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A study on the hepatoprotective activities of Methanol Extract of *Spinacia oleracea* (Linn.) to the Induced Hepatotoxicity in wistar rat models

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ABSTRACT

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A study on the hepatoprotective effects of methanol extract of *Spinacia oleracea* (**MESO**) against carbon tetrachloride induced hepatotoxicity in rats (150 to 180g) was done. Thirty male albino rats of wistar strain were divided into 5 groups with six individual each. The group-I served as normal control, group II as negative control intoxicated with carbon tetrachloride (1.9 ml/kg, Intra Peritoneal), groups III, IV & V with carbon tetrachloride and methanol extract of *Spinacia oleracea* at 100µgm/ml, 200µgm/ml & 300µgm/ml *per os*, respectively, for 14 days. At the end, the animals were sacrificed, blood samples were collected and analysed. The results showed carbon tetrachloride administration was associated with considerable increase in the activities of Alanine amino transferase, Aspartate amino transferase and Bilirubin (P<0.05) in comparison with the respective mean values of the control. The antioxidants such as, serum catalase, reduced glutathione, glutathione peroxidase, vitamin E, vitamin C & superoxide dismutase, blood parameters such as total WBC, RBC, Hb, and Haematocrit were observed to be lower than the control. The hepatic chord architecture was disturbed. Upon treatment with **MESO** the antioxidant enzymes and blood parameters were restored to significantly near normal level. This research work showed that **MESO** was successful at 300µgm/ml, in counteracting carbon tetrachloride induced Hepatotoxicity and hepatic architecture remediation to significantly near normal level. It may also be inferred that, the level of progressive amelioration may be due to the total phenolics, flavonoids and other antioxidants composition in the *Spinacia oleracea* extract.

Keywords: Antioxidants, Alanine amino transferases, Aspartate transaminases, Glutathione, Hepatotoxicity, Haematocrit.

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1. INTRODUCTION

Plants have been investigated for their medicinal properties throughout the world, mainly due to their potent pharmacological activities, low toxicity and economic viability¹. In the last decade the hunt for naturally occurring antioxidants has grown tremendously since free radicals are known to quench various ailments that affect human health. Consumption of herbal antioxidants improves health². This necessitates the search for natural plant products which could effectively intervene in the onset and morbidity of the disorders and diseases. It was well reported that expression of many disorders or ailments / diseases is associated with the generation of reactive oxygen species (ROS) or free radicals.

The liver is the largest exocrine gland in our body. It is the vital organ undertaking wide range of functions, such as detoxification, Protein – Fat – Carbohydrate metabolism, storage of iron and vitamins. The liver also plays major role in decomposition of RBCs, hormone production, plasma protein synthesis, glycogen storage and synthesis of urea. The organ liver is inevitable for survival and one cannot live without it for long period. The liver acts as the chief site for intense metabolism and excretion⁴. The liver is the key organ of metabolism and detoxification. Regular exposure to a variety of abusive measures adds on to hepatic injury.

During liver injury, the liver marker enzymes like Alanine amino transaminase (ALT), Aspartate aminotransferase (AST), Bilirubin are present in blood serum in very low concentration and thus provide us indications on hepatic health. Liver diseases were among the first disorders to which serums test were applied and have proved to be useful in diagnostic purposes⁵. Free radicals or

Reactive oxygen species (ROS) are often produced as byproducts of biological reactions or as a result of intake of exogenous elements which foster oxidative damage, leading to a wide range of biological malfunctions prompting DNA damage, carcinoma, cardiovascular, metabolic and neuro degenerative disorders, as well as acceleration of senescence⁶.

Carbon tetrachloride (CCl₄) is one of the most potent hepatotoxins and is widely used in scientific research to evaluate hepatoprotective agents. The hepatotoxic effect of CCl₄ is largely due to its active metabolite, trichloromethyl free radical (CCl₃[•] and / or CCl₃OO[•]) by chronic or acute vehicles. The CCl₄ is shown to induce hepatocellular carcinomas in rodents by oral, inhalation, and parenteral (Intraperitoneal) exposure⁷.

