the condition from getting worse. Treatment can include some dietary changes as well as medication. In more serious cases, surgery may also be necessary to transplant a new liver (Bergmeyer, 1974).

2.7.1. Main symptoms

In an initial stage, liver inflammation does not usually cause any type of symptoms. However, as the condition worsens and progresses into cirrhosis, symptoms may start appearing. These include:

- Weakness and fatigue;
- General malaise;
- Frequent nausea;
- Loss of appetite;
- Red patches on the skin;
- Weight loss.

In more advanced stages of cirrhosis, it's common to observe other signs such as yellow skin and eyes, bloated stomach, dark urine, white faeces and itchiness in the whole body (Bergmeyer, 1974).

2.7.2. Temperature Programmed Desorption to study the Behavior of Arsenite

Temperature Programmed Desorption (TPD) is a powerful technique employed to study surface reactions, desorption processes, and the chemical nature of materials. It is particularly useful in understanding the behavior of contaminants such as sodium arsenite (NaAsO_2) and the protective effects of antioxidants like butylated hydroxytoluene (BHT). TPD can analyze the interactions between NaAsO_2 and various surfaces, including biologically relevant ones like liver tissues, providing insights into its bioavailability and toxicity. Furthermore, TPD elucidates the decomposition pathways of NaAsO_2 on catalytic surfaces, essential for understanding its breakdown products and potential detoxification strategies. By studying how BHT interacts with oxidative species generated by NaAsO_2 , TPD can reveal the binding sites

and reaction mechanisms of BHT, highlighting its role in mitigating oxidative stress and stabilizing reactive surfaces (Onivefu, 2023)

3. Materials and Methods

3.1. Experimental animals

Forty-two male Wistar rats (7-8 weeks old) with a body weight of between (90-100) g were obtained from the animal house of the Department of Veterinary Anatomy, University of Ibadan, and Ibadan, Nigeria. The animals were housed in plastic boxes in an experimental room under controlled conditions of temperature (22 ± 2 °C) and humidity (50 ± 10 %) on a 12 hour light dark-cycle and water would be available ad libitum. They were fed with a grower rat pellet purchased from Ladokun Feeds, Sango, Ibadan.

3.2. Chemicals, reagents and kits

Most chemicals and reagents that were used during this research work were obtained from the Laboratory. Reagents used during this project include Sodium Arsenite (NaAsO_2), Butylated Hydroxytoluene (BHT), Absolute ethanol, Hydrochloric Acid, Sodium Hydroxide, Potassium Chloride, Tris Buffer, Hydrogen Peroxide, Sodium Carbonate, Sodium-Potassium Tartrate, Copper Sulphate Pentahydrate, Folin-C reagent, Dipotassium Hydrogen Phosphate Dihydrate, Epinephrine, Potassium Dihydrogen Phosphate, 1-Chloro-2,4-dinitrobenzene (CDNB), Reduced Glutathione, Sulphosalicylic Acid, Trichloroacetic Acid, 2',7'-dichlorodihydrofluorescein Diacetate (DCFH-DA), O-Dianisidine, Griess Reagent, Manganese Azide, Thiobarbituric Acid, Xanthine. Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Urea, Creatinine, Alkaline Phosphate (ALP), Alkaline Phosphatase (ALP) kits were obtained from Randox Laboratories, UK.

3.3. Apparatus/ equipments

The apparatuses used during the course of this project includes conical flasks, beakers, measuring cylinders, glass stirrer, Ependorf tubes, pipette tips, micropipette, oral cannulas, syringes and needles, dissecting set, dissecting board, plain bottles, universal bottles, spatula, capillary tubes, cotton wool, hand gloves, cuvettes, centrifuge, cold centrifuge, microplate reader, spectrophotometer, pH meter, weighing balance, incubator, and freezer.

3.4. Experimental drugs

Sodium arsenite, the toxicant and Butylated Hydroxytoluene (BHT), the antioxidant, used during the course of the experiment was obtained from the lab we were experimenting in, Cancer Research and Molecular Biology Unit, Department of Biochemistry, University of Ibadan.

3.5. Experimental design

The animal were divided into seven groups of six rats each and administered as follows;

- Group A: Control rats received corn oil at 2 ml/kg body weight.
- Group B: Control rats received distilled water at 2 ml/kg body weight.
- Group C: Rats were administered sodium arsenite at 2.50 mg/kg body weight.
- Group D: Rats were administered butylated hydroxytoluene (BHT) at 25 mg/kg body weight.
- Group E: Rats were administered butylated hydroxytoluene (BHT) at 50 mg/kg body weight.
- Group F: Rats were co-administered to sodium arsenite (2.50 mg/kg per body weight) and butylated hydroxytoluene (BHT) (at 25 mg/kg per body weight).
- Group G: Rats were co-administered to sodium arsenite (2.50 mg/kg per body weight) and butylated hydroxytoluene (BHT) (at 50 mg/kg per body weight).

3.6. Animal sacrifice and preparation of serum

The daily weights of the animals were recorded to ascertain the change in body weight during the period of treatment. The final weights of the animals were taken 24 hours after the treatment, and they were fasted overnight. The animals' blood was collected through the infra orbital sinus by ocular puncture using capillary tubes into plain sample bottles and they were sacrificed by cervical dislocation.

To obtain the serum, the clotted blood was centrifuged at 3000g per 20 minutes after which the liver of the animals were immediately excised, weighed and processed for biochemical, inflammatory and histological analyses.

3.7. Homogenization of the organs

Harvested liver samples were rinsed in ice cold 1.15% KCL solution, blotted with filter paper and weighed. Thereafter, the liver samples were sectioned for histological examination and submerged in Formalin. The remaining parts of the harvested liver were homogenized with 0.1M phosphate buffer (pH 7.4) using a Teflon homogenizer. The homogenates gotten were then centrifuged at 15,000 Revolution per minute for 10 minutes in an ultracentrifuge (4 degrees) to obtain the post mitochondrial fraction. After centrifugation, the supernatants were collected and used for biochemical and inflammatory assays.

3.7.1. Preparation of reagents

Potassium chloride (1.15%)

1.15g of potassium chloride (KCl) was dissolved in 60ml of distilled water and the volume made up to 100ml. The prepared potassium chloride was used as a rinsing buffer for the harvested organs.

Formalin (10%)

10mls of formaldehyde was dissolved in distilled water and made up to 100mls with distilled water.

Homogenizing buffer (0.1M Phosphate buffer, pH 7.4)

3.497 g of dipotassium hydrogen phosphate trihydrate and 1.318 g of potassium dihydrogen phosphate were dissolved in 200 ml of distilled water, the pH adjusted to 7.4 and the volume made up to 250 ml with distilled water.

3.8. Determination of total body and relative organ weights

The total body weight of each rat was determined using a digital balance before and after the experimental period. Weights recorded before the commencement of treatments were tagged initial body weights whereas weights recorded after the experimental periods were tagged final body weights. Thereafter, the mean body weights for each group were calculated. Changes in weights were also expressed as percentage weight increase.

Percentage weight increase was calculated from the formula:

$$\frac{W_y - W_x}{W_x} \times 100$$

Where W_x = Initial mean total body weight

W_y = Final mean total body weight

The weights of harvested organs of respective rats was measured with a digital balance and presented as percentage kidney weight per total body weight. This was calculated from the formula:

$$\frac{\text{Liver Weight}}{W_y} \times 100$$

3.9. Liver function test

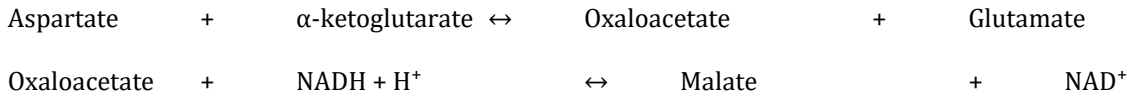
3.9.1. Measurement of Aspartate Aminotransferase (AST) Activity

Aspartate aminotransferase, which is otherwise also called serum glutamic oxaloacetic transaminase (GOT, SGOT), is a pyridoxal phosphate (PLP)-dependent transaminase enzyme. AST catalyzes the reversible transfer of an amino group between aspartate and glutamate and, accordingly, is a significant enzyme in amino acid metabolism. AST is found in the liver, heart, skeletal muscle, kidneys, brain, and red blood cells. Serum AST level, alongside serum ALT levels are usually estimated clinically as biomarkers for liver health. AST is normally found in most body fluids, but not in urine except in instances of kidney lesions. The greatest concentrations of AST are found in heart, liver, muscle, and kidney tissues. Damage to these tissues can greatly elevate serum AST levels. Elevated levels of AST have been implicated in acute myocardial infarction, severe angina, hepatitis, liver necrosis, cancer of the liver, alcoholism, musculoskeletal disease, heat stroke, acute pancreatitis, strenuous exercise, cerebral infarction, trauma, and intramuscular injection,

among others. Exhausted AST levels have likewise been found related with certain conditions which include uraemia and deficiency in vitamin B.

Principle

AST in the sample catalyzes the reversible transamination of L-aspartate and α -ketoglutarate to oxaloacetate and L-glutamate in the presence of pyridoxal-5'-phosphate. The oxaloacetate is then reduced to malate in the presence of malate dehydrogenase with the concurrent oxidation of NADH to NAD. The reaction is monitored by measuring the rate of decrease in absorbance at 340 nm due to the oxidation of NADH to NAD. The rate of change in absorbance is directly proportional to the AST activity in the sample. Randox Laboratories limited kits was used to determine AST activity following the procedure described by Reitman and Frankel, 1957.



Reagents

- **Reagent 1 (Buffer)**
 - 100 mmol/l of phosphate buffer pH 7.4
 - 100 mmol/l of L-aspartate
 - 2 mmol/l α - oxoglutarate.
- **Reagent 2**
 - 2,4 dinitrophenylhydrazine
- **0.4mol/L sodium hydroxide solution**
 - 4g of sodium hydroxide was dissolved in distilled water and made up to 100mL.

Table 1 Procedure For Determination Of AST Levels In Serum Samples

	Reagent blank(μ l)	Sample(μ l)
Sample	-----	5
Reagent 1 (R1)	25	25
Distilled water	5	-----
Thoroughly mix and incubate for 30mins at 37°C		
Reagent 2 (R2)	25	25
Mix, allow to stand for 20mins at 20 - 25°C		
Sodium hydroxide	250	250
Samples were mixed and absorbance was read against blank after 5mins at 546nm with a microplate reader.		

