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DETERMINATION AND PREVENTION OF MERCURY INDUCED OXIDATIVE STRESS USING *THEOBROMA CACAO* IN *POECILIASPHENOPS (BLACK MOLLY) FISH*

D. Parvathavardhini*[1] and R. Amithkar[2]

[1]Assistant Professor, [2]PG Student

[1,2]Department of Biochemistry, Rathnavel Subramaniam College of Arts & Science, Coimbatore, Tamilnadu, India.

***Corresponding Author: D. Parvathavardhini**

Assistant Professor, Department of Biochemistry, Rathnavel Subramaniam College of Arts & Science, Coimbatore, Tamilnadu, India.

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ABSTRACT

This study aimed to investigate the potential use of Theobroma cacao in preventing oxidative stress in Poeciliasphenops (black molly) fish. The experiment was conducted by exposing the fish to sub-lethal concentrations of mercury and treating them with different doses of T. cacao extract. The levels of oxidative stress biomarkers, such as lipid peroxidation, glutathione peroxidase, and catalase, were measured in the fish tissues. The results showed that mercury exposure caused a significant increase in lipid peroxidation and a decrease in the activity of antioxidant enzymes, indicating oxidative stress in the fish tissues. However, treatment with T. cacao extract resulted in a dose-dependent decrease in lipid peroxidation and an increase in the activity of antioxidant enzymes, suggesting a protective effect against mercury-induced oxidative stress. In conclusion, this study demonstrates the potential of T. cacao as a natural antioxidant agent that can protect against mercury-induced oxidative stress in black molly fish. Further research is needed to explore the mechanism of action of T. cacao and its potential use in preventing oxidative stress in other aquatic organisms.

KEYWORDS:

1. Mercury
2. Oxidative stress
3. Theobroma cacao
4. Poeciliasphenops
5. Fish
6. Antioxidants
7. Lipid peroxidation
8. Glutathione peroxidase
9. Catalase
10. Environmental toxicity

INTRODUCTION

Mercury is an important pollutant of water worldwide. A variety of human activities are connected with mercury pollution (silver and gold mining, coal combustion, dental amalgam Organic methylmercury and inorganic (mercurous, mercuric) forms exist in nature. Organic forms are the result of methylation of inorganic mercury by microorganisms in sediments and water. Methylmercury is generally more toxic to fish than the inorganic forms. Mercury reacts with the thiol groups of GSH, which can induce GSH depletion and oxidative stress in tissue. Metallothioneins also play a protective role in response to mercury exposure. The mRNA expression of two MT genes was noted by in the liver of feral carp *Cyprinuscarpio*from a mercury-contaminated river.

Chemically, oxidative stress is associated with increased production of oxidizing species or a particularly destructive aspect of oxidative stress is the production of reactive oxygen species, which include free radicals and peroxides. Some of the less reactive of these species (such as superoxide) can be converted by oxido reduction reactions with transition metals or other redox cycling compounds (including quinones) into more aggressive radical species that can cause extensive cellular damage. The major portion of long term effects is inflicted by damage on DNA. Most of these oxygen-derived species are produced at a low level by normal aerobic metabolism and the damage they cause to cells is constantly repaired. However, under the severe levels of oxidative stress that cause necrosis, the damage causes ATP depletion, preventing controlled apoptotic death and causing the cell to simply fall apart.

The activation of oxidative manifestations leads to the response of antioxidants, activation of expression of genes encoding antioxidant enzymes, elevation of the concentration of ROS scavengers. The cocoa bean is one of nature's most amazing **superfoods**, offering high antioxidant value, mineral benefits and **natural energy**. Doctors and dieticians have espoused the benefits of dark chocolate for years, teaching us that the cacao beans used to make this healthy chocolate can become a critical part of a healthy diet.

The present study is about to determine the activity of *Theobroma cacao* to prevent mercury induced oxidative stress in black molly (poeciliasphenops) fish by considering the parameters such as Catalase, Glutathione Peroxidase and Protein estimation. The plant is also undergone the preliminary phytochemical screening along with the Invivo study.

AIM AND OBJECTIVE

The current study entitled "Determination and Prevention of Mercury Induced Oxidative Stress using *Theobroma cacao*in Black molly Fish" was focussed on the following Objectives,

- To Determine the presence of Phytochemicals in *Theobroma cacao.*
- To Determine the activity of *Theobroma cacao*to prevent mercury induced oxidative stress in Black molly (Poeciliasphenops) fish.
- To find out the activity of the Enzyme Glutatione peroxidase and Catalase in Mercury induced Oxidative stress in Black molly fish.
- To Estimate the Protein in the experimental groups.

MATERIALS AND METHODS

COLLECTION OF PLANT MATERIAL

Fresh beans of Theobroma cacao were collected from local areas of Calicut, Kerala.

PREPARATION OF EXTRACT

The collected fresh beans of Theobroma cacao was washed with running tap water and then washed with distilled water. Then the beans were dried that is shadow drying. After proper drying takes place the beans were powdered. 5g of powder was taken and soaked in 50ml of various solvents i.e., ethanol, methanol, benzene and distilled water for 72 hours. After 72 hours, the mixtures were filtered through Whatmann No.1 filter paper. The filtrate obtained were centrifuged at 5000rpm for 5 minutes. The supernatant was collected in the beaker and the solvents were evaporated to dryness. The residue left over was stored at 4^0C in refrigerator. At the time of phytochemical screening the residues were used.

QUALITATIVE PHYTOCHEMICAL SCREENING

The presence of possible phytochemical constituents in the extracts were evaluated qualitatively.

1. Detection of Alkaloids

Hanger's test

To 2ml of extract, a few drops of Hanger's reagent (saturated picric acid) were added. Formation of a bright yellow colored precipitate indicates the presence of alkaloids. *(P.Archana et al., Jul 2012*)

2. Detection of Carbohydrates

a) Molisch's test

To 2ml of the extract, a few drops of Molisch's reagent was added, followed by 1ml of concentrated H_2SO_4 slowly down the sides of the test tube so that the acid forms an immiscible reddish brown layer with the extract solution.

b) Benedict's test

To 2ml of the extract, add Benedict's reagent and heated gently. Appearance of orange red precipitate shows the presence of carbohydrate. (*Cannell, 1998*).

3. Detection of Proteins

a) Xanthoproteic test

The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

b) Ninhydrin test

To the extract, add Ninhydrin reagent and boiled for few minutes. Formation of yellow colour indicates the presence ofamino acids.

4. Detection of Flavanoids

a) Alkaline Reagent test

To 1-2 ml of the extracts, a solution of sodium hydroxide is added. Appearance of yellow to red colour indicates the presence of flavanoids.

b) Lead-acetate test

To 1-2 ml of the extracts, add few drops basic lead acetate solution. Formation of reddish brown precipitate indicates the presence of flavonoids. *(P. Archana et al., Jul 2012)*

5. Determination of Saponins

5ml of the extract is taken in a test tube and add sodium bicarbonate. then the miwture was shaken vigorously to obtain a stable froth. This indicates the presence of saponins. *(P.Archana et al., Jul 2012*)

6. Determination of Tannin

Ferric chloride test

The extracts were treated separately with few drops of FeCl3 solution. Formation of blackish precipitate indicates the presence of tannins. *(P. Archana et al., Jul 2012)*

7. Determination of Phenols

To 2 ml of added few drops of 10% FeCl3. Appearance of blue or green colour indicates presence of phenols. *(Haris et al., 2014).*

8. Determination of Steroids

To 1-2 ml of all the extracts, a few drops of acetic anhydride solution was added. To this mixture, a few drops of Conc. H2SO4 was added carefully along the walls of the test tube. Formation of reddish brown ring at the junction of two layers indicates the presence of steroids. *(P. Archana et al., Jul 2012)*

DETERMINATION AND PREVENTION OF MERCURY INDUCED OXIDATIVE STRESS USING *Theobroma cacao* IN *poeciliasphenops(black molly)FISH*

FISH COLLECTION

The *Poeciliasphenops* (black molly) fishwas purchase from commercial aquarum in Sulur, coimbatore.

KINGDOM : Animalia
FAMILY : Poeciliidea

GENUS : Poecilia
SPECIES : sphenops

KINGDOM *Plantae*

ORDER *Malvales*
FAMILY *Malvaceae*
GENUS *Theobroma*
SPECIES *T.cacao*

Mercuric chloride

Mercury was obtained in the form of Mercuric chloride from Biochemistry laboratory in Rathnavel Subramaniam College of Arts and Science, Sulur, coimbator and dissolved in acid and add to the fish tank for different dosage.

- **Stock solution preparation (1M)**

0.272 g of mercuric chloride was dissolved in hydrochloric acid.

- **Working standard preparation(20mM)**

20µl of the stock solution was made up to 1ml with distilled water.

From this working standard 30µl concentration was added in our experiment.

Experimental design

Each group we used duplicate sample for first group control (C), second Test I (T1), Third group Test II (T2), Fourth group T III (T3). Initially all the groups were kept in separate bowls and maintain for 5 days in environmental condition. Initially we add 10µM/L followed 20µM/L. For all the dosage we gave time for sacrifaction. After fish was sacrifice and collect the muscle sample to keep on 0.1M Phosphate buffered saline.

Sample preparation

The muscle sample was homogenized with glass tissue homogenizer and the sample was transfer to fresh microfuge tube. The tube was centrifuge at 12000 rpm 10minutes at 4^{O}C. collect the supernatant and transfer to fresh microfuge tube. The tube was keeping it on -20^{O}C until processing of enzyme assay.

1. ENZYME ASSAY

1. ANALYSIS OF CATALASE ACTIVITY

The CAT activity in the tissues was determined by measuring the decompositon of hydrogen peroxide at 240 nm, according to the method of Aebi. *(Aebi, 1984).*

Reagent grade water	: 1.9ml
0.059M Hydrogen peroxide	:1.0ml
Enzyme	:0.1ml

Incubate in spectrophotometer for 4-5 minutes to achieve temperature equilibration and to establish blank rate if any. Add 0.1 ml of diluted enzyme and record decrease in absorbance at 240 nm for 2-3 minutes. Calculate ΔA_{240}/min from the initial (45 second) linear portion of the curve.

Calculation

$$\text{Units/mg} = \frac{\text{A240/minX1000}}{\text{43.6Xmg enzyme/ml reaction mixture}}$$

43.6= Coefficient value

2. ANALYSIS OF GLUTATHIONE PEROXIDASE

Glutathione peroxidase activity was measured following the method of *Flohe* and *Gunzler (1984*).

The GSSG generated by GPX was reduced by GR, and NADPH oxidation was monitored at 340 nm. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.1), 3.6 mM GSH, 3.6 mM sodium azide, 1 IU/mL glutathione reductase, 0.2 mM NADPH, and 1.2 mM H2O2.

Glutathione peroxidase

2 GSH + H2O2---------- > GSSG + 2 H2O

Glutathione Reductase

GSSG + ß-NADPH---------- > ß-NADP + 2 GSH

CALCULATIONS

$$\text{Units/ml enzyme} = \frac{(\text{r A 340nm/min Test - rA340nm/ min Blank})(2)(3.1)(df)}{(6.22)\ (0.05)}$$

2 = 2 µmoles of GSH produced per µmole of ß-NADPH oxidized
3.1 = Total volume (in milliliters) of assay
Df = Dilution factor
6.22 = Millimolar extinction coefficient of ß-NADPH at 340 nm
0.05 = Volume (in milliliters) of enzyme used

3. PROTEIN ESTIMATION BY LOWRY'S METHOD

Lowry's method is one of most commonly used methods of protein estimation.

PRINCIPLE

In alkaline condition, copper is bind to peptide nitrogen of proteins and the reduction under alkaline conditions of Folin- Ciocalteu reagent. Copper ions facilitate the reduction process. The formation of intense blue colour is measured at 750nm.

MATERIALS

1. Stock reagents:
Solution A : 1%w/v copper sulfate
Solution B : 2% w/v sodium potassium tartarate
Solution C : 0.2 M NaOH
Solution D : 4% Sodium carbonate
Lowry's reagent (Folin- Ciocalteu reagent)
2. To 49ml of solution C add 49ml solution D. Then add 1ml of solution A and 1ml of solution B. this is the copper alkali reagent (Lowry's reagent), which must be prepared freshly for each lab session.
3. Dilute the Folin- Ciocalteu reagent (dilute with an equal volume of water).

PROCEDURE

Make up a set of standards using BSA or some other protein source in the range of 5-500µg/ml.

PHYTOCHEMICAL ANALYSIS

phytochemicals found in the plant extracts have beenidentified. The experiment showed the presence of secondary metabolites such as alkaloid, glycoside, flavonoid, saponins, steroids, tannins and phenol. Tabulation of phytochemicals screening is as follows.

Table:2

SL NO	TEST	ETHANOL	METHANOL	WATER	BENZENE
1	ALKALOIDS	+	+	+	_
2	CARBOHYDRATES	+	+	+	+
3	GLYCOSIDES	+	+	_	+
4	SAPONINS	+	+	_	+
5	PHENOLS	+	+	+	+
6	TANNINS	+	+	+	+
7	FLAVONOIDS	+	+	+	+
8	PROTIEN AND AMINO ACIDS	+	+	+	+
9	DITERPENES	+	+	+	+

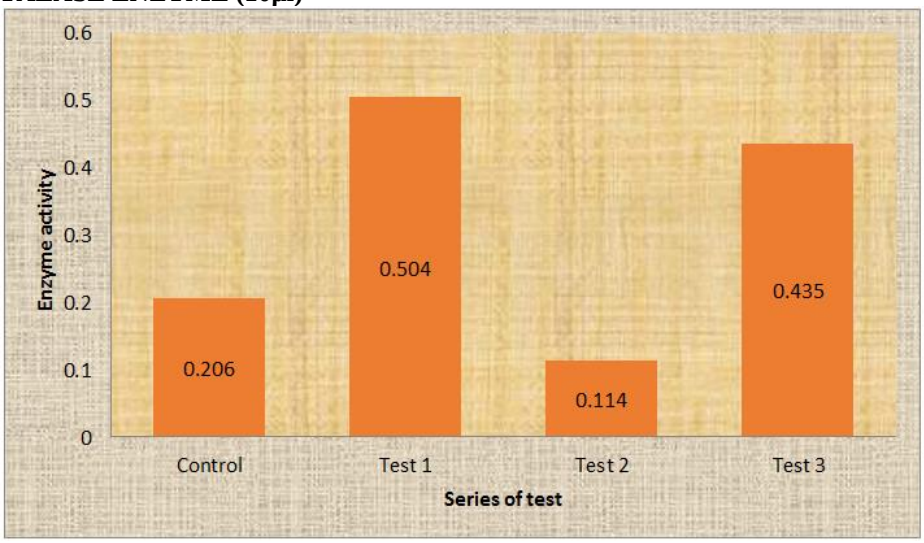
After accumulation of environmental condition, to start the experiment on four different group.
Ist group: control
IInd group: T1 for mercuric chloride
IIIrd group: T2 for Mercuric chloride with Theobroma Cacao plant
IVth group: T3 for Theobroma Cacao plant.

Based on above group we gave 2 different dosages, initially we gave 10µl and 20µl concentration for 5 days. For the fifth day all fish were sacrificed and collect the muscle protein and then estimate the catalase and glutathione peroxidase activity. For this study table 3 (**a** and **b**) shows the initial dosage of 10µM concentration significant level of increased in Catalase and decreased in glutathione Peroxidase level in test1. For the comparative of Test 2 decrease the level of expression system of test 1.

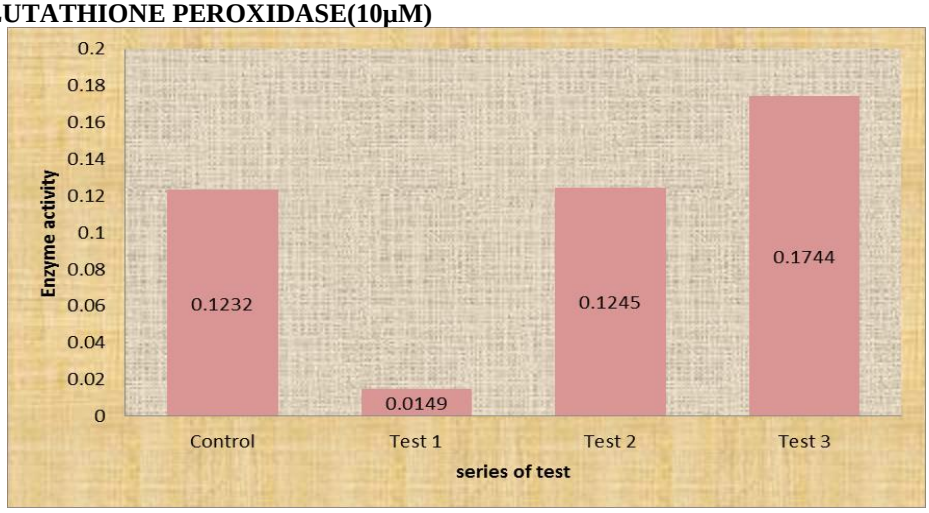
- To 0.5 ml of unknown sample and to 0.5 ml of the standards add 2.5ml of Lowry reagent. Mix well and stand for 10 minutes.

- For each sample, add 0.25 ml of Lowry reagent and mix well immediately. Stand for 30 minutes.
- Measure the absorbance at 750 nm against blank consisting of 0.5 ml sample buffer.

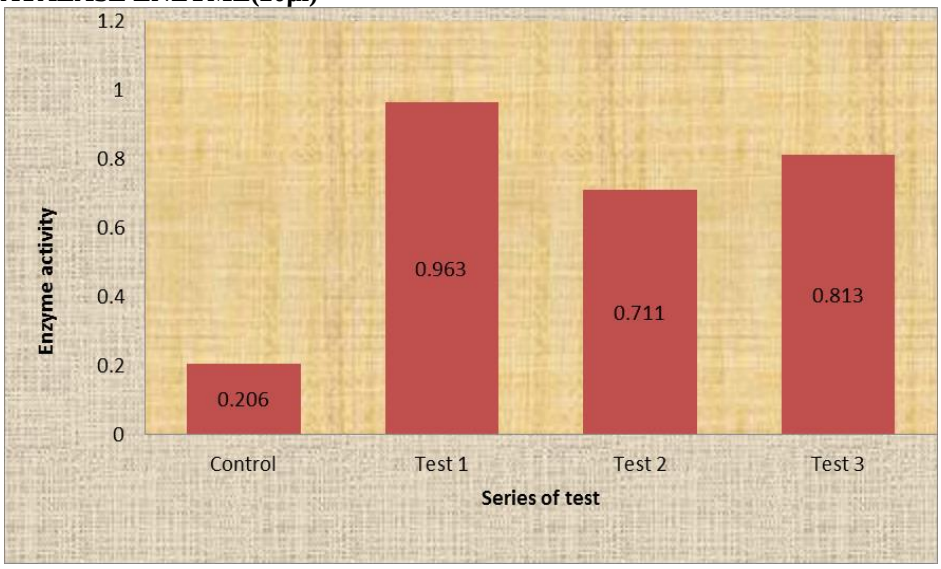
ASSAY OF CATALASE ENZYME (10µl)



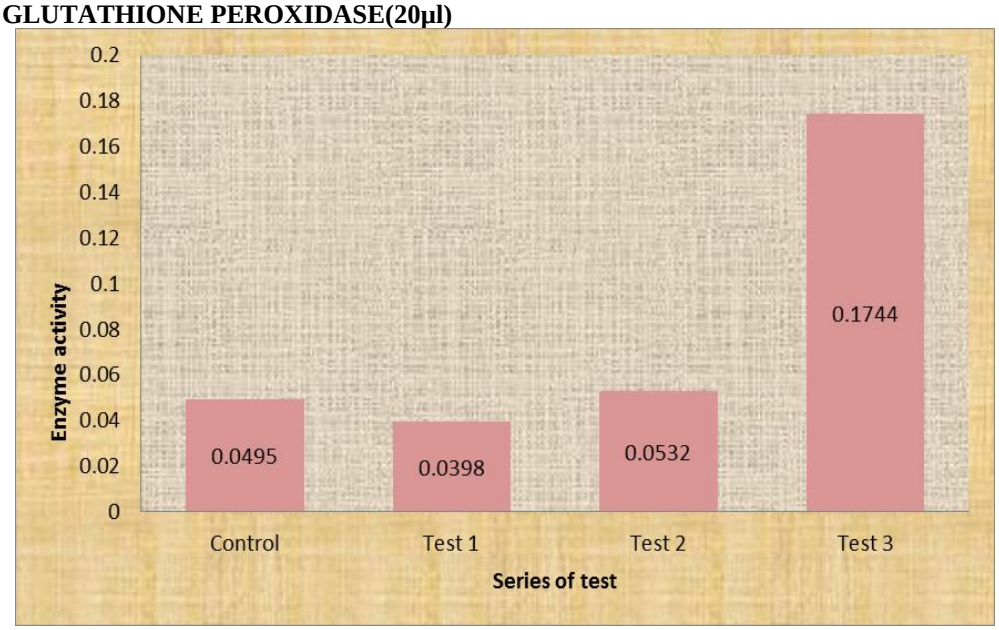
ASSASY OF GLUTATHIONE PEROXIDASE(10µM)



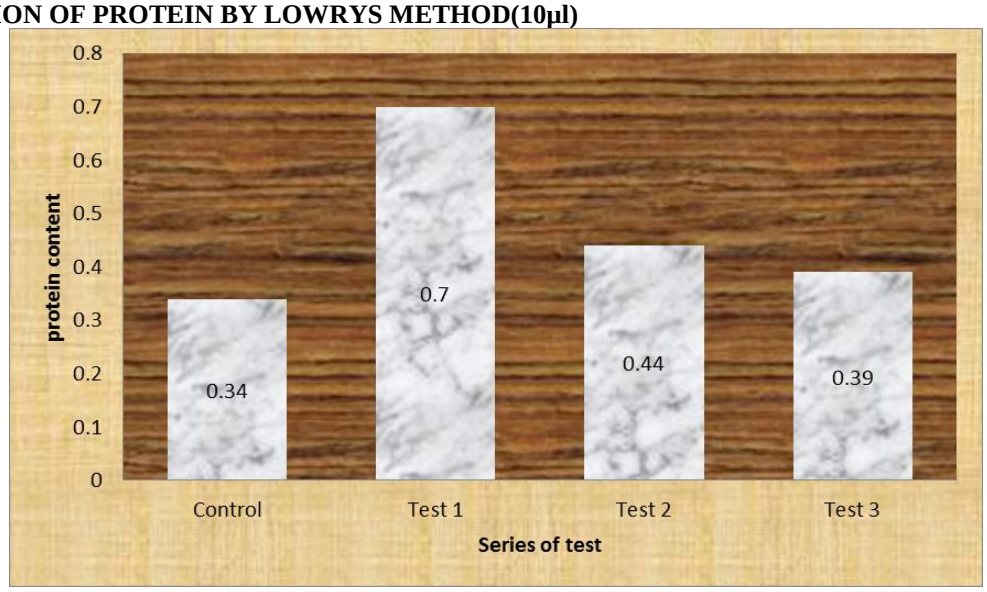
5ASSAY OF CATALASE ENZYME(20µl)



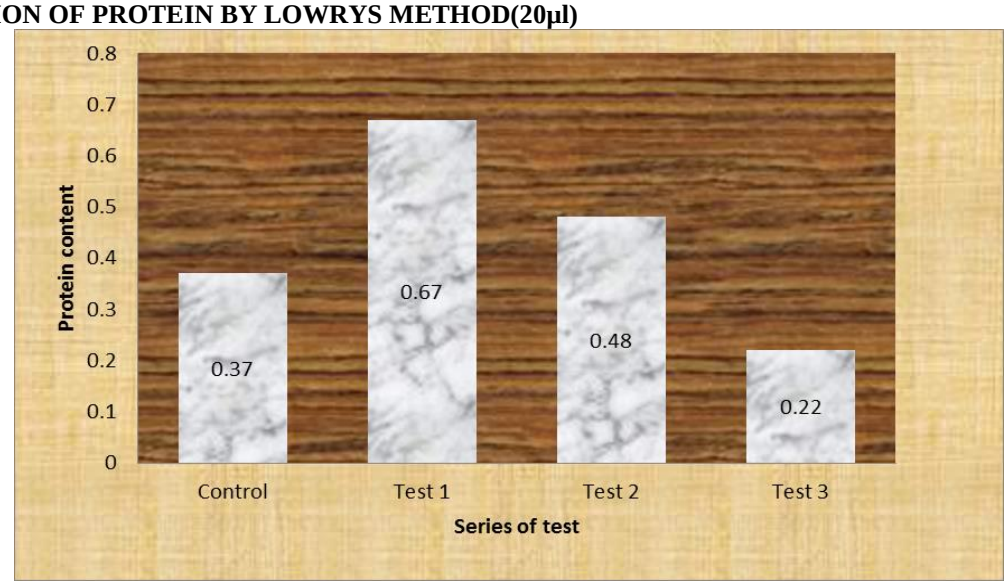
ASSAY OF GLUTATHIONE PEROXIDASE(20µl)



7ESTIMATION OF PROTEIN BY LOWRYS METHOD(10µl)



ESTIMATION OF PROTEIN BY LOWRYS METHOD(20µl)



From this study, it is clear that the mercury induced oxidative stress can be able to prevent by using the plant *Theobroma cacao* in black molly fish.

SUMMARY AND CONCLUSION

In the present study investigations prevention of mercury inducing oxidative stress in black molly fish using *Theobroma cacaoplant*, we gave 2 different dosages, initially we gave 10µM and 20µm concentration for 5 days. For the fifth day all fish were sacrificed and collect the muscle protein and then estimate the catalase and glutathione peroxidase activitythe initial dosage of 10µM concentration significant level of increased in Catalase and decreased in glutathione Peroxidase level.

Finally found out that the both dosage level 10µM and 20µM studies, the separation of protein was significantly changed. Based on our result we discuss about the work, the aquatic contamination of mercuric chloride to induce the oxidative stress. This oxidative stress is inhibiting the breeding pattern and also risk factor of surviving in aquatic animals.

Fish can be used as bio indicators of metals in the environment by studying the induction of oxidative stress, however, the specific forms of biomarkers and mechanisms of their action still need to be investigated.

SCOPE OF THE STUDY

- Aquarium fish Vs River fish oxidative stress and prevention using this Theobroma cacao plant.
- Prolong usage of abuse drugs can cause oxidative stress.
- The further study of above mentioned cases can be prevented by the action of Theobroma cacao plant.
- The oxidative stress of smokers and dunkers can be studied by using Theobroma cacao plant.
- Further more studies can be done on this plant Theobroma cacao because of its Medicinal uses.

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