High consumption of fruits and vegetables is associated with low risk for these diseases, which is attributed to the antioxidant vitamins and other phytochemicals. **Spices** and **herbs** are recognized as sources of natural antioxidants and thus play an important role in the chemoprevention of diseases and aging. The phytochemicals tests indicated the presence of alkaloids, glycosides, tannins, and flavonoids in the crude methanolic extract of plants. Several of such compounds are known to possess potent antioxidant activity⁸. Phenolic compounds are commonly found in both edible and other traditional medicinal plants, and they have been reported to have multiple biological activities, including free radical scavenging activity.

The spinach leaves, (*Spinacia oleracea* L.), a flowering plant of family 'Chinopodiaceae', is widely available commonly consumed vegetable, recognized as 'palak' in vernacular. It is reported

to have phyto-nutrients such as Carotene- β , Crypto-xanthin- β , Lutein-zeaxanthin, Vitamins such as, Folic acid, Niacin, Pantothenic acid, pyridoxine, Riboflavin, Thiamin, Vitamin-A, Vitamin-C, Vitamin-E and Vitamin-K, Electrolytes such as Sodium, Potassium, minerals such as, Ca, Cu, Fe, Mg, Mn and Zn^{9,10,11}.

As per many investigations on the activity of spinach parts such as leaves, seeds *in vivo*, the present research study was aimed at an evaluation of methanolic extract of *Spinacia oleracea* (**MESO**) and its anti-oxidants in ameliorating the induced hepatotoxicity to CCl₄ exposure and circulating liver marker enzyme levels and proteins using wistar albino rat models

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

The Anthrone Reagent, Folin-Ciocalteau Reagent, Ninhydrin, thiobarbituric acid (TBA), the carbon tetrachloride (CCl₄), 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), Methanol, 2,4-dinitrophenyl hydrazine-thiourea-CuSO₄ reagent, and isobutyl alcohol were procured from Sigma-Aldrich Chemicals (Bangalore). Remaining analytical grade chemicals and estimation kits were purchased from commercial sources and used.

2.2 Plant Material

The spinach leaves (*Spinacia oleracea* L.) were purchased from a local market, Vellore, Tamil Nadu, India and the plants were authenticated by the Botany Department, Govt. Arts and Science College, Thiruvannamalai (Thiruvalluvar University), Tamil Nadu, India. The plant materials were washed in tap water, shade dried and powdered.

2.3 Experimental animals

The experimental animals used for the study were male Wistar rats *Rattus norvegicus*, weighing (160–180 g) and procured from Kings Institute of Preventive Medicine, Guindy, Chennai, and fed with standard rat chow (Amrut Laboratory Animal Feed, Bangalore, India) and water ad libitum. Forty eight animals were used for the experiment and maintained as per the Committee for the purpose of control and supervision of Experiments on Animals (CPCSEA) guidelines. Animals were acclimatized for one week prior to experiment. The animals were maintained under standard laboratory conditions of temperature (25 \pm 2%) and humidity (55 \pm 5%) with 12 h light- dark cycle.

2.4 Extraction and fractionation

The powdered leaves material (800 g) was successively extracted with 95% methanol using soxhlet extractor. The marc left after the methanol extraction was macerated with distilled water for 24 h. The solvents were distilled off under reduced pressure below 45°C to afford *Spinacia oleracea* a methanol extract (**MESO**, 12.4% w/w).

2.5 Preliminary photochemical screening

Preliminary phytochemicals analysis was performed as per standard methods¹² in order to identify the nature of phyto-constituents in the methanol extract of spinach leaves (*Spinacia oleracea* L.)

2.6 Antioxidant enzymes assay (DPPH scavenging assay)

The in vitro free radical scavenging activity of methanol extract of *Spinacia oleracea* (**MESO**) was measured by 2, 2-diphenyl-1-picrylhydrazyl (DPPH)¹³. Various concentrations of the extract (0.8, 4, 20 and 100 μ g/ml) were added to a solution of 1.5 x 10⁻⁴M DPPH (sigma, Bangalore) in methanol and the reaction mixture was shaken vigorously. Quercetin, a known antioxidant was

used as normal control. The amount of DPPH remaining was determined at 520nm and the radical scavenging activity was obtained from the equation.

$$\text{Radical Scavenging activity (\%)} = \frac{(\text{OD Control} - \text{OD Sample})}{\text{OD Control}} \times 100$$

2.7 Determination of total phenolics and flavonoids content

In studying phenolics antioxidants, the total phenolics and flavonoids content of plant extracts were determined as per Folin-Ciocalteu assay method¹⁴. The total phenolics content was expressed as milligrams of Gallic acid equivalents/g extract (mg GAE/g of dry mass) and the total flavonoids content was expressed in milligrams of quercetin equivalents/g of extract (mg QE/g of dry mass).

2.8 Determination of LD₅₀ of CCl₄ for Male wistar rats

The acute oral toxicity studies of *Spinacia oleracea* extract was carried out as per OECD guidelines. The rats were orally fed with different doses of CCl₄ and the LD₅₀ value was calculated as per the method of OECD – 423 guidelines and was found to be 2400ml/kg body weight for a period of 14 days (OECD – 423, 2001). The rats were treated with 1.9ml/kg of CCl₄ daily intra-peritoneal (IP) to induce hepatotoxicity¹⁵.

2.9 In vivo hepatoprotective activity

The rats were randomly divided into five groups, each consisting of five rats and treated as follows:

Group-I as normal control, (fed with normal diet and distilled water).

Group II received CCl₄ 100mM (IP), (as negative control)

Group III received CCl₄ 100mM (IP) and 100µgm/ml (*MESO*) p.o.,

Group IV received CCl₄ 100mM (IP) and 200µgm/ml (*MESO*) p.o.,

Group V received CCl₄ 100mM (IP) and 300µgm/ml (*MESO*) p.o.,

The rats were maintained in the above condition for 14 days and on the 15th day, the rats were anesthetized and sacrificed. Then the blood and liver samples were collected, processed, analysed for various biochemical factors and antioxidant enzymes in the blood samples and they were duly reported.

2.10 Blood sampling assay and liver marker enzymes

The whole blood samples were collected into heparinized capillary tubes, filled up to 2/3 of the tube, sealed and centrifuged at 10,000rpm in a haematocrit centrifuge for 10 minutes and analyzed for the erythrocytes count, leucocytes count and packed cell volume (PCV %) also known as Hematocrit (HCT)¹⁶. Packed cell volume was determined using a haematocrit reader and PCV was expressed as percentage erythrocytes blood contained and the haemoglobin concentration was estimated as per using the formula

$$\text{Hb concentration} = \frac{1}{3} \times \text{PCV concentration}$$

Liver marker enzymes such as Alanine transaminase (ALT), Aspartate aminotransferase (AST) and Bilirubin levels were determined in serum proteins as per standard methods. However, the level of tissue toxicity is tested as Level of lipid peroxidation (LPO) and expressed in terms of GPx, (Glutathione peroxidase), Glutathione (GSH), Superoxide dismutase (SOD) and catalase (CAT)¹⁶. They were determined by the standard methods to assess oxidative stress, on the 'in vivo' subjects.

2.11 Histopathological Examination

For the histopathological observations at the light microscopic level, fresh tissue pieces of liver were fixed in Bouin's fluid¹⁷. Following two days of

fixation, the specimens were washed and dehydrated through an ascending series of ethanol (70 – 100%). Then, they were cleaned with xylene and embedded in paraffin wax, then sectioned at 5µm thickness using a rotary microtome. Sections were rehydrated in distilled water and stained with Hematoxylin-Eosin (H&E) and then examined under light microscopy.

2.12 Statistical analysis

The results were expressed as mean ± SE of six individuals and analyzed by one-way analysis of variance (ANOVA)¹⁸. The SPSS software Version 22 was also used to analyse the obtained data. Post hoc - SNK test was done for variation analysis in addition to student t-test for comparative study; the significantly different value was considered at $p < 0.05$, with normal control. However, $p < 0.001$ was considered to be significantly lower with normal control.

3. RESULTS AND DISCUSSIONS

3.1 Preliminary phytochemicals screening

Screening plant extracts for their antioxidant potency in order to identify their ability in scavenging free radicals (ROS) and binding with metal ions of oxidative reactions is considered as an important step prior to the isolation of antioxidant phytochemicals¹⁹.

In the preliminary phytochemicals analysis, methanol extract of *Spinacia oleracea* (**MESO**) showed the presence of various phytoconstituents as listed in **Table 1**. It was observed that, carbohydrate, protein, fat and total amino acid content is moderately high. The carbohydrates were 40.5g/100g, protein was 18.4g/100g, fat was 1.20g/100g and total amino acid content was (12.5g/100g), indicating the phytoconstituents are moderately available in spinach leaves.

The Vitamins and Minerals Factors of the leafy parts of the plant *Spinacia oleracea* are presented

in **Table-2**. All the non – enzymatic antioxidants such as total phenolics, β- carotene, ascorbic acid, thiamine, flavonoids etc., were found in high content. The β- carotene level was at 30.2 µg/100g, ascorbic acid (vitamin C) is measured to be at 355mg/100g, thiamine at 0.21 µg/100g, total ash at 4.2g/100g, potassium at 412 mg/100g, sodium at 511mg/100gm, Iron at 9.7mg/100g, and calcium at 345mg/100g.

3.2 Effect of MESO on the Antioxidant Activity (DPPH assay)

The DPPH assay is the widely used as screening method for antioxidant activity of plant extracts¹⁹. DPPH is relatively stable, nitrogen-centered free radical which produces violet colour in ethanol solution. It will be reduced to a yellow coloured product, diphenylpicryl hydrazine, with the addition of the plant extracts in a concentration dependent manner. The reduction in the number of DPPH molecules can be correlated with the number of available hydroxyl groups. The Detoxification of excess Reactive Oxygen Species (ROS) produced during stress is important as it may induce membrane lipid peroxidation, enzyme inhibition and nucleic acid damage²⁰.

Table-3 shows the antioxidant properties which include the (1, 1-diphenyl-2- picrylhydrazyl) DPPH assay, the antioxidant enzymes such as catalase and superoxide dismutase (SOD) and the non enzymatic antioxidant glutathione. The DPPH assay shows that **MESO** has a 69% antioxidant activity. Similarly the other antioxidant enzymes such as SOD, GSH and Catalase showed decent levels of content [**MESO** = SOD - 225µgm/minute/ mg protein; the catalase activity was 64.2µmol/min/g; and Glutathione of **MESO** was 79mg/g;] the extracter showed significant inhibition percentage (stronger hydrogen donating ability)

and may be positively correlated with total phenolics content (TPC) ²¹.

The phytochemicals analysis showed the presence of total phenolics content (TPC) as 32.15mg GAE/g and total flavonoids content (TFC) as 18.3QE/g in *MESO*. The amount of total phenolics was determined with the Folin-Ciocalteu reagent. Gallic acid was used as a standard compound. The total phenols were expressed as mg/g Gallic acid equivalent (GAE). The maximum flavonoids content in the metholic extracts of *Spinacia oleracea* was determined with the Quercetin reagent. Quercetin was used as a standard compound and the total flavonoids were expressed as mg/g Quercetin equivalent ²².

3.3 Effect of *MESO* on the liver function analysis

Administration of CCl₄ for 14 days caused the level of ALT to get raised at 101 ±1.11 IU/L which was considerably reduced to 40 ±1.01 IU/L by the administration of *MESO*, at 300µg/ml which is close to control value of 39.5 ±1.11 IU/L. (**Table-4**). Similarly the level of AST was elevated to 1288±1.02 (IU/L) by CCl₄ (Group-II), was effectively reduced to 345 ±1.02 IU/L by *MESO*, at 300µg/ml; thus reducing the AST level to near normal value close to the control 359.5 ±1.01 IU/L (Group-I). The elevated Bilirubin level of 0.94 ±1.01mg/dL due to CCl₄ toxicity was reportedly reduced by *MESO* at 300µg/ml, to 0.49±1.02mg/dL which was almost close to the normal control value of 0.48 ±1.01mg/dL. The ALT and the Bilirubin levels were reported to be significantly higher (p<0.05) Vs normal control at *MESO* of 300µg/ml. However, the AST level at 300µg/ml of *MESO* was significantly lower (p<0.05) Vs negative control.

3.4 Effect of *MESO* on the level of non-enzymatic

The level of non-enzymatic antioxidants (GPx, GSH, SOD, CAT, Vit.C and Vit.E respectively) as observed in CCl₄ control group rats (**Table-5**), were significantly (p<0.05) low, indicating the rise of ROS group and significant damage to the antioxidant system. However, the *MESO* treated groups, recorded with rising values close to the normal control levels, indicating remarkable antioxidant effect. Inactivation, detoxification, removal of ROS and other free radicals depend on enzymatic and non-enzymatic antioxidants. The important enzymatic antioxidants in the tissues are SOD, CAT, glutathione peroxidase (GPx), Vit. C and Vit. E. These antioxidants together with GSH act to prevent the formation of free radicals and thereby prevent oxidative stress ²³.

Upon exposure to methanol extract of *Spinacia oleracea* (*MESO*), in this study, they have got improved to near normal levels as shown by the control. (The values at 300µg/ml are given as per below;) the GPx of *MESO* was 5.31±1.05, against CCl₄ impacted value of 2.08±1.02µg/ml; and the values were close to the control value of 5.71±1.02 µg/ml. The GSH of *MESO* = 4.38±1.01mg/mg, against CCl₄=1.42±1.05mg/mg; and the value was near the normal value of 4.55±0.11mg/mg; The value for SOD of *MESO* was 7.42±1.01u/min/mg, as against CCl₄ impacted value of 4.1±1.01u/min/mg; whereas the value of control was 7.88±1.05µg/ml; and the Catalase (CAT) levels of *MESO* at 82.7±1.01 u/min/mg, as against CCl₄ exposed rats level of 42±1.04 u/min/mg liver protein and the value of the control group was 90.5±1.05 u/min/mg. The ascorbic acid of the CCl₄ diseased is measured to be 1.15±1.01mmol/mg tissue, which has got improved to 1.52±1.03mmol/mg tissue as against

control rats value of 1.63 ± 1.01 mmol/mg tissue. The vitamin E content of CCl_4 intoxicated rats was measured at 0.52 ± 1.02 mmol/mg tissue, whereas the control group was measured at 1.24 ± 1.01 mmol/mg and the effect of *MESO* at $300 \mu\text{g}/\text{ml}$ was 1.02 ± 1.11 mmol/mg, which is obviously a significant recovery.

Reduction in the enzyme activities towards the respective normal values by *MESO* at different dose levels (100, 200 and 300 mg/kg) is an indication of stabilization of plasma membranes and repair of liver tissue damage. The first line of defence against ROS is displayed by the antioxidant enzymes, such as SOD and CAT. The GSH, a non-enzymatic antioxidant, plays a pivotal role in protecting organ against toxicity. These antioxidants mainly convert active oxygen molecules into non-toxic compounds. It was observed that, Glutathione peroxidase (Gpx), Glutathione (GSH), Superoxide dismutase (SOD) and Catalase (CAT) levels of liver protein were low in the CCl_4 diseased rats. And it may be due to the accumulation of superoxide radicals and hydrogen peroxide²⁴.

3.5 Effect of *MESO* on the blood parameters

As summarized in **Table-6**, injection of CCl_4 in rats with significant ($P \leq 0.05$) reduction of WBC, RBCs counts, Hb content as compared with control. Oral administration of *MESO* for 14 days along with CCl_4 ameliorated the suppressive effect of CCl_4 in group III, IV and V at $300 \mu\text{g}/\text{ml}$ when compared with $100 \mu\text{g}/\text{ml}$ and $200 \mu\text{g}/\text{ml}$. The amelioration by *MESO* is reported to the near normal values that were exhibited in control group. The WBC count of 12.2 thousand in a unit blood was raised to the level of 21.5 thousands which was very close to the level of control (group-I) at 22.1 thousands per unit. Similarly the RBC count is raised from 5.7 millions to 8.5

million which is close to the control values of 8.7 million per unit blood. The hemoglobin content in group-V was raised to 13.5 g/dL from 8.2 g/dL of CCl_4 group-II which was close to 14.2 g/dL the value of the control. The hematocrit value too have improved in group-V (44.9%) when compared with group-II (30%) which is near normal value of 47% in group-I.

3.6 Histopathological findings

The oral administration of methanol extract of *Spinacia oleracea* (*per os*) for 14 days in addition to 1.9ml of CCl_4 through intraperitoneal (IP) route did offer remarkable hepatic remediation at $300 \mu\text{g}/\text{ml}$ level and restored the hepatic architecture close to the normal control group of rats.

Figure 1 showed (normal control, untreated normal liver) the composition of hexagonal or pentagonal lobules with central veins and peripheral hepatic triads or tetrads embedded in connective tissue; Hepatocytes were regular and contain a large spheroidal nucleus with a distinctly marked nucleolus and peripheral chromatin distribution. (figures at a resolution of $\times 300$). However, the CCl_4 exposed negative control group (**Figure 2**) revealed that, there was dilation of blood sinusoids, single cell necrosis which affected most of the cell cords. But in **Figure 3(c)**, the restoration of hepatocytes, close to normal arrangement, at $300 \mu\text{g}/\text{ml}$ of *MESO* although cytoplasmic vacuoles were observed. **Figure 3(a) & (b)** show the hepatic architecture at 100 and 200 $\mu\text{g}/\text{ml}$ of *MESO*, respectively, where, there is no response in the mentioned concentrations.

But, it was evidenced that the $300 \mu\text{g}/\text{ml}$ of *MESO* was efficient enough in regenerating the affected hepatic cells from their organization and also showed the dilated blood sinusoids and

cytoplasmic vacuoles. The above results support the view on stabilization of plasma membranes of hepatocytes and thus reduced ROS levels by the activity of *MESO*. Previous phytochemicals investigations on *Spinacia oleracea* reported the presence of flavonoids and phenolics²⁴. The antioxidant activity, flavonoids and phenolics may be responsible for hepatoprotective activity¹⁹.

4. DISCUSSIONS

The screening for 'biochemical factors' in the methanol extract of *Spinacia oleracea* (*MESO*) showed that fairly higher content of carbohydrates, proteins, total amino acids, fats, mono unsaturated fatty acids, polyunsaturated fatty acids, prolines and negligible amount of glycosides were present. These were reported to improve higher hepato-glycogen level between carbohydrate metabolism and antioxidant systems. Amino acids can directly scavenge super oxide, hydroxyl radicals and singlet oxygen and reduce H₂O₂ to water via ascorbate peroxidase reaction. Amino acids regenerate tocopherol from tocopheroxyl radical providing membrane protection and also carries out a number of non-antioxidant functions in the cell²⁵. The dietary fats that are present in the vegetable oils and animal based diet can influence the extent of the formation of lipid radicals and hