Hepatoprotective effect and Anti-mutagenic activity of Zeamays (Maize) leaves

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Abstract

The non enzymic antioxidant activities in the leaves of Zeamays were identified in four different time periods of their growth namely 10th, 15th, 20th, 25th day after their sowing. The results showed that all the non enzymic antioxidants were found to increase with the age of the leaves. The hepatoprotective effect of Zeamays leaves were analyzed by using in vivo model and inferred that CCl4 induces the liver cirrohosis and it was controlled by Zeamays leaves extract group and Vitamin-E group . The antimutagenic activity of Zeamays leaves were confirmed in Ames test

Keywords: Zeamays, Non- enzymic antioxidants, Antimutagens, Vitamin-E, CCl4, Liver cirrohosis, Hepatotoxic

1. Introduction

Antioxidants can prevent/retard the oxidation caused by free radicals and sufficient intake of antioxidants is supposed to protect against diseases (Celikta et al., 2007).When this natural defense becomes imperfect, many diseases ensure such as aging, cardiovascular diseases, cancer, cataract, diabetes, nervous degeneration etc., (Zakaria, 1979). Antioxidants comprises of minerals, vitamins, enzymes, phytochemicals like phenolics and flavanoids. There is an increased quest to obtain natural antioxidants with broad spectrum actions (Aquil et al., 2006). Antioxidants in our diet help to prevent and repair the damage caused by free radical attack. The best-known antioxidants are ascorbic acid, Vitamin-C and β-carotene found in fruits, vegetables, cereals and vegetable oils. Antioxidants like lycopene and other carotenoids, phenotic compounds, ascorbic acid and vitamin-E lower the incidence of a numbe of diseases (Palmer and Pavlson, 1997). Cereals the "food of poor", have a high nutritive value and in the food pyramid they occupies area, with 6-11 servings per day followed by fruits and vegetables (Suchalatha and Srinivasalu, 2005). The important cereal ranks high nutritive value is maize often called as "mother maize" or "king corn". Zeamays, Linn-popularly called "Indian Corn" or "maize" or "makka cholam" in Tamil- belongs to the family gramineae and subfamily maydeae. Maize grass is used to cure kidney problems, kidney stones, bladder trouble. The maize grass is used as fodder and siliage. Now maize grass not only as fodder but also used as human consumption. In the present study the maize grass of different stages were taken and analyse the Non enzymic antioxidants which was confirmed by *in vivo* and *in vitro* study.

2. Methodology

2.1 Assessment of Components Present in Maize Leaf

The The maize seeds are brought from Agricultural college, Madurai. These leaves have therefore been analyzed for the biochemical parameters at four different time intervals namely 10th, 15th , 20th and 25th day after sowing or the appropriate tests.

2.2 Estimation Of Non-Enzymic Antioxidants

2.2.1 Estimation of Chlorophylls (Witham et al., 1971)

Principle

The chlorophylls are the essential components for photosynthesis, and occur in chloroplasts as green pigments in all photosynthetic plant tissues. Chlorophyll a and b occur in higher plants, ferns and mosses. Chlorophyll c, d and e are only found in algae and in certain bacteria.

Methods

Weighted 1g of leaves and mixed with the addition of 20 ml of 80% accetone. Centrifuged (5,000 rpm for 5 min) and transferred the supernatant to a 100ml volumetric flask. Ground the residue with 20ml of 80% acetone, centrifuged and transferred the supernatant to the same volumetric flask. Repeated this procedure until the residue was colourless. Washed the mortar and pestle thoroughly with 80% acetone and collected the clear washings in the volumetric flask. Made up the volume to 100ml with 80% acetone. Read the absorbance of the solution at 645 and 663nm against the solvent (80% acetone) blank.

2.2.2 Estimation of Triglycerides (Bucolo And David, 1973)

Principle

Triglycerides are determined after enzymatic hydrolysis with lipases. Peroxidase catalyzes the conversion of hydrogen peroxide, 4-amino-antipyrine and N-ethyl-N-sulphopropyl-N-anisidine (ESPAS) to a purple coloured quinonine complex, which can be measured at 546nm.