U/I = Units per litre

Calculation

$$U/I = 17646 * \Delta A \text{ 340 nm/min}$$

$$\text{Time} = 5 \text{ mins}$$

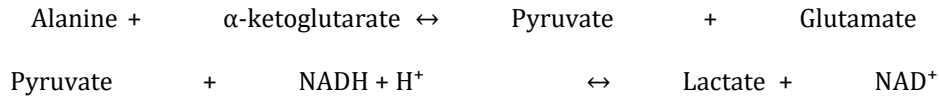
3.9.2. Measurement of Alanine Aminotransferase (ALT) Activity

Alanine aminotransferase (ALT) is a transaminase enzyme that was formerly known as serum glutamate pyruvate transaminase (SGPT). Alanine aminotransferase (ALT) is the most widely used clinical biomarker of hepatic health (Ozer *et al.*, 2008). As its name implies, Alanine aminotransferase catalyzes the transfer of an amino group from alanine to alpha-ketoglutarate in the alanine cycle to form pyruvate and glutamate. The ALT enzyme is found in serum and organ tissues, especially liver, although significant concentrations are also found in kidney, skeletal muscle, and myocardium. Lower levels of ALT are present in pancreas, spleen, and lung. Alanine aminotransferase is elevated in serum under conditions of significant cellular necrosis and is used as a measure of liver function. Leakage of ALT from the hepatocyte into the blood occurs following hepatocellular injury, where it is cleared with a plasma half-life of

approximately 42 hours. Levels of ALT may be elevated in cases of hepatitis, congestive heart failure, liver or biliary duct damage, or myopathy.

Principle

ALT catalyses the reversible transamination of L-alanine and α-ketoglutarate to pyruvate and L-glutamate. Pyruvate formed is then reduced to lactate in the presence of lactate dehydrogenase (LDH). This is accompanied with the simultaneous oxidation of NADH to NAD. Pyridoxal -5- phosphate is necessary for the commencement of this reaction, where it functions as a cofactor, by binding to the enzyme using Schiff-base linkage. The change in absorbance is monitored and is directly proportional to the ALT activity in the sample. . Randox Laboratories limited kit was used to determine AST activity following the procedure described by Reitman and Frankel, 1957. (Reitman et al., 1957)



Reagents

- **Reagent 1 (Buffer)**
 - 100mmol/L of phosphate buffer (pH 7.4)
 - 100 mmol/L of L-alanine
 - 2 mmol/L of α-oxoglutarate
- **Reagent 2**
 - 2 mmol/L of 2, 4-dinitrophenylhydrazine
- **0.4mol/l sodium hydroxide solution**
 - 4g of sodium hydroxide was dissolved in distilled water and made up to 100mL.

Table 2 Procedure for determination of ALT level in serum samples.

	Reagent blank(μl)	Sample(μl)
Sample	-----	5
Reagent 1 (R1)	25	25
Distilled water	5	-----
Mix and incubate for 30mins at 37°C		
Reagent 2 (R2)	25	25
Samples were mixed and allowed to stand for 20mins at 25 - 25°C		
Sodium hydroxide	250	250
Samples were mixed and absorbance was read against blank after 5mins at 546nm with a microplate reader.		

U/I = Units per litre

Calculation

$$U/I = 17646 * \Delta A \text{ 340 nm/min}$$

$$\text{Time} = 5 \text{ mins}$$

3.9.3. Measurement of Alkaline phosphatase (ALP) Activity

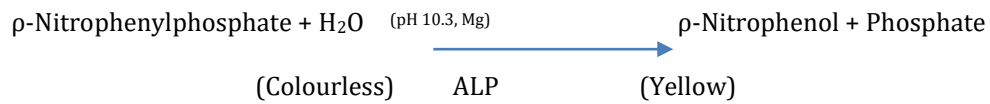
Alkaline Phosphatase is a metallodependent enzyme that hydrolyzes the phosphomonoesters from number of organic molecules like ribonucleotides, deoxy-ribonucleotides, proteins, alkaloids, phosphate esters and anhydrides of phosphoric acid. Alkaline Phosphatase shows its catalytic activity optima at alkaline pH. Alkaline phosphatase is divided into four isozymes depending upon the site of tissue expression which include Intestinal ALP, Placental ALP, Germ cell ALP and tissue nonspecific alkaline phosphatase or liver/bone/kidney (L/B/K) ALP. Alkaline phosphatases are widely distributed in nature, including prokaryotes and higher eukaryotes with the exception of some higher plants. The activity of liver and bone alkaline phosphatases in serum has been applied extensively in routine diagnosis. The enzyme alkaline phosphatase is an important serum analyte and its elevation in serum is correlated with the presence of bone,

liver, and other diseases. High ALP levels can show that the bile ducts are obstructed. Elevated ALP also indicate active bone formation as ALP is a by-product of osteoblast activity (such as the case in Paget’s disease of bone) or a disease that affects blood calcium level (hyperparathyroidism), vitamin D deficiency, or damaged liver cells. ALP Levels are also elevated in people with untreated Celiac Disease. Placental alkaline phosphatase is elevated in seminomas. Decreased alkaline phosphatase levels are also seen in some conditions or diseases such as hypophosphatasia, malnutrition, magnesium deficiency, hypothyroidism, severe anemia, children with achondroplasia and cretinism, children after a severe episode of enteritis, pernicious anemia, aplastic anemia, chronic myelogenous leukaemia and Wilson’s disease. In addition, some drugs such as oral contraceptives have been demonstrated to reduce alkaline phosphatase.

Principle

Alkaline phosphatase catalyzes the hydrolysis of colourless p-nitrophenyl phosphate (p-NPP), yielding p-nitrophenol and inorganic phosphate. At the pH of the assay (alkaline), the p-nitrophenol is in the yellow phenoxide form. Optimized concentrations of zinc and magnesium ions are usually present to activate the alkaline phosphatase in the sample. The rate of p-nitrophenol formation is directly proportional to the activity of alkaline phosphatase. Randox Laboratories Limited kits was used to determine ALP level following the principle described by Englehardt, *et al.* (1970).

The principle of this assay is according to the following reaction:



Reagents

- **Reagent 1a (Buffer)**
 - 1 mol/l of Diethanolamine buffer pH 9.8
 - 0.5 mmol/l of MgCl₂.
 - 2.0 mmol/l of α-oxoglutarate.
- **Reagent 1b**
 - 10 mmol/l of p-nitrophenylphosphate. Substrate

Table 3 Procedure for determination of alp level in serum samples

	Reagent blank(μl)	Sample(μl)
Sample	-----	5
Reagent(25 ⁰ C, 30 ⁰ C, 37 ⁰ C)	250	250
Distilled water	5	-----

The resultant solutions were mixed and absorbance was read against blank at 405nm with a microplate reader. The timer was then started simultaneously. Readings were taken again exactly after 1, 2 and 3 minutes.

(Englehardt, 1970)

Calculation:

To Calculate the ALP activity, use the following formulae:

$$\text{U/l} = 2760 \times \Delta A_{405\text{nm}/\text{min}}$$

3.10. Total protein

The protein concentration of the various homogenates was determined by means of the Lowry method described by (Lowry *et al.*, 1951).

Principle

The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen(s) with copper (ii) ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteu phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalysed oxidation of aromatic acids. The Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10-10.5.

Reagents

- **2% Na₂CO₃ in 0.1M NaOH**

500ml of 0.1M NaOH was prepared by dissolving 2g of NaOH in distilled water and making up to 500ml. 10g of Na₂CO₃ was then dissolved in the 500ml of 0.1M NaOH and the solution was labelled reagent A.

- **2% Na-K Tartrate**

0.2g of Na-K Tartrate was dissolved in 10ml of distilled H₂O and labelled as reagent B.

- **1% CuSO₄.5H₂O**

0.1g of CuSO₄.5H₂O was dissolved in 10ml of distilled H₂O and labelled as reagent C.

- **Alkaline CuSO₄ Solution**

Alkaline CuSO₄.5H₂O was prepared by mixing reagent A, B and C in the ratio 100:1:1. To a clean 500ml volumetric flask, 490.19mls of reagent A, 4.9mls of reagent B and 4.9mls of reagent C were mixed, giving 500mls of alkaline CuSO₄ solution.

- **Folin C solution**

A solution of 1ml/5ml of Folin-C was prepared. This was done by adding 40mls of water to 8mls of Folin-C, to give 48mls of Folin-C solution.

Standard Protein (BSA) Curve

2 mg/ml stock solution of Bovine Serum Albumin was prepared. Serial dilutions of the solutions were placed in 5 different test tubes into 1ml of each protein standard solution in a test tube was added 3ml of alkaline CuSO₄. The mixture was allowed to stand at room temperature for 10 minutes after which 0.3 ml of Folin-C was added and allowed to stand at room temperature for 30 minutes. The optical densities of the resulting solutions were read in a spectrophotometer 750 nm against a blank of 1ml of distilled water and 3ml of biuret reagent. A curve of absorbance against protein concentration was plotted.

Table 4 Protocol for protein standard curve preparation

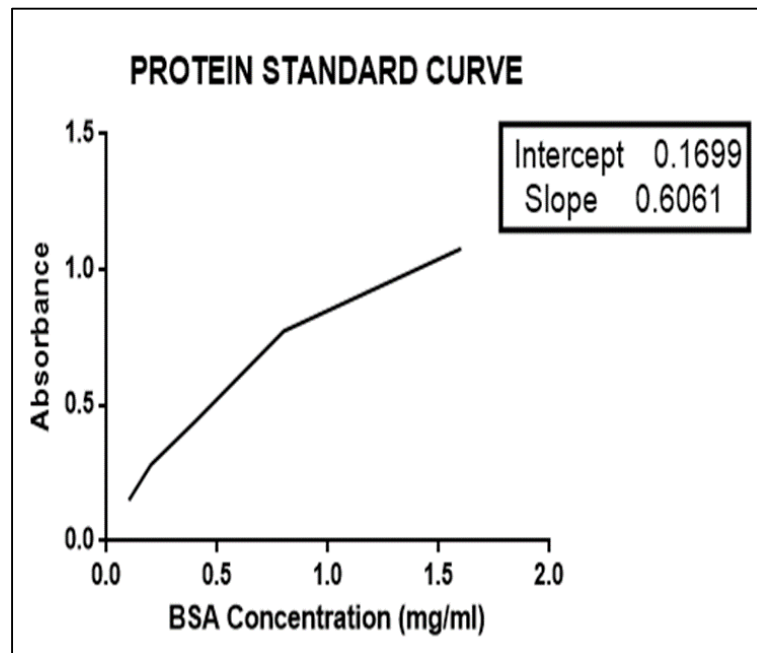
Test tubes No.	1	2	3	4	5
Stock BSA (ml)	0.05	0.10	0.20	0.40	0.80
Distilled water (ml)	0.95	0.90	0.80	0.60	0.20
Alkaline CuSO ₄ (ml)	3.00	3.00	3.00	3.00	3.00
Folin-C reagent (ml)	0.30	0.30	0.30	0.30	0.30

Procedure for Sample Preparation

7µl of sample was added to 23 µl of distilled water in a microplate, to which 150 µl of alkaline CuSO₄ solution was added and left to incubate at room temperature for 10 minutes. 15 µl of folin-c solution was then added and the mixture was incubated at room temperature for 30 minutes, after which the absorbance was read at 750 nm using the reagent blank (Lowry *et al.*, 1951).

Calculation

The total protein levels in liver homogenates was extrapolated from a standard curve of the values below



(Lowry *et al.*, 1951)

Figure 6 Protein Standard Curve

3.11. Antioxidant assays

3.11.1. Determination of superoxide dismutase (sod) activity

The activity of SOD was determined by the method of (Misra *et al.*, 1972).

Principle

The ability of SOD to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide radical causes the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per superoxide radical introduced increases with increasing pH and concentration of epinephrine.

Reagents

- **0.05M Carbonate buffer (pH 10.2)**

3.9325 g of $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$ and 1.47g of NaHCO_3 were dissolved in 450 ml of distilled water. The pH was adjusted to 10.2 and then made up to 500 ml with distilled water.

- **0.3M Epinephrine**

0.0125 g of epinephrine was dissolved in 50 ml of distilled water containing 0.5 ml of concentrated HCl (37%).

Procedure

50 μl of sample was added to 2.5 ml of 0.05M carbonate buffer (pH 10.2) and 0.3 ml of epinephrine in a cuvette, mixed by inversion and change in absorbance monitored every 30 sec for 2 minutes at 480 nm. The reference cuvette was the same as for the samples with water replacing the samples. (Misra *et al.*, 1972)

Calculation

$$\% \text{ inhibition} = \frac{100 - (100 \times \text{Increase in absorbance per min for sample})}{\text{Increase in absorbance per min for blank}}$$

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the autoxidation of epinephrine

3.11.2. Determination of catalase activity

Catalase activity was determined according to the method of Claiborne (1985).

Principle

The method is based on the loss of absorbance observed at 240 nm as catalase splits hydrogen peroxide. Despite the fact that hydrogen peroxide has no absorbance maximum at this wavelength, its absorbance correlates well enough with concentration to allow its use for a quantitative assay. An extinction coefficient of $0.0436 \text{ mM}^{-1}\text{cm}^{-1}$ (Noble *et al.*, 1970) was used.

Reagents

- **Phosphate buffer (0.05 M, pH 7.4)**

Dipotassium hydrogen phosphate trihydrate (0.696 g) and potassium dihydrogen phosphate (0.265 g) were dissolved in 90 ml of distilled water, the pH adjusted to 7.4 and the volume made up to 100 ml with distilled water.

- **Hydrogen peroxide (19 mM)**

194 μl of 30% H_2O_2 was added to 50 ml of 0.05 M phosphate buffer, pH 7.4 and the volume made up to 100 ml with the same.

Procedure

Hydrogen peroxide (2.95 ml of 19 mM solution) was pipetted into a 1 cm quartz cuvette and 50 μl of sample was added. The mixture was rapidly inverted to mix and placed in a spectrophotometer. Change in absorbance was read at 240 nm every minute for 5 min.

Calculation

$$\begin{aligned} \text{Catalase activity} &= \frac{\Delta A_{240}/\text{min} \times \text{dilution factor}}{0.0436 \times \text{mg protein/ml}} \\ &= \mu\text{mole H}_2\text{O}_2/\text{min/mg protein (Claiborne, 1985)} \end{aligned}$$

3.11.3. Determination of glutathione S-transferase activity

Glutathione S-transferase activity was determined according to (Habig *et al.*, 1974).

Principle

The assay is based on the principle that all known glutathione S-transferase isotypes demonstrate a relatively high activity with 1-chloro-2, 4-dinitrobenzene (CDNB) as the second substrate. When CDNB is conjugated to reduced glutathione, its absorption maximum shifts to a longer wavelength and the absorption increase at the new wavelength of 340 nm provides a direct measurement of the enzymatic reaction.

Reagents

- Chloro-2, 4-dinitrobenzene (20 mM)

16.85 mg of 1-chloro-2, 4-dinitrobenzene (CDNB) was dissolved in 5 ml of absolute ethanol.

- Reduced Glutathione (0.1 M)

30.73 mg of glutathione (GSH) was dissolved in 1 ml of 0.1 M phosphate buffer (pH 6.5).

- Phosphate buffer (0.1 M, pH 6.5)

Dipotassium hydrogen phosphate trihydrate (0.381 g) and potassium dihydrogen phosphate (1.134 g) were dissolved in 90 ml of distilled water, the pH adjusted to 6.5 and the volume made up to 100 ml with distilled water.

Procedure

The medium for the estimation was prepared as shown in the table below and the reaction was allowed to run for 3 min with readings taken every 60 seconds against the blank at 340 nm.

Table 5 Determination of GSH S- Transferase Activity

Reagent	Blank	Test
CDNB (20 mM)	30 μ l	30 μ l
Reaction mixture (20mls phosphate buffer, 0.5mls Reduced glutathione and 10.5mls distilled water)	510 μ l	510 μ l
Sample	-	60 μ l

Calculations

The extinction coefficient of CDNB at 340 nm = 9.6 mM⁻¹cm⁻¹

$$\text{GSH S – transferase activity} = \frac{\Delta A_{340}/\text{min} \times \text{reaction volume}}{9.6 \times \text{sample volume} \times \text{mg protein/ml}}$$

$$= \text{ } \mu\text{mole/min/mg protein}$$

3.11.4. Determination of xanthine oxidase activity

The activity of xanthine oxidase was assessed by the method of (Bergmeyer *et al.*, 1974).

Principle

The assay is based on the measurement of uric acid absorbance at 290 nm as it is produced from xanthine by the action of xanthine oxidase.

Reagents

- **Phosphate buffer (0.05 M, pH 7.5)**

Dipotassium hydrogen phosphate trihydrate (0.760 g) and potassium dihydrogen phosphate (0.227 g) were dissolved in 90 ml of distilled water, the pH adjusted to 7.5 and the volume made up to 100 ml with distilled water.

- **Xanthine solution (0.15 mM)**

2.3 mg of xanthine was dissolved in a few drops of 0.1 M NaOH, 90 ml of distilled water added, the solution adjusted to pH 7.5 with dilute acid or base, and the solution made up to 100 ml.

Procedure

Into a micro plate, 150 μ l of phosphate buffer, 80 μ l of xanthine solution and 8 μ l of sample were pipetted. The sample was added first, then the xanthine solution. The mixture was mixed and absorbance was taken every minute for 3 minutes at 290 nm. A blank was made by replacing 8 μ l of sample with distilled water.

Calculation

The extinction coefficient of uric acid at 290 nm = $12.1 \text{ mM}^{-1}\text{cm}^{-1}$

$$\text{Xanthine oxidase activity} = \frac{\Delta A_{290}/\text{min} \times \text{reaction volume}}{12.1 \times \text{sample volume} \times \text{mg protein/ml}}$$

$$= \text{μmole/min/mg protein}$$

(Bergmeyer, 1974)

3.11.5. Determination of lipid peroxidation

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) present in the test sample according to the method of Varshney and Kale (1990).

Principle

Under acidic conditions, malondialdehyde (MDA) produced from the peroxidation of fatty acids reacts with the chromogenic reagent 2-thiobarbituric acid to yield a pink coloured complex with maximum absorbance at 532 nm and is extractable into organic solvent such as butanol. Malondialdehyde is often used to calibrate this test and thus the result is expressed as the amount of free MDA produced.

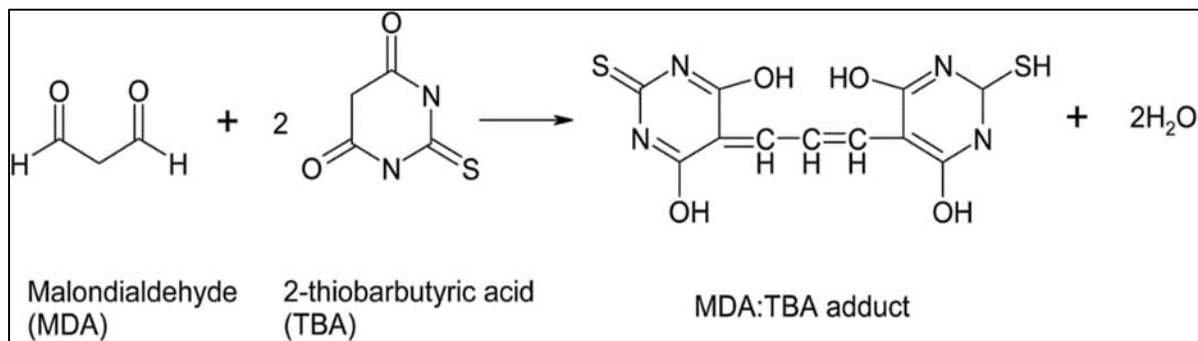


Figure 7 Chemical Reaction For Lipid Peroxidation (Varshney *et al.*, 1990)

Reagents

- 30% Trichloroacetic acid (TCA)

4.5 g of TCA (CCl_3COOH) was dissolved in distilled water and made up to 15ml with the same.

- 0.1M Hydrochloric acid (HCl)

13 μl of concentrated HCl (36.5-38%) was added to distilled water and the volume made up to 15 ml with the same.

- 0.75% Thiobarbituric acid (TBA)

0.1125 g of TBA was dissolved in 0.1 M HCl and made up to 15 ml with the same. Dissolution was aided by stirring in a hot water bath (50°C).

- 0.15 M Tris-KCl buffer (pH 7.4)

0.559 g of KCl and 0.909 g of Tris base were dissolved in 45 ml of distilled water, the pH was then adjusted to 7.4 with HCl and the volume made up to 50 ml with the same.

Procedure

An aliquot of $40\mu\text{l}$ of the test sample was mixed with $160\mu\text{l}$ of Tris-KCl buffer to which $50\mu\text{l}$ of 30% TCA was added. Then $50\mu\text{l}$ of 0.75 % TBA was added and placed in a water bath for 45 minutes at 80°C . This was then cooled in ice to room

temperature and centrifuged at 3000 rpm for 10 min. 200 μL of the clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532 nm using a microplate reader. (Varshney *et al.*, 1990)

Calculation

$$\text{MDA (units/mg protein)} = \frac{\text{Absorbance} \times \text{Volume of mixture}}{\text{E532nm} \times \text{Volume of Sample} \times \text{mg Protein}}$$

3.12. Inflammatory biomarkers assays

3.12.1. Determination of nitric oxide (NO) level

The NO Radical plays an important role as a physiological messenger. NO is formed from L-arginine (Palmer *et al.*, 1988) by NO synthase, which exists in several isoforms (Griffith and Stuehr, 1995). Constitutive calcium-dependent isoforms (cNOS) modulate the control of vascular tone in endothelial cells or the neurotransmission in neurons, whereas inducible calcium-independent isoforms (iNOS) are located in macrophages, chondrocytes and hepatocytes and are induced by cytokines and endotoxin (Bredt and Snyder, 1994; Nathan, 1992). Pathological conditions associated with increased release of cytokines and endotoxin, e.g. inflammation or sepsis can therefore increase NO production. Upon coming into the blood stream, nitrite reacts immediately with oxy-haemoglobin to form methaemoglobin. The level of nitric oxide was determined by the method of Green *et al.*, (1982).

Principle

Nitrite mediates the nitrosative modification of sulphanilic acid which then reacts with N-naphthylethylenediamine dihydrochloride forming a pink orange coloured products with maximum absorbance at 550nm.

Reagent

Griess Reagent [0.1 % N-(1-naphthyl) ethylenediamine dihydrochloride; 1 % sulphanilamide in 5 % phosphoric acid 1:1].

Procedure

The amounts of nitrite in supernatants or in serum were measured following the Griess reaction by incubating a 100 μL of sample with 100 μL of Griess reagent [0.1 % N-(1-naphthyl) ethylenediamine dihydrochloride; 1 % sulfanilamide in 5 % phosphoric acid; 1:1 purchased a] at room temperature for 20 min. The absorbance at 550 nm (OD 550) was measured spectrophotometrically. Nitrite concentration was calculated by comparison with the OD 550 of a standard solution of known sodium nitrite concentrations.

Calibration curve

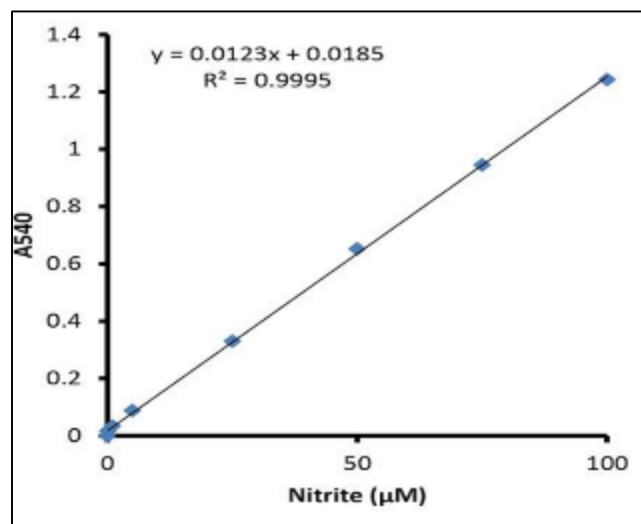


Figure 8 NO Calibration Curve

Calibrator at various concentrations was prepared by diluting stock 20 mmol/L solutions of NaNO₂ with distilled water. The nitrate calibrator was diluted with glycine buffer just as the serum samples were. Calibration curve was made over a linear range of nitrate between 0 and 100 µmol/L (Green *et al.*, 1982).

3.12.2. Determination of myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) is a lysosomal enzyme present in the azurophilic granules of polymorphonuclear leukocytes (PMNs) and is unique to neutrophils and monocytes. However, monocytes contain only one-third of the MPO found in PMNs. MPO utilizes H₂O₂ produced by the neutrophils to oxidize a variety of aromatic compounds to give substrate radicals for bacterial activity (Hampton *et al.*, 1998). This enzyme is unique however in that it can oxidize chloride ions to produce a strong non-radical oxidant, hypochlorous acid (HOCl). HOCl is the most powerful bactericidal compound produced by neutrophils. Excessive production of these radicals can cause oxidative stress leading to oxidative tissue injury. Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was determined by the modification of the method described by Trush *et al.*, (1994).

Principle

The activity of MPO was measured spectrophotometrically using o-dianisidine (Sigma-Aldrich) and hydrogen peroxide. In this presence of H₂O₂ as an oxidizing agent, MPO catalyses the oxidation of o-dianisidine yielding a brown coloured product, oxidized O-dianisidine, with a minimum absorbance at 470 nm, according to the following overall reaction:



Procedure

200 µl of O-dianisidine mixture [containing 16.7 mg of o-dianisidine dihydrochloride (3, 3-Dimethoxybenzidine, Fast Blue B, C₁₄H₁₆N₂O₂ Mol. Wt 244.3) in 100 ml of 50 mM phosphate buffer, pH=6.0, plus 50 µl of dilute H₂O₂ (4 µl of 59% H₂O₂ diluted in 96 µl of dH₂O)] was added to 7 µl of tissue homogenate (in triplicate). Absorbance readings were taken at 30 seconds interval for 4 minutes at 460 nm using a spectrophotometer. The MPO activity is in unit (U) of MPO/mg tissue, where one unit of MPO is defined as the amount needed to degrade 1 µmol of H₂O₂ per minute at room temperature. Considering that one unit (U) of MPO = 1 µmol of H₂O₂ split and that 1 µmol of H₂O₂ gives a change of absorbance of 1.13 x 10⁻² nm/min, units of MPO in each sample is determined as change in absorbance, that is:

$$[\Delta\text{Abs} (t_2 - t_1)] / \Delta\text{min} \times (1.13 \times 10^{-2}).$$

To get unit mg of tissue, the tissue: buffer ratio is used. For example, if a tissue: buffer ratio of 50 mg/ml is used, in 7 µl (0.007 ml) of homogenate, there is 0.35 mg of tissue. Therefore, to get units per mg tissue, the units MPO divided by 0.35.

Reagents

- 0.5 M Phosphate buffer (pH 6.0)

Dipotassium hydrogen phosphate trihydrate (K₂HPO₄·12H₂O (0.052g)) and Potassium dihydrogen phosphate (K₂HPO₄·12H₂O (0.64g)) were dissolved in 100 ml of distilled water, pH was adjusted to 6.0.

- Hydrogen peroxide

µl of 59% H₂O₂ diluted in 96 µl distilled water.

- O-Dianisidine

16.7 mg of o-dianisidine was dissolved in 100 ml 0.5M phosphate buffer.

NOTE: Add a drop of HCl to obtain clear mixture, if working with O-dianisidine instead of O-dianisidine dihydrochloride. However, do not calibrate pH back to 6.0 after dissolving O-Dianisidine with HCl in buffer.

Procedure

200 µl of combined solutions (buffered O-dianisidine and H₂O₂) and 7 µl of sample were pipetted into a microplate and absorbance was measured at 30 seconds interval for 4 minutes at 460 nm.

Calculation

$$\text{MPO activity} = \frac{\Delta \text{Abs (t}_2 - \text{t}_1) / \text{min} \times \text{volume of mixture} \times \text{dilution factor}}{(11.3 \times 10 - 3) \times \text{volume of sample} \times \text{mg protein}}$$

(Hampton *et al.*, 1998)

3.13. Statistical analysis of results

Graph pad prism was the application used to analyse the differences between the groups. Data were expressed as mean standard deviation. Statistical analyses were carried out using one-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered to be significant and post-hoc tests were carried out using the least significant difference (LSD).

4. Experiments and results

4.1. Effects of sodium arsenite (NaAsO₂) and butylated hydroxytoluene (bht) on the animal body weight and relative organ weight in male wistar rats

Table 6 Effects Of NaAsO₂ And BHT on the Body And Organ

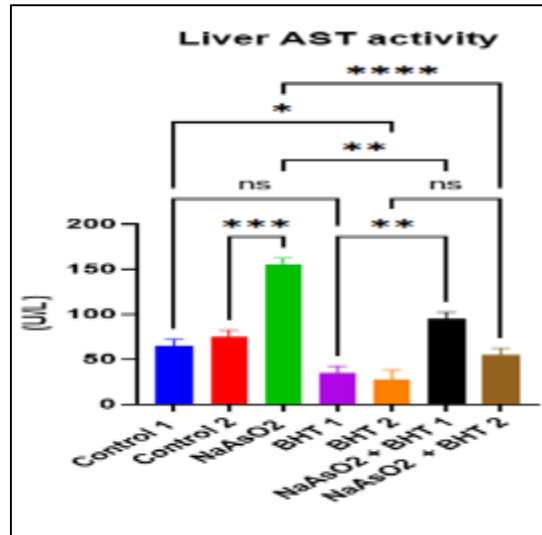
(g)	Control 1	Control 2	NaAsO ₂	BHT1	BHT 2	NaAsO ₂ + BHT1	NaAsO ₂ +BHT2
Final Body Weight	149.2±30.10	141.8±12.64	153.2±20.88	144.2±4.025	149.8±6.047	137.2±15.79	123.0±20.45
Initial Body Weight	126.8±24.29	111.3±15.51	127.5±12.50	117.2±6.181	115.7±7.528	111.5±9.524	112.8±11.27
Body Weight Gain	22.33±12.27	30.50±14.15	25.67±11.52	27.00±4.637	34.14±6.646	25.67±11.36	10.17±18.35***
Relative Liver Weight	3.254 ±0.4466	3.611 ±0.6093	3.682 ±0.3543	3.082 ±0.3180	3.521 ±0.1906	3.603±0.2996	3.919 ±0.3896

Values are expressed as mean ± SD; n=6; Control 1= Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day);NaAsO₂=2.5mg/kg/day of NaAsO₂ alone; BHT 1=25mg/kg/day of Butylated Hydroxytoluene alone; ; BHT2=50mg/kg/day of Butylated Hydroxytoluene alone; NaAsO₂+ BHT 1=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; NaAsO₂+ BHT 2=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; ***: Significant as compared with BHT1 group; $p \leq 0.05$; ****: Significant as compared with BHT2 and co-administered low dose group (NaAsO₂+ BHT 2); $p \leq 0.05$.

Generally, in table 5 above, there were no significant decrease ($p < 0.05$) in relation to the control group compared to the groups with administered doses. However, the co-administered high dose (BHT=50mg) showed a decreased body weight gain compared to the singly administered.

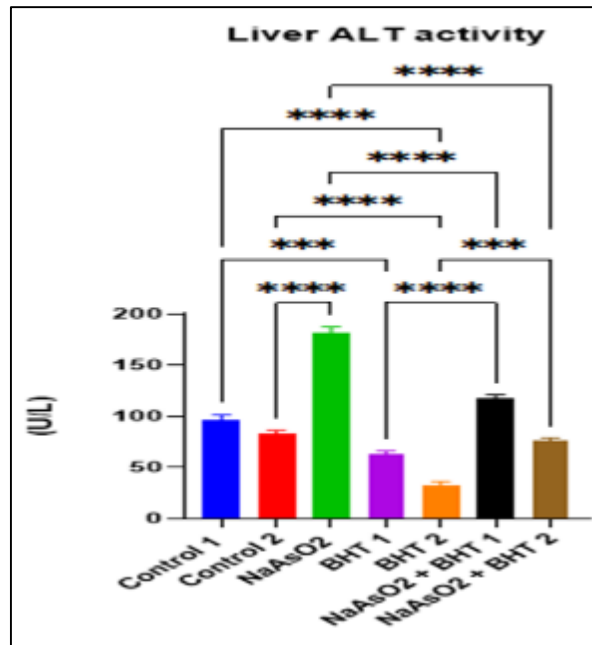
There were no significant increase or decrease ($p < 0.05$) in the kidney weight of the BHT administered group compared to control and all other groups. Sodium Arsenite administered group showed a non-significant decrease ($p < 0.05$) in the kidney weight across all the groups.

4.2. Effects of sodium arsenite (NaAsO₂) and butylated hydroxytoluene (BHT) on aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in male wistar rats



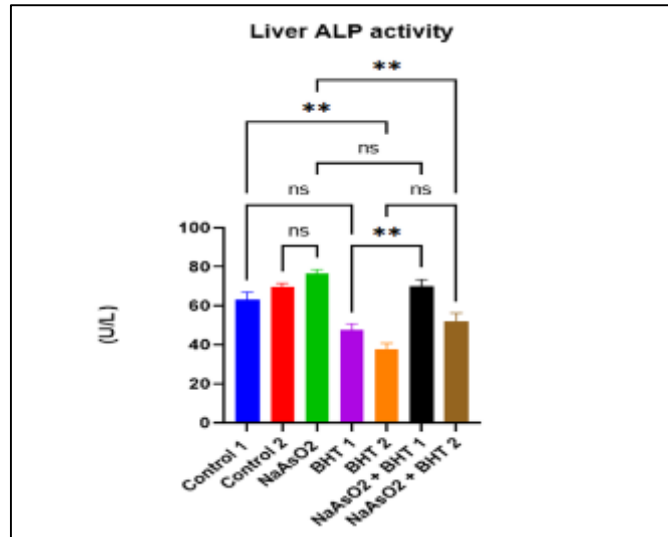
Values are expressed as mean \pm SD; n=6; Control 1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); NaAsO₂=2.5mg/kg/day of NaAsO₂ alone; BHT 1=25mg/kg/day of Butylated Hydroxytoluene alone; BHT 2=50mg/kg/day of Butylated Hydroxytoluene alone; NaAsO₂ + BHT 1 =25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; NaAsO₂ + BHT 2=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; *: significant as compared with control 1 group; p \leq 0.05; **: significant as compared with control 2 group; p \leq 0.05; ***: significant as compared with NaAsO₂ only group; p \leq 0.05

Figure 9 Impact of Sodium Arsenite (NaAsO₂) and Butylated Hydroxytoluene (BHT) on Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), and Alkaline Phosphatase (ALP) Levels in Male Wistar Rats



Values are expressed as mean \pm SD; n=6; Control 1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); NaAsO₂=2.5mg/kg/day of NaAsO₂ alone; BHT 1=25mg/kg/day of Butylated Hydroxytoluene alone; BHT 2=50mg/kg/day of Butylated Hydroxytoluene alone; NaAsO₂ + BHT 1 =25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; NaAsO₂ + BHT 2=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; *: significant as compared with control 1 group; p \leq 0.05; **: significant as compared with control 2 group; p \leq 0.05; ***: significant as compared with NaAsO₂ only group; p \leq 0.05

Figure 10 Impact of Sodium Arsenite (NaAsO₂) and Butylated Hydroxytoluene (BHT) on Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), and Alkaline Phosphatase (ALP) Levels in Male Wistar Rats

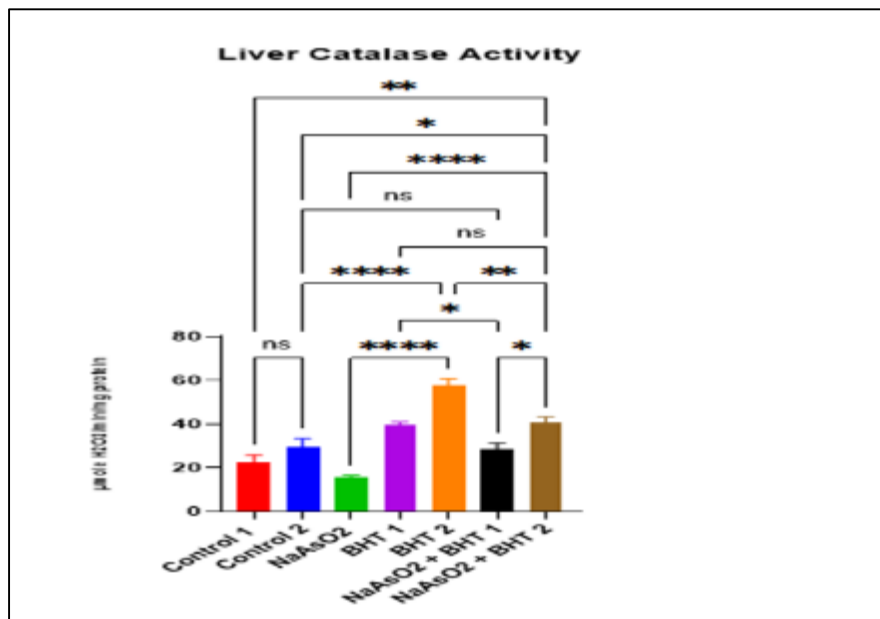


Values are expressed as mean \pm SD; n=6; Control 1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); NaAsO₂=2.5mg/kg/day of NaAsO₂ alone; BHT 1=25mg/kg/day of Butylated Hydroxytoluene alone; BHT 2=50mg/kg/day of Butylated Hydroxytoluene alone; NaAsO₂+ BHT 1 =25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; NaAsO₂+ BHT 2=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; *: significant as compared with control 1 group; p \leq 0.05; **: significant as compared with control 2 group; p \leq 0.05; ***: significant as compared with NaAsO₂ only group; p \leq 0.05

Figure 11 Effects Of Sodium Arsenite And Butylated Hydroxytoluene (BHT) on AST, ALT, And ALP In Male Wistar Rats

In figure 3.2, AST, ALT, and ALP levels in the liver show a significant difference (p \leq 0.05) in the BHT 2 administered compared to those treated the control groups. Also, AST, ALT, and ALP levels in the liver show a significant difference (p \leq 0.05) in the NaAsO₂ administered compared to those treated the control groups. The two co-administered groups for AST, ALT and ALP shows a level of significant difference (p \leq 0.05) when compared to those treated with NaAsO₂.

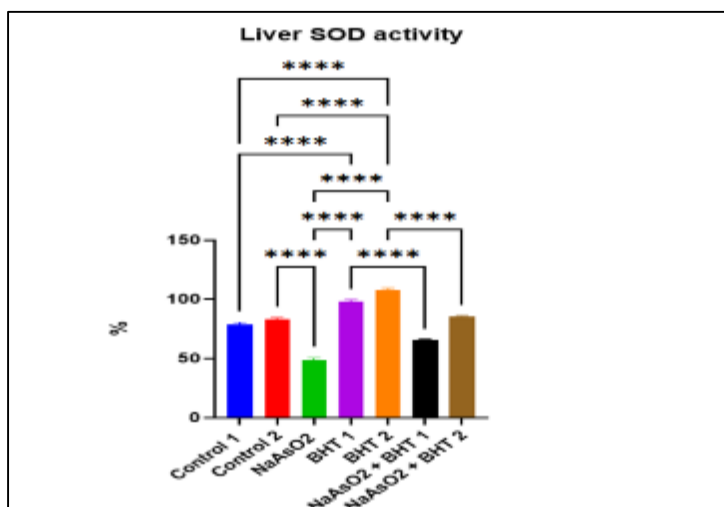
4.3. Effects of sodium arsenite (NaAsO₂) and butylated hydroxytoluene (BHT) on catalase, and superoxide dismutase (SOD) in male Wistar rats



Values are expressed as mean \pm SD; n=6; Control1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); NaAsO₂=2.5mg/kg/day of NaAsO₂ alone; BHT 1=25mg/kg/day of Butylated Hydroxytoluene alone; BHT2=50mg/kg/day of Butylated Hydroxytoluene alone; NaAsO₂+ BHT 1=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; NaAsO₂+ BHT 2=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; *: significant as compared with control 1 group; p \leq 0.05; **: significant as compared with control 2 group; p \leq 0.05; ***: significant as compared with NaAsO₂ only group; p \leq 0.05

Figure 12 Impact of Sodium Arsenite (NaAsO₂) and Butylated Hydroxytoluene (BHT) on Catalase and Superoxide Dismutase (SOD) Levels in Male Wistar Rats

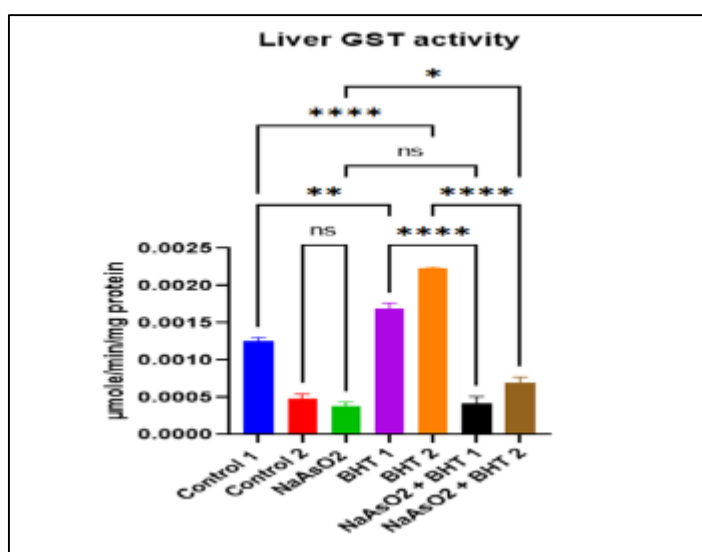
The table illustrates a significant difference ($p \leq 0.05$) and a very low activity of SOD in the liver in the rats treated with Sodium Arsenite alone compared with the control group 2. The liver SOD activities in animals treated with BHT showed a significant difference ($p \leq 0.05$) compared with control group. Also, the liver SOD activities in animals treated with BHT showed a significant difference ($p \leq 0.05$) compared with the NaAsO₂ group. The group co-treated with BHT 2+ NaAsO₂ showed a significant difference ($p \leq 0.05$) and a high CATALASE activity in liver compared with the rats in the control groups and NaAsO₂. There was an observed increase, the largest increase ($p \leq 0.05$) in CAT activity in the group treated with BHT2 in the liver compared with the control group (control 2) and NaAsO₂.



Values are expressed as mean \pm SD; n=6; Control 1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); NaAsO₂=2.5mg/kg/day of NaAsO₂ alone; BHT 1 =25mg/kg/day of Butylated Hydroxytoluene alone; BHT2 =50mg/kg/day of Butylated Hydroxytoluene alone; NaAsO₂+ BHT 1=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; NaAsO₂+ BHT 2=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; *: significant as compared with control 1 group; p \leq 0.05; **: significant as compared with control 2 group; p \leq 0.05; ***: significant as compared with NaAsO₂ only group; p \leq 0.05

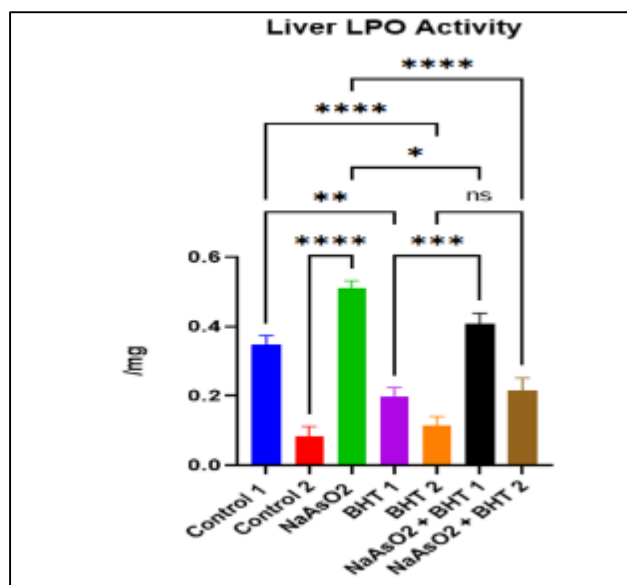
Figure 13 Effects of Sodium Arsenite and Butylated Hydroxytoluene (BHT) on Catalase (CAT) and Superoxide Dismutase (SOD) Activity in Male Wistar Rats

4.4. Effects Of Sodium Arsenite (NaAsO₂) And Butylated Hydroxytoluene (BHT) On Glutathione -S- Transferase (GST), Xanthine Oxidase And Lipid Peroxidation (LPO) Activity In Male Wistar Rats



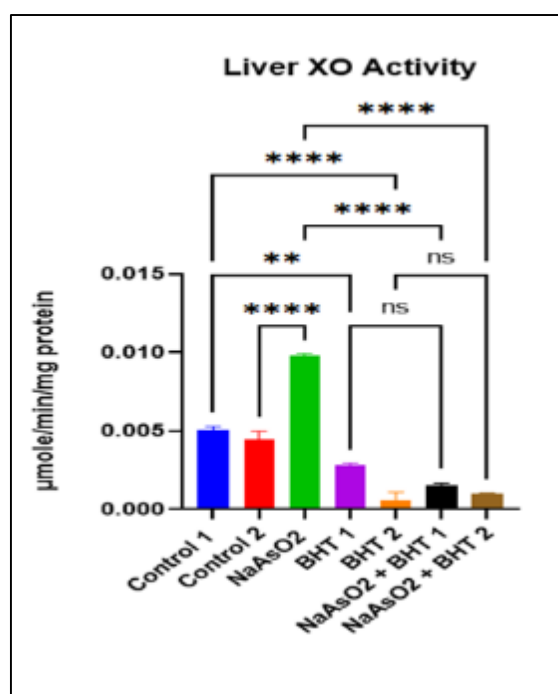
Values are expressed as Mean \pm SD; n=6; Control 1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); NaAsO₂=2.5mg/kg/day of NaAsO₂ alone; BHT 1=25mg/kg/day of Butylated Hydroxytoluene alone; BHT2=50mg/kg/day of Butylated Hydroxytoluene alone; NaAsO₂+ BHT 1=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; NaAsO₂+ BHT 2=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; *: significant as compared with control 1 group; p \leq 0.05; **: significant as compared with control 2 group; p \leq 0.05; ***: significant as compared with NaAsO₂ only group; p \leq 0.05

Figure 14 Impact of Sodium Arsenite (NaAsO₂) and Butylated Hydroxytoluene (BHT) on Glutathione-S-Transferase (GST), Xanthine Oxidase, and Lipid Peroxidation (LPO) Activity in Male Wistar Rats



Values are expressed as Mean \pm SD; n=6; Control 1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); NaAsO₂=2.5mg/kg/day of NaAsO₂ alone; BHT 1=25mg/kg/day of Butylated Hydroxytoluene alone; BHT2=50mg/kg/day of Butylated Hydroxytoluene alone; NaAsO₂+ BHT 1=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; NaAsO₂+ BHT 2=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; *: significant as compared with control 1 group; p \leq 0.05; **: significant as compared with control 2 group; p \leq 0.05; ***: significant as compared with NaAsO₂ only group; p \leq 0.05

Figure 15 Liver LPO Activity - Effects of Sodium Arsenite and Butylated Hydroxytoluene (BHT) on Glutathione S-Transferase (GST), Xanthine Oxidase and LPO activity in male wistar rats



Values are expressed as Mean \pm SD; n=6; Control 1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); NaAsO₂=2.5mg/kg/day of NaAsO₂ alone; BHT 1=25mg/kg/day of Butylated Hydroxytoluene alone; BHT2=50mg/kg/day of Butylated Hydroxytoluene alone; NaAsO₂+ BHT 1=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; NaAsO₂+ BHT 2=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; *: significant as compared with control 1 group; p \leq 0.05; **: significant as compared with control 2 group; p \leq 0.05; ***: significant as compared with NaAsO₂ only group; p \leq 0.05

Figure 16 Liver XO Activity - Effects of Sodium Arsenite and Butylated Hydroxytoluene (BHT) on Glutathione S-Transferase (GST), Xanthine Oxidase and LPO activity in male wistar rats

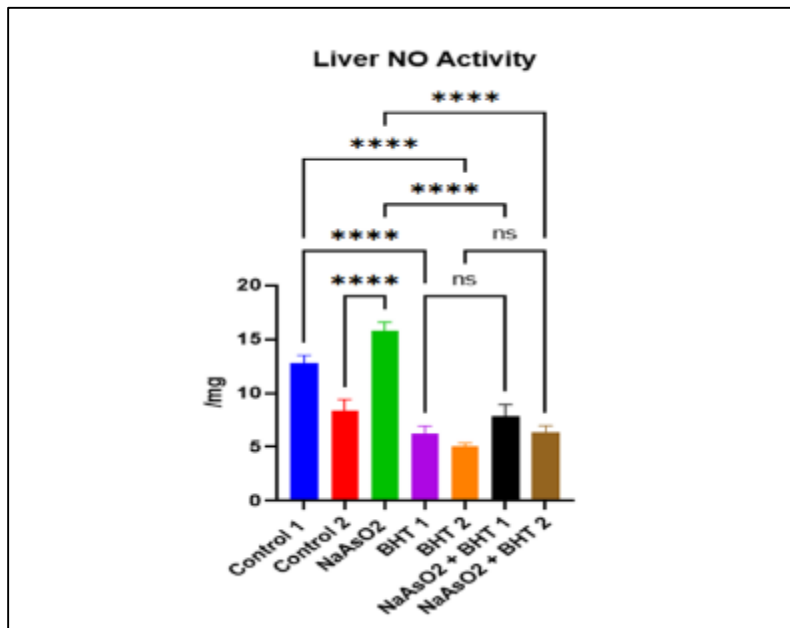
There was a significant difference ($p \leq 0.05$) and increase in the activities of GST in the liver of rats treated with BHT (low dose and high dose) only when compared to the control group. The group co-treated with NaAsO_2 +BHT2 showed a significant difference ($p \leq 0.05$) and a decrease in the GST activity in liver compared with the control group.

The XO activity decreased significantly difference ($p \leq 0.05$) and an increase in the liver the group treated with NaAsO_2 only compared to the control. The rest of the groups show a decrease in liver XO activity when compared with the former group with the co-administered group showing significant decrease in order of the dose of BHT.

The table vividly manifests a significant difference ($p \leq 0.05$) and a very high activity of LPO in the liver in the rats treated with Sodium Arsenite alone compared with the control group 2. The liver LPO activities in animals treated with BHT showed a significant difference ($p \leq 0.05$) a decrease in LPO activity compared with control group. Also, the liver LPO activities in animals treated with BHT showed a significant difference ($p \leq 0.05$) compared with the NaAsO_2 group.

The co-treated groups showed a significant difference ($p \leq 0.05$), an increase in activity (low dose of BHT) and a decrease in activity (high dose of BHT) in liver compared with the NaAsO_2 group.

4.5. Effects of sodium arsenite (NaAsO_2) and butylated hydroxytoluene (BHT) on inflammatory bio markers in male Wistar rats

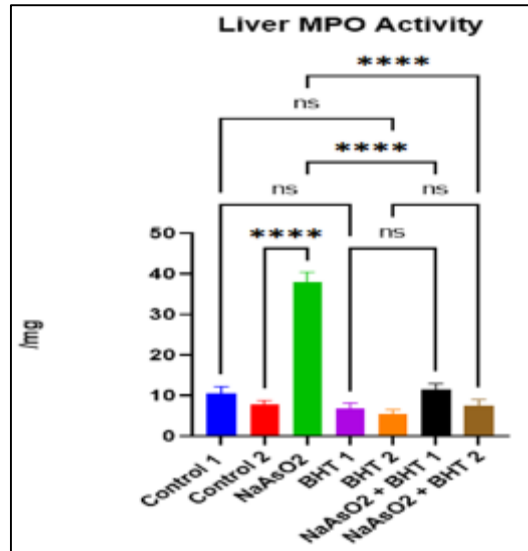


Values are expressed as Mean \pm SD; n=6; Control 1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); $\text{NaAsO}_2 = 2.5\text{mg/kg/day}$ of NaAsO_2 only; BHT 1 = 25mg/kg/day of Butylated Hydroxytoluene only; BHT2=50mg/kg/day of Butylated Hydroxytoluene only; NaAsO_2 + BHT 1= 25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO_2 after 1 hour; NaAsO_2 + BHT 2= 25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO_2 after 1hour; *: significant as compared with control 1 group; $p \leq 0.05$; **: significant as compared with control 2 group; $p \leq 0.05$; ***: significant as compared with NaAsO_2 only group; $p \leq 0.05$

Figure 17 Effects of Sodium Arsenite (NaAsO_2) and Butylated Hydroxytoluene (BHT) on Inflammatory Biomarkers in Male Wistar Rats

In figure 3.5, NO levels in the liver show a significant difference ($p \leq 0.05$) and increase in activity NO levels when the NaAsO_2 administered is compared to those treated the control groups. Overall, the NO levels of the administered groups in the liver decrease ($p \leq 0.05$) in relation to the control group especially in groups administered with BHT

MPO activity in the liver generally shows significant difference ($p \leq 0.05$) and increase in activity when the NaAsO_2 administered is compared to the control group. The group co-treated with NaAsO_2 +BHT2 showed a significant difference ($p \leq 0.05$), an increase in activity (low dose of BHT) and a decrease in activity (high dose of BHT) in liver compared with the control group.

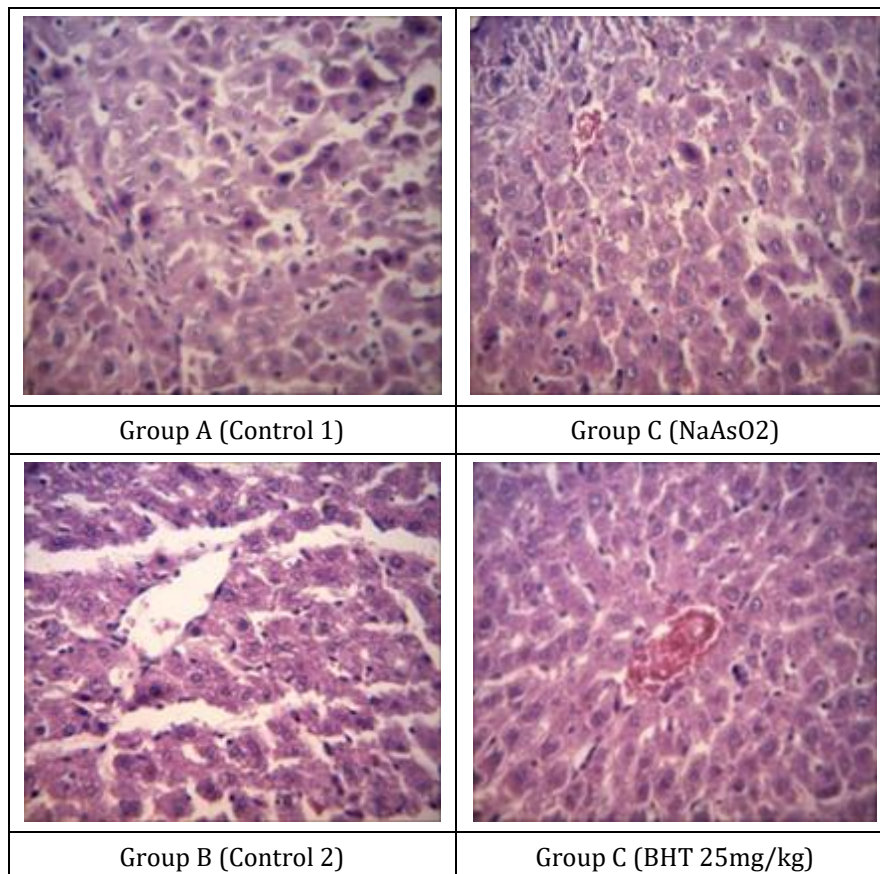


Values are expressed as Mean \pm SD; n=6; Control 1 =Corn oil (2ml/kg/day); Control 2 =Distilled water (2ml/kg/day); NaAsO₂= 2.5mg/kg/day of NaAsO₂ only; BHT 1= 25mg/kg/day of Butylated Hydroxytoluene only; BHT2 =50mg/kg/day of Butylated Hydroxytoluene only; NaAsO₂+ BHT 1= 25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; NaAsO₂+ BHT 2= 25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour; *: significant as compared with control 1 group; p \leq 0.05; **: significant as compared with control 2 group; p \leq 0.05; ***: significant as compared with NaAsO₂ only group; p \leq 0.05

Figure 18 Liver MPO Activity - Effects of Sodium Arsenite and Butylated Hydroxytoluene (BHT) on inflammatory biomarkers in male wistar rats

4.6. Histology

4.6.1. Liver



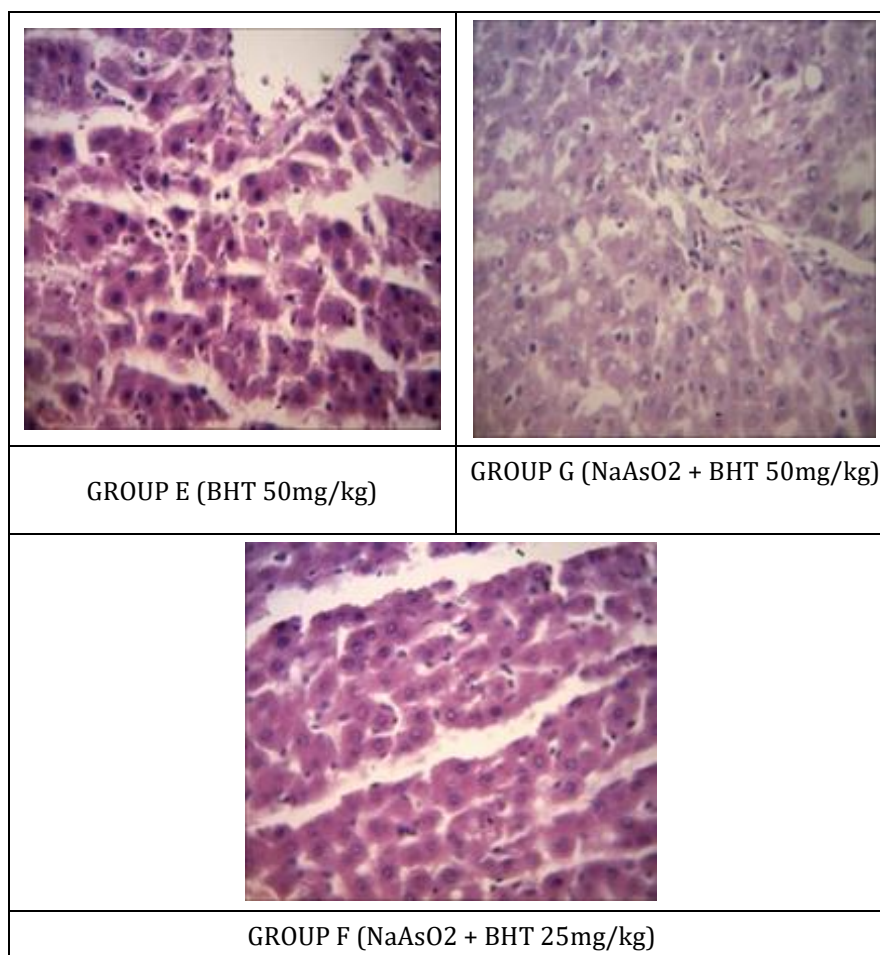


Figure 19 Histological representation of the liver organ from each group from group A-G

In group A, few hepatocytes appeared rounded and individualized. In groups B, E, and F, we have no lesions seen. For group C, there is a very mild diffuse hydropic degeneration of hepatocytes. Then, as regards group D, there is a very mild central venous congestion.. Lastly, in group G, there is a mild diffuse vacuolar degeneration of hepatocytes.

5. Discussion

In this study, we examined the effects of exposure to NaAsO₂ on specific concentrations in conjunction with the intake of the proper dosage of Butylated hydroxytoluene (BHT) at varying concentrations. Our findings confirm that NaAsO₂ is a hepatotoxic compound and that BHT has positive effects on liver organ.

The ALT, ALP, and AST are enzymes found majorly in the liver. AST, ALT, and ALP activity have significant increase in the NaAsO₂ treated group when compared to the control suggesting damage to the liver and consequently leakage of these enzymes from the liver to the serum. Also, when compared to control 1 and control 2, the AST, ALT, ALP activity in BHT 1 and BHT 2 group experienced a significant decrease when compared to the group of control 1 and control 2. However, when the low dose co-administered group, NaAsO₂+ BHT1 was compared to the group taking NaAsO₂ alone and BHT1 alone, it is found out that the level of activity of these enzymes are lower in the NaAsO₂+ BHT1, when compared to the NaAsO₂ group alone but higher when compared to BHT 1.

Also, when NaAsO₂+ BHT 2 group was compared to the group taking NaAsO₂ alone and BHT1 alone, it appeared that the levels of these enzymes are significantly lower in the NaAsO₂+ BHT 2 group when compared to the NaAsO₂ group alone but higher when compared to BHT 2 group.

The CAT and SOD are important enzymes used in removing reactive oxygen species. Their activities were decreased ($p \leq 0.05$) in the group administered with the dose of NaAsO₂. It was observed to have a strong negative influence in the liver.

GST has similar antioxidant properties. GST activity generally increased ($p < 0.05$) in the liver at both co-administered concentrations (25mg/kg and 50mg/kg) compared to the NaAsO₂ group.

XO activity generally increases compared to the control group. The liver of NaAsO₂ administered rats were observed to have increased in the activity of XO compared to those treated with BHT only and the co-administered. This suggests a prominent inflammatory effect on XO activity by sodium arsenite.

LPO is a marker of oxidative stress. LPO levels increase in liver of animals exposed to NaAsO₂ and decreased when BHT doses were administered compared to NaAsO₂.

NO and MPO are inflammatory molecules. That is, they suggest the presence of inflammation in the organ tested as an inflammatory response. NO levels are increased in the liver and more pronounced effect is observed in kidneys of induced with sodium Arsenite. The administration of BHT to such rats ameliorated this effect significant especially when used in high dose. MPO activity is higher at all concentrations in the liver and this was furthermore enhanced by the administration of the toxicant under study, Sodium Arsenite.

And finally, the use of BHT alone in high dose was observed to have caused in the damage of the kidney as seen in group treated only with its high dose having reduced activity in the antioxidant assays.

Histologically, sodium arsenite administration suggests toxicity while that of BHT showed ameliorative potentials of the chemical on sodium arsenite toxicity.

Histological analysis of liver organs showed that in the control group assigned with distilled water (Group A), there were a few mild aggregates of mono-nuclear inflammatory cells. In the control group (Group B), the group treated with 50 mg/kg BHT (Group E), the co-administered group of NaAsO₂ + 25 mg/kg (Group F), the histological analysis showed that there was no lesion in the liver organ of these groups mentioned.

Lastly, in the groups administered with NaAsO₂ (Group C), there is a very mild diffuse hydropic degeneration of hepatocytes. The ones administered with 25 mg/kg BHT (Group D) have a very mild central venous congestion. The group co-administered with NaAsO₂ + 50 mg/kg BHT (Group G), there is a mild diffuse vacuolar degeneration of hepatocytes.

All these results are consistent with earlier reports on the hepatotoxicity of sodium arsenite (Sally *et al.*, 2023).

6. Conclusion

According to the results of the present study, exposure of rats to NaAsO₂ and BHT increased antioxidant activity and decreased the levels of free radicals, including reactive oxygen species. This suggests that liver damage from sodium arsenite exposure can be mitigated by using antioxidant compounds, such as butylated hydroxytoluene (BHT). Therefore, co-exposure to these compounds is confirmed to reduce toxicity in the liver and liver tissues. In addition, this study examining the chemo-protective effect of BHT on NaAsO₂-induced hepatotoxicity in male Wistar rats, the experimental design involved grouping the rats into seven treatment categories. These included controls (corn oil and distilled water), NaAsO₂ alone (2.5mg/kg), BHT alone (25 mg/kg), and various combinations of NaAsO₂ and BHT. Over 14 days, markers of liver function, inflammation, oxidative stress, and antioxidant profiles were measured. Results indicated that NaAsO₂ exposure significantly increased AST, ALT, and ALP activities and oxidative stress markers such as xanthine oxidase and lipid peroxidation. It also elevated inflammatory markers like myeloperoxidase and nitric oxide while decreasing antioxidant markers, including superoxide dismutase, glutathione-S-transferase, and catalase. However, co-exposure to BHT significantly mitigated these adverse effects, demonstrating its protective role. This study confirms BHT's potential as an effective antioxidant against NaAsO₂-induced hepatotoxicity. Integrating TPD into this context offers deeper insights into the molecular interactions of NaAsO₂ and BHT with biological surfaces, aiding in the development of more effective detoxification strategies (Onivefu, 2023).

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no conflicts of interest.

Statement of ethical approval

All experimental procedures involving Wistar rats were conducted in accordance with the ethical standards and guidelines set forth by the University of Ibadan and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Ibadan. The approval number for this study is 8466879009. All efforts were made to minimize the number of animals used and to reduce suffering.

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Appendices

Table Effects of Sodium Arsenite and Butylated Hydroxytoluene (BHT) on AST, ALT, and ALP in male Wistar rats.

(g)	Control 1	Control 2	NaAsO ₂	BHT1	BHT 2	NaAsO ₂ + BHT1	NaAsO ₂ +BHT2
AST	65.00±7.071	75.00±7.071	155.0±7.071**	35.00±7.071	27.50±7.071*	95.00±7.071***	55.00±7.071***
ALT	96.50±4.950	82.50±3.536	182.0±5.292**	62.50±3.536*	32.50±3.536* **	117.5±3.536***	76.00±2.828***
ALP	63.00±4.243	69.50±2.121	76.82±1.952**	47.50±3.536*	37.50±3.536*	70.49±3.097***	52.10±4.384***

Values are expressed as Mean ± SD; n=6; Control 1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); NaAsO₂= 2.5mg/kg/day of NaAsO₂ only; BHT 1= 25mg/kg/day of Butylated Hydroxytoluene only.; BHT2=50mg/kg/day of Butylated Hydroxytoluene only; NaAsO₂+ BHT 1= 25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour. ;NaAsO₂+ BHT 2= 25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1hour; *: significant as compared with control 1 group; p≤ 0.05; **: significant as compared with control 2 group; p≤ 0.05; ***: significant as compared with NaAsO₂ only group; p≤ 0.05

Table Effects of Sodium Arsenite and Butylated Hydroxytoluene (BHT) on catalase (CAT) and superoxide dismutase (SOD) activity in male Wistar rats

Antioxidant Assays	Control 1	Control 2	NaAsO ₂	BHT1	BHT2	NaAsO ₂ + BHT1	NaAsO ₂ +BHT 2
SOD	78.50 ±2.121	83.00±1.414	48.50 ±2.121**	97.90±1.729* ***	108.0±1.414* ** ***	65.50±0.7071	85.50±0.7071
CAT	22.69±3.163	29.61±3.703	15.54±1.016	39.53±1.544	57.55±3.226* ** ***	28.42±2.966	40.63±2.704* ** ***

Values are expressed as Mean ± SD; n=6; Control 1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); NaAsO₂= 2.5mg/kg/day of NaAsO₂ only; BHT 1= 25mg/kg/day of Butylated Hydroxytoluene only.; BHT2=50mg/kg/day of Butylated Hydroxytoluene only; NaAsO₂+ BHT 1= 25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour. ; NaAsO₂+ BHT 2=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1hour; *: significant as compared with control 1 group; p≤ 0.05; **: significant as compared with control 2 group; p≤ 0.05; ***: significant as compared with NaAsO₂ only group; p≤ 0.05

Table Effects of sodium arsenite and butylated hydroxytoluene (BHT) on glutathione s-transferase (GST) and xanthine oxidase activity in male Wistar rats.

Antioxidant indicators	Control 1	Control 2	NaAsO ₂	BHT1	BHT 2	NaAsO ₂ + BHT1	NaAsO ₂ +BHT 2
GST	0.001261 ± 0.00004071	0.0004782 ± 0.00006596	0.003774± 0.00006001	0.001697± 0.00006661*	0.002237± 0.00001392*	0.0004223± 0.00008854	0.0004223± 0.00008854**
XO	0.005085± 0.0001909	0.004484± 0.0004999	0.009777±	0.002844±	0.0005911± 0.0005184*	0.001501±	0.0009935±

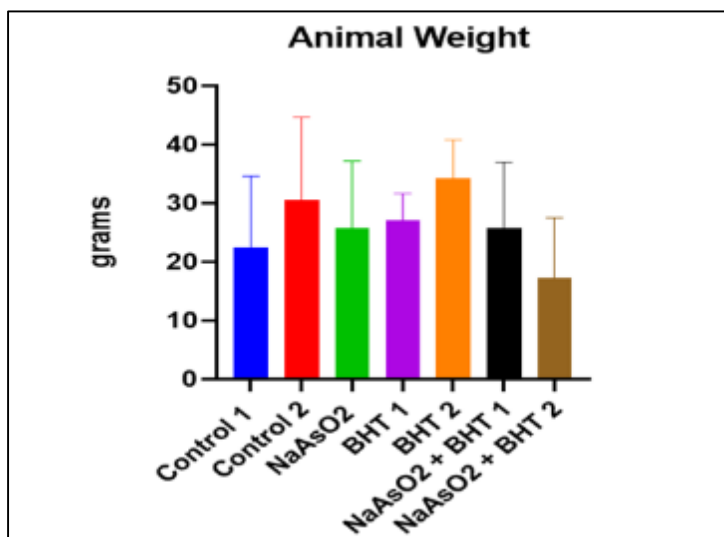
			0.0001428* *	0.00008415 *		0.0001704** *	0.00005162* **
NO Level	12.74± 0.7530	8.393± 0.9912	15.80± 0.7799**	6.215± 0.7317*	5.069± 0.2979*	7.895± 1.051***	6.352± 0.5994***
MPO	10.59± 1.627	7.827± 1.048	38.12± 1.358**	6.912± 1.281	5.522± 1.027	11.66± 1.313**	7.617± 1.506**

Values are expressed as Mean ± SD; n=6; Control 1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); NaAsO₂= 2.5mg/kg/day of NaAsO₂ only; BHT 1= 25mg/kg/day of Butylated Hydroxytoluene only.; BHT2 =50mg/kg/day of Butylated Hydroxytoluene only; NaAsO+BHT1=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour. ;NaAsO₂+ BHT 2=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1hour; *: significant as compared with control 1 group; p≤ 0.05; **: significant as compared with control 2 group; p≤ 0.05; ***: significant as compared with NaAsO₂ only group; p≤ 0.05

Table Effects of Sodium Arsenite and Butylated Hydroxytoluene (BHT) on oxidative stress biomarkers in male Wistar rats

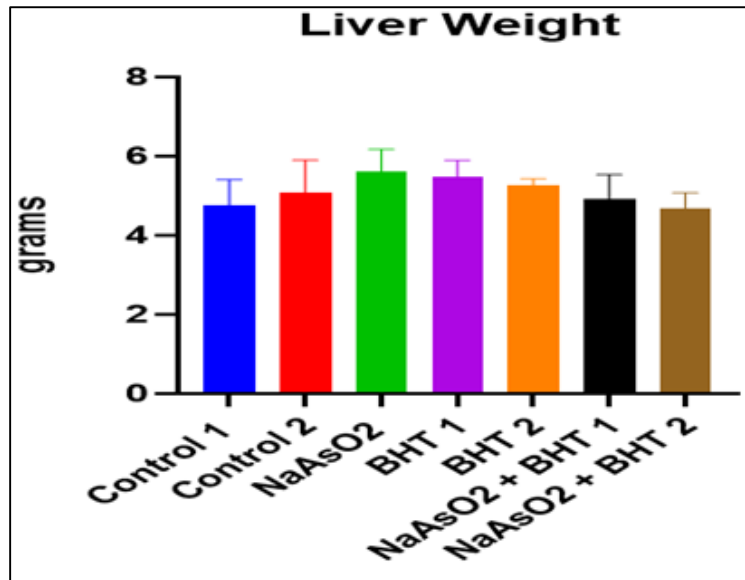
Oxidative Biomarker	Stress	Control 1	Control 2	NaAsO ₂	BHT1	BHT 2	NaAsO ₂ + BHT1	NaAsO ₂ +BHT2
LPO		10.59 ± 1.627	7.827 ± 1.048	38.12 ± 2.353**	6.912 ± 1.281*	5.522 ± 1.072*	11.66 ± 1.313***	7.617 ± 1.506***

Values are expressed as Mean ± SD; n=6; Control 1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); NaAsO₂=2.5mg/kg/day of NaAsO₂ only; BHT 1= 25mg/kg/day of Butylated Hydroxytoluene only.; BHT2=50mg/kg/day of Butylated Hydroxytoluene only; NaAsO+BHT1=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour. ; NaAsO₂+ BHT 2=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1hour; *: significant as compared with control 1 group; p≤ 0.05; **: significant as compared with control 2 group; p≤ 0.05; ***: significant as compared with NaAsO₂ only group; p≤ 0.05



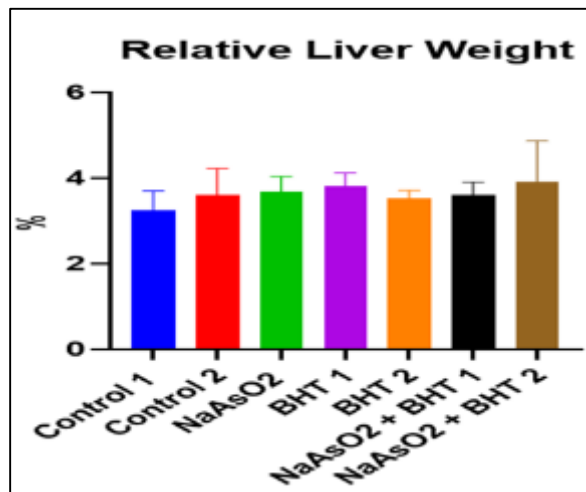
Values are expressed as Mean ± SD; n=6; Control 1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); NaAsO₂=2.5mg/kg/day of NaAsO₂ only; BHT 1= 25mg/kg/day of Butylated Hydroxytoluene only.; BHT2=50mg/kg/day of Butylated Hydroxytoluene only; NaAsO+ BHT 1=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour.;NaAsO₂+ BHT 2=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1hour; *: significant as compared with control 1 group; p≤ 0.05; **: significant as compared with control 2 group; p≤ 0.05; ***: significant as compared with NaAsO₂ only group; p≤ 0.05

Figure 20 Animal Weights considered in this research



Values are expressed as Mean \pm SD; n=6; Control 1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); NaAsO₂= 2.5mg/kg/day of NaAsO₂ only; BHT 1= 25mg/kg/day of Butylated Hydroxytoluene only.; BHT2=50mg/kg/day of Butylated Hydroxytoluene only; NaAsO₂+BHT1= 25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour.;NaAsO₂+ BHT2=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1hour; *: significant as compared with control 1 group; p \leq 0.05; **: significant as compared with control 2 group; p \leq 0.05; ***: significant as compared with NaAsO₂ only group; p \leq 0.05

Figure 21 Animal Liver Weights considered in this research



Values are expressed as Mean \pm SD; n=6; Control 1=Corn oil (2ml/kg/day); Control 2=Distilled water (2ml/kg/day); NaAsO₂= 2.5mg/kg/day of NaAsO₂ only; BHT 1= 25mg/kg/day of Butylated Hydroxytoluene only.; BHT2 =50mg/kg/day of Butylated Hydroxytoluene only; NaAsO₂+BHT1= 25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1 hour.;NaAsO₂+ BHT 2=25mg/kg/day of Butylated Hydroxytoluene +2.5mg/kg/day of NaAsO₂ after 1hour; *: significant as compared with control 1 group; p \leq 0.05; **: significant as compared with control 2 group; p \leq 0.05; ***: significant as compared with NaAsO₂ only group; p \leq 0.05

Figure 22 Relative Animal Liver Weights considered in this research