Procedure

The reagent kit contained triglycerides mono reagent and standard (200mg/dL). To 0.1ml of the triglyceride mono reagent taken in three tubes marked as blank, standard and test, 0.01ml of standard and serum were added in the respective tubes and incubated at 37˚C for 10 min. All the tubes of the test and standard were read against blank at 546 nm. The values were expressed as mg/dL.

2.2.3 Estimation of Cholesterol (Allain et al., 1974)

Principle

Cholesterol esters are hydrolyzed by cholesterol ester hydrolase to free cholesterol and fatty acids. The free cholesterol produced and the pre-existing ones are oxidized by cholesterol oxidase to 3-cholesterone and H2O2. H2O2, in the presence of peroxidase, oxidizes the chromogen (4-amino antipyrene and phenol) to a red coloured compound, which can be read at 510nm.

Procedure

The reagent kit contained solution 1 (buffer/enzyme/chromogen), solution 2 (phenol) and standard cholesterol (200mg/dL). The working reagent was prepared by mixing equal volumes of solutions 1 and 2. An accurate amount (0.01ml) each of the serum and standard and 1.0ml of the working reagent was added, mixed and kept at 37˚C for 5 minutes. The colour developed was read at 510nm against a reagent blank. The serum cholesterol was expressed as mg/dL.

2.2.4 Ames *Salmonella* Microsome Assay

The mutant bacterial strain of Salmonella typhimurium, T100 was obtained from the Institute of Microbial Technology, Microbial type culture collection and gene bank, Chandigarh.

2.2.5 Preparation of Leaf Extract for Assay

For all estimation 0.5 g of maize leaves was weighed accurately and grinded well with mortar and pestle with phosphate buffers and then centrifuge. Take the supernent solution and analyse the nonenzymic activity in maize leaves.

2.3 Animal Study

2.3.1 Selection of animal model

Ten male albino rats weighing 180-200 gm were brought from the Madurai Medical College. These were divided into five groups are as follows. The initial weight of all these animals was recorded and they were groups according to their weight and kept in the separate plastic cages. They were adopted in the laboratory condition and maintained in a controlled temperature (27 ± 2˚C). These were given weeks time to get acclimatized with the laboratory.

2.3.2 Experimental Induction of Hepatic Damage

Liver damage was induced in rats (by administrating CCl4 subcutaneously) by giving 50% alcohol orally along with water and also for CCl4 inducing group, administrating CCl4 subcutaneously (sc) in the lower abdomen in a suspension of liquid paraffin (LP) in the ratio of 1 : 10 v/v at the dose of 0.1 ml CCl4 / kg body weight of each animal. Both the hepatotoxic group and herb treated group 50% alcohol and ethanolic extract of maize leaf orally along with water adlibitum for about 21 days. Whereas on 21st day, CCl4 was administrated for the group III & VI. All the animals were kept starved overnight on the 21st day. On the next day, after recording the weight in each case, they were sacrificed by decapitation by making an incision on jugular vein to collect blood. The liver tissue was dissected out, blotted of blood, washed in saline and weighed simultaneously. This was kept in frozen containers and preceded for biochemical estimations.

2.4 Biochemical Analysis

Serum was collected and subject to biochemical estimations of different parameters.

Liver homogenate was prepared which is taken from different groups of rat. 0.5 g of liver was weighed which is homoge -nate by using 5 ml saline and this liver homogenate undergoes various biochemical estimation of different parameters

2.4.1 Alkaline Phosphatase (ALP)

Principle

The substrate, p-nitrophenyl phosphate (PNPP) is hydrolysed by ALP to p-nitrophenol and phosphoric acid. Some divalent ions like Mg^{++} are added to the system which act as activators. PNPP is colourless in acid or alkaline medium while PNP is yellow in colour in the alkaline medium and colourless in the acid medium. Two types of buffers can be used for maintaining the pH of the reaction medium-glycine and MAP (2-methyl-2-aminopropanol-1). The latter is recommended but difficult to get in the laboratories of developing countries and in addition, it is cumbersome to prepare and has a short shelf-life. Glycine buffer, however, inhibits the ALP reaction and hence the values are lower than the activity measured in the MAP buffer. In the following pages only the used of glycine buffer will be discussed.

Reagents

1. Stock substrate of PNPP (4 mg/ml or 15.2 mmol/ml)- Dissolve 0.4 gm of p-nitrophenyl disodium phosphate 100 ml water. The PNPP should be of highly pure quality and correct for hydration if it is a hydrated salt. The solution is unstable, prepare only as much as needed. Preweighed dry substrate can be kept in small vials for ready use. If the solution is refrigerated, it stays for a few days without any appreciable change.
2. Sodium hydroxide solutions
   a. 1 N NaOH:
      Dissolve 40 gm sodium hydroxide in about 800 ml of water placed in a 1-litre volumetric flask, dilute the solution to 1000 ml volume with water.
   b. Other strengths (0.1 N, 0.05 N, 0.02 N)
      Dilute 1 N sodium hydroxide 1:10, 1:20 and 1:50 for getting 0.1 N, 0.05 N and 0.02 N sodium hydroxide solutions. Take three 10 ml, 5 ml and 2 ml of 1 N NaOH and dilute each to 100 ml. This will yield sodium hydroxide solutions of above strengths in the same sequence.
3. Glycine buffered substrate
   a. Glycine buffer (alkaline): Mix 7.5 gm of glycine, 0.095 gm of magnesium chloride, 750 ml water, and 85 ml 1 N sodium hy droxide in a 1-litre volumetric flask. Dilute the solution to the 1000 ml mark. Keep in a refrigerator.
   b. Working substrate
      Mix equal volumes of glycine buffer and stock substrate of PNPP. Adjust the pH to 10.3 to 10.4 if necessary. The use of a pH meter is recommended. Use dilute HCl or NaOH for adjusting the pH. If the reagents are of good quality, this may not be necessary.
4. Standard solution of p-nitrophenol (PNP)
   a. Stock standard (1 mmole/L): Dissolve 139.1 mg of high purity PNP in water to make 1000 ml of solution in a 1-litre volumetric flask. This solution is stable if keep in the dark. If high purity PNP is not available commercially, make a batch of purified PNP by recrystallization from hot water, dry it overnight in a vacuum desiccators over silica gel or any other desiccant.
   b. PNP working standard (0.04 mmol/L)
      Pipette out 1.0 ml of the stock standard into a 25-ml volumetric flask and dilute the volume with 0.05 N NaOH solution. Mix thoroughly. This should be prepared daily for the test.

Procedure

Pipette 1.0 ml of buffered substrate into each of two test tubes, labelled a "T" and "B" corresponding to test and blank. Use one pair of tubes for each specimen. The blank ("B") is the serum blank. Place the tubes in a water bath set at 370C for 5 to 7 minutes to equilibrate the temperature. With the timer set, add 0.05 ml serum to the "T" tube and mix. Incubate at 370C for exactly 30 minutes. At the end of 30 minutes, add 10 ml of 0.05 M NaOH to both tubes to stop the reaction and dilute the PNP formed. Mix well. Add 0.05 ml of serum to the B tube (serum blank), and mix the contents thoroughly. Pour the contents of the B and T tubes into appropriate cuvettes and read absorbances on the solutions at 405 nm against water as an instrument blank. Consult the calibration curve to determine the enzyme activity in International unit (U/L) or calculate as follows.

Calculation

a. Determine the absorptivity factor (F) in terms of International unit for ALP enzyme as given below:
   i. Dilute 1.0 ml of working standard of PNP (0.04 mmole) to 4.88 ml with 0.05 N NaOH and read the absorbance (As) against 0.05 N NaOH which is used for setting the zero absorbance.
   ii. The absorbance (A) is equivalent to 60 U/L (see comments).

iii. The absorptivity factor (F) = 60/As U/L.

b. Calculate the enzyme activity by the following formula:

DA = At – Ab

ALP activity (U/L) = DA x F

At = Absorbance of solution in tube "T" under experimental conditions, after 30 minutes of enzymatic reaction.

Ab = Absorbance of solution in tube "B" at 0 minute (serum blank)

DA = Change in absorbance following enzymatic reaction = At – Ab

F = Absorptivity factor that corresponds to the absorption scale in terms of International units (U/L).

2.4.2 ALT (SGPT)

Principle:

ALT catalyzed the transfer to the amino group from L-alanine to a-Ketoglutarate resulting in the formation of pyruvate and L-glutamate. Lactate dehydrogenase catalyzes the reduction of pyruvate and the simultaneous oxidation of NADH to NAD. The resulting rate of decrease in absorbance is directly proportional to ALT activity.

Reagents

Take α ketoglutaric acid acid 13mM, D-Alanine 400mM, NADH 0.2 mM, LDH 1200 U/L, Tris Buffer, pH 7.5±0.1, non reactive fillers and stabilizers.

Procedure

Reconstitute reagent according to instructions. Pipette 1.0 ml of reagent into appropriate tubes and prewarm at 370C for five minutes. Zero spectrophotometer with water at 340 nm. Transfer 0.10 ml (100 µl) of sample to reagent, mix and incubate at 370C for one minute. After one minute, read and record absorbance. Return tube to 370C. Repeat readings every minute for the next two minutes. Calculate the average absorbance difference per minute (Δabs/min). The Δabs / min multiplied by the factor 1768 will yields results in IU/L. Samples with values above 500 IU/L. should be diluted 1:1 with saline, re-assayed and the results multiplied by two.

Calculations

One international unit (IU /L) is defined as the amount of enzyme that catalyzed the transformation of one micromole of substrate per minute under specified conditions.

$$ALT(IU/L) = \frac{\Delta Abs/\min \times 1.10 \times 1000}{6.22 \times 0.10 \times 1.0} \times \Delta Abs/\min \times 1768$$

where

ΔAbs / min = Average absorbance change per minute

1.10	=	Total reaction volume (ml)
1000	=	Conversion of IU / ml to IU / L
6.22	=	Millimolar absorptivity of NADH
0.10	=	Sample volume (ml)
1.0	=	Light path in cm.

Expected values

Up to 26 IU / L (300C)

Up to 38 IU / L (370C)

2.4.3 AST (SGOT)

Principle

Aspartate aminotransferase (AST) catalyzes the transfer of the amino group from L-aspartate to a-ketoglutarate to yield oxalacetate and L-glutamate. The oxalacetate undergoes reduction with simultaneous oxidation of NADH to NAD in the malate dehydrogenase (MDH) catalyzed indicator reaction. The resulting rate of decrease in absorbance at 340 nm is directly proportional to the AST activity. Lactate dehydrogenase (LDH) is added to prevent interference from endogenous pyruvate which is normally present in serum.

Reagents

Take α-Ketoglutarate 12nM, L-aspartic acid 200mM, NADH 0.2mM, LDH 800U/L, MDH 600 U/L, Tris Buffer, pH 7.8±0.1. Non-reactive fillers and stabilizers.

Procedure

Reconstitute reagent according to instructions. Pipette 1.0 ml of reagent into appropriate tubes and prewarm at 370C for five minutes. Zero spectrophotometer with water at 340nm. Add 0.100 ml (100 µl) of sample to reagent, mix and incubate at 37˚C for one minute. After one minute read and record the absorbance. Return tube to 37˚C. Repeat readings every minute for the next two minutes. Calculate the average absorbance difference per minute (Δbs / min). The Δabs / min multiplied by the factor 1768 will yield results in IU/L. Samples with values above 500 IU / L should be diluted 1:1 with saline, reassayed and the results multiplied by two.

Calculations

One international Unit (IU /L) is defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under specified conditions.

$$AST(IU/L) = \frac{\Delta Abs/\min \times 1.10 \times 1000}{6.22 \times 0.10 \times 1.0} \times \Delta Abs/\min \times 1768$$

where

ΔAbs / min	=	Average absorbance change per minute
1.10	=	Total reaction volume (ml)
1000	=	Conversion of IU / ml to IU /L
6.22	=	Millimolar absorptivity of NADH
0.10	=	Sample volume (ml)
1.0	=	Light path in cm

Expected values

Up to 28 IU / L (370C), Up to 40 IU / L (370C), VLDL (mg / dl) = Triglycerides / 5

LDL (mg / dl) = Total cholesterol - (Triglycerides / 5) – HDL.

3. RESULTS

The antioxidant activities/levels were estimated in the leaves of Zeamays at four different stages of its growth namely.

1.10 days after Sowing.

2.15 days after Sowing.

3.20 days after Sowing.

4.25 days after Sowing.

The earlier days of the growth were alone selected because as the plant matured the fiber content of the leaf increased resulting in decreased palatability.

3.1 Non-enzymic antioxidants

The leaves of Zeamays were screened for the levels of non-enzymic antioxidants such as chlorophyll, Ascorbic acid, reduced glutathione at four different stages of growth. The Vitamin C levels in maize leaves at different stages of their growth are shown in Figure I. The ascorbate levels found maximum in the 15th day leaves. The reduced glutathione levels were estimated and the results obtained in Figure II. Glutathione function as an important antioxidant in the destruction of H2O2 and lipid peroxide by acting as a substrate for GPx . The leaves on 15th day shows maximum level of reduced glutathione. The levels of chlorophyll were analyzed and expressed in Figure –III showed that the leaves had moderate levels of chlorophylls and increased in 15th day leaves.

Figure-I: *Levels of Ascorbic Acid In The Leaves of Zeamays At Four Different Stages Of Growth.*

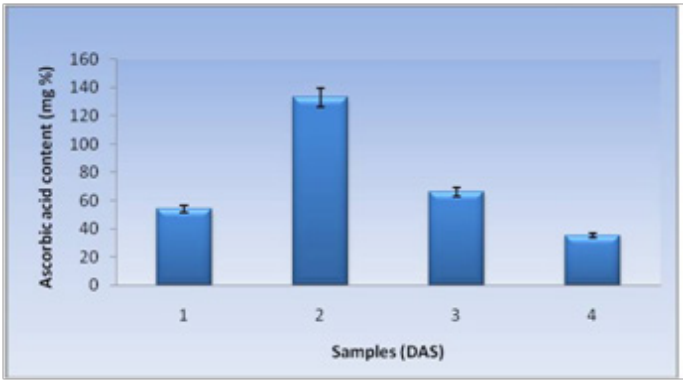


Figure II: *Levels of Reduced Glutathione in the Leaves Zeamays at Four Different Stages Of Growth*

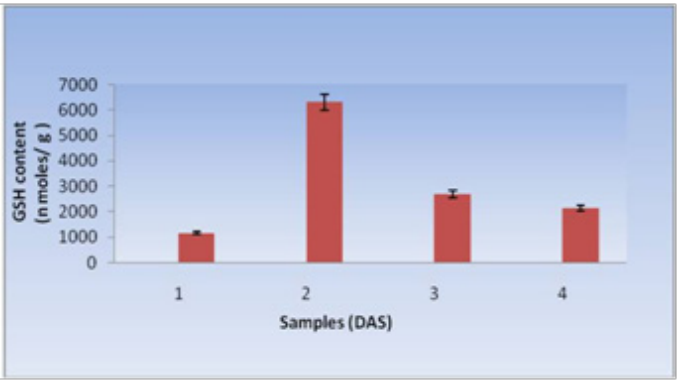


Figure III: *Levels of Chlorophyll in the Leaves Of Zeamays At Four Different Stages Of Growth*

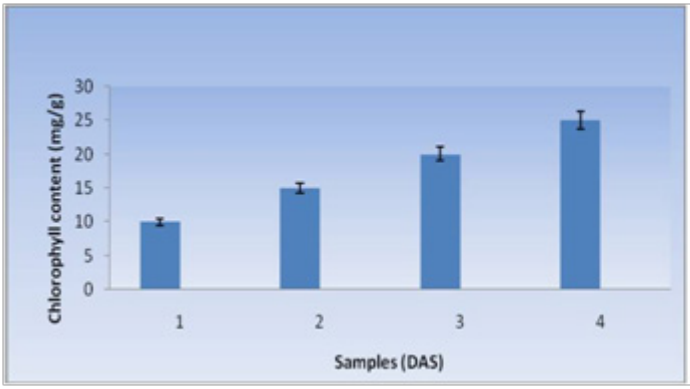
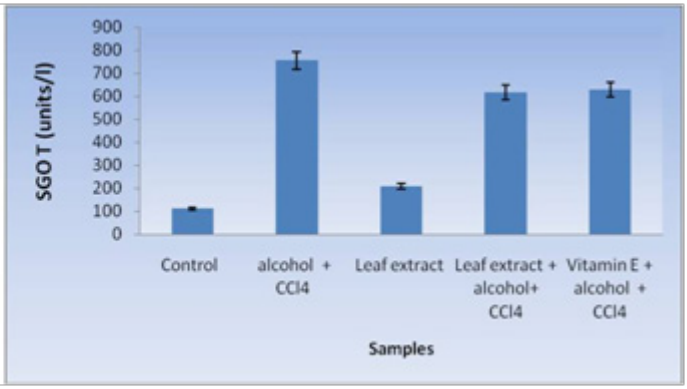


Figure-IV : *Levels of SGOT in Serum at Different Groups*



3.2 Animal Study

The animals are grouped according to the given table. There are five groups and the animals were sacrificed and the serum was collected and analyse the following parameters

3.3 Grouping and supplementation of animals

Group	Administration of drug
I	Control + Water only
II	50% alcohol orally + subcutaneous CCl4
III	Leaf extract orally
IV	Leaf extract orally + 50%alcohol+ Subcutaneous CCl4
V	Vitamin E + 50% alcohol + Subcutaneous CCl4

The levels of AST, ALT, and ALP were shown in Figure IV, V and VI. The Total cholesterol, LDL, TGL values, VLDL and HDL were shown in Figure VII, VIII, IX, X and XI. The normal level of SGOT and SGPT enzymes in the serum increases in CCl4 group showed that the liver get damaged and the serum enzymes get increased. Administration of CCl4¬ caused significant increase in the activity of serum ALT and AST but maize leaf reduces the hepatotoxic activity of CCl4. In analyzing the lipid profile test, decreases in the activity of the enzymes was due to the action of vitamin E but the CCl4 has maximum level of activity. The antimutagenic activity of the liver and leaf extract was detected in Ames test and the results was shown in Table-I .The Salmonella typhimurium was used as the culture. By comparing the number of colonies in standard revertant, standard mutagens and leaf extract groups and detect the antimutagenic activity. The standard mutagen plate has increased number of colonies than standard revertants due to the presence of mutagenic activity. In the leaf extract and standard mutagen plates showed the decreased number of colonies which inferred that Zeamays leaf has antimutagenic activity.

Figure- V: *Levels of SGPT in Serum At Different Groups*

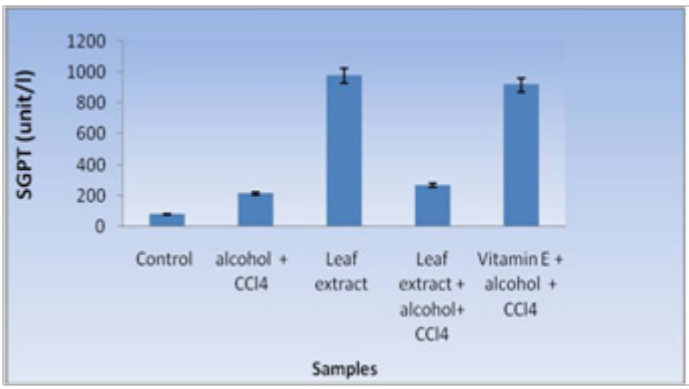


Figure - VI *Levels of ALP in Serum at Different Groups*

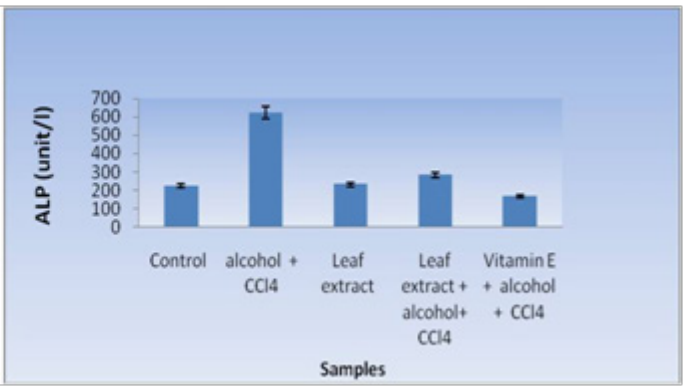


Figure- VII: *Level of Total Cholesterol in Serum at Different Groups*

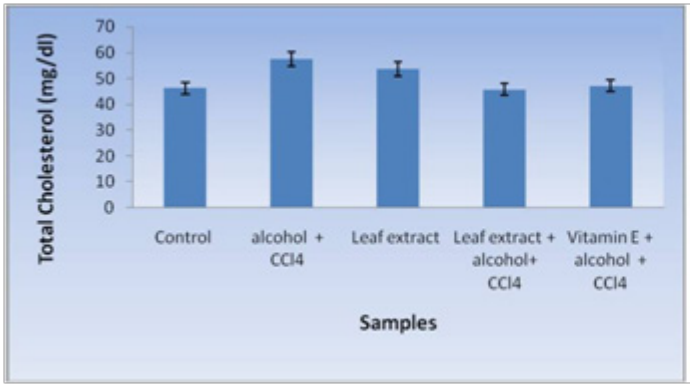


Figure - VIII: *Level of LDL in Rat Serum at Different Group*

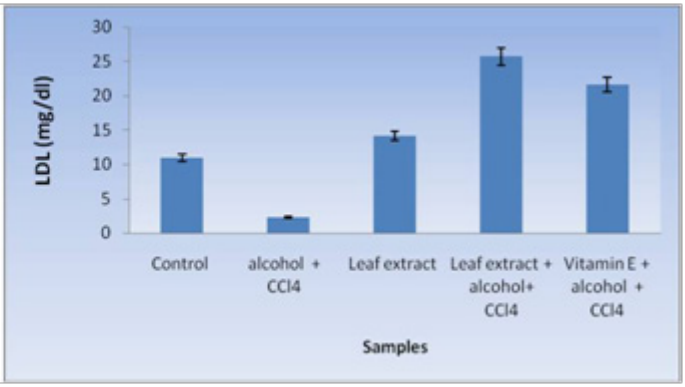


Figure- IX: *Level of TGL Values in Serum At Different Groups*

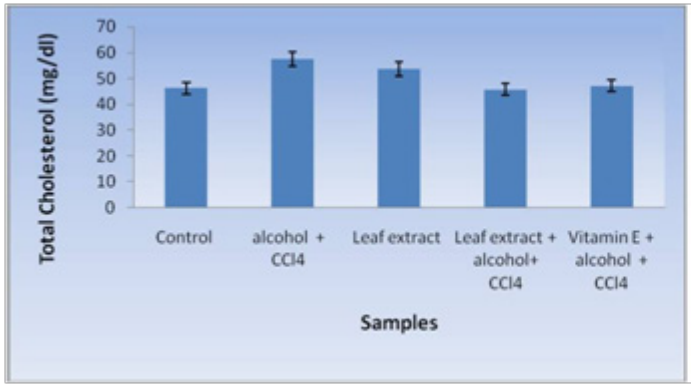


Figure- X: *Level of VLDL in Serum at Different Groups*

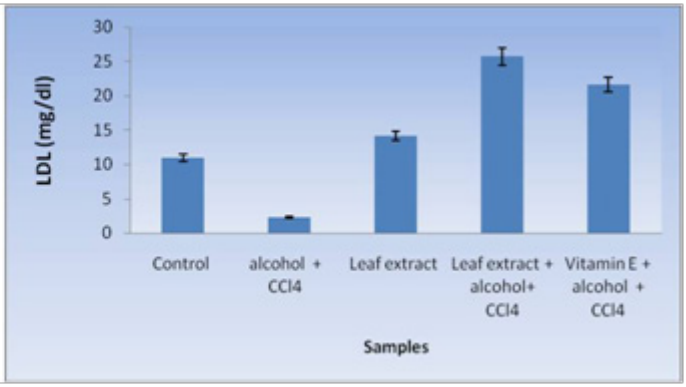


Figure-XI: *Level of HDL in Serum At Different Groups*

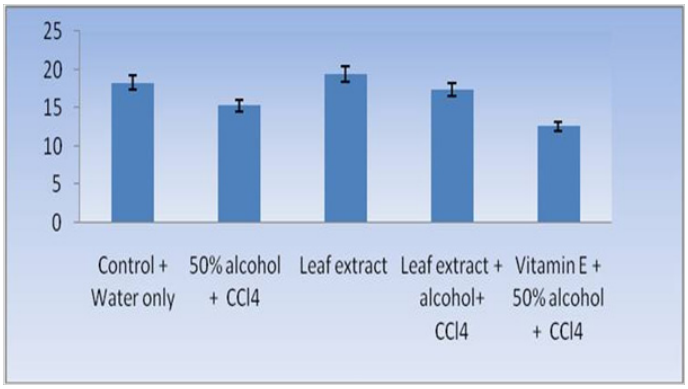


TABLE I : *Effect of Zeamays leaf Extract on the Reversibility of Standard Tester Strain TA 100*

	Number of His+ revertant in TA 100	
	Plant extract –SM	Plant extract + SM
SM (control)	15	20
L1	-	-
L2	15	25
L3	18	77
L4	-	2
L5	3	5

	Number of His+ revertant in TA 100	
	Plant Extract –SM	Plant extract + SM
SM (control)	22	28
15th day maize grass	20	32

4. DISCUSSION

Ascorbic acid is a terminal water soluble antioxidant that protects lipids against peroxidation (Maneesh et al., 2005). It readily oxidizes the dehydro ascorbic acid and interferes with the process of LPO by scavenging the superoxide anion (Jariyapongskul et al., 2002). Vitamin C supplementation in humans increases plasma ascorbate and improves the resistance of plasma lipids to LPO (Polidori et al., 2004). GSH maintains functional and structural integrity of cells and is often involved in the defense against tissue injury from administered or metabolically generated toxic agents (Ortman et al., 2000). Reduced glutathione levels decreased in alloxan treated rats, which was efficiently counteracted by the administration of Boerhavia diffusa leaf extracts (Satheesh and Pari, 2004). Ethanol is known to increase the hepatic CYP2El upto ten folds. This induction is responsible for oxidative damage in hepatocytes (Lieber, 2004). CCl4 is a chemical with hepatotoxic and nephrotoxic effects, and has been used as a standard compound in many studies to induce liver injury (Chen et al., 2005). Metabolic activation of CCl4 by CYP2El to the free radicals, namely trichloro methyl and trichloro methyl peroxy radical is reported to enhance lipid peroxidation and protein oxidation in the liver resulting in widespread membrane damage and liver injury (Sheweita et al., 2001). Many medicinal plants have been reported for the hepatoprotective activity against CCl4 induced liver damage in rats (Jeganathan and Nalini, 2001; Achuthan et al., 2003; Umadevi et al., 2004; Aniya et al., 2005). The administration of ethanol increased the activity of liver marker enzymes such as AST, ALT, ALP and γ-GT, which was nullified by the administration of grape leaf extracts (Pari and Suresh, 2008). The administration of plant extracts of Sarcostemma brevistigma (Sethuraman et al., 2003), Murraya koenigii (Gurgune et al., (Jaiprakash et al., 2003) and Glycirrhiza glabra (Rajeshwar et al., 2004) have been reported for their efficacy in controlling the CCl4 induced hepatic damage, as reflected by the serum marker enzymes. Administration of ethanol decreased the activity of HMG CoA reductase; so, increased serum cholesterol in ethanol toxicity may be due to the impairment in esterification and utilization than increased cholesterogenesis in the liver (Ginsberg and Goldberg, 1998). Shah et al. (2004) and Venukumar and Latha (2004b) have reported that serum total cholesterol level was altered during CCl4 intoxication. On the administration of plant extracts, serum cholesterol level was normalized in these studies. Dolichos biflorus (Muthu et al., 2005) and Cassia auriculata leaf extracts (Kumar et al., 2002) significantly decreased the level of serum triglycerides, free fatty acids and phospholipids in rats with alcoholic liver injury. A similar effect was also observed by the administration of beta carotene in CCl4 induced hepatic inflammation and fibrosis in rats (Seifert et al., 1995) and Asteracantha longifolia (Shailajan et al., 2005),Cassia fisfula Linn (Pradeep et al., 2005) Pterocarpus santalinus (Manjunatha, 2006) and Duzhong leaves (Hung et al., 2006) in CCl4 induced hepatotoxicity in the liver of rats.

5. References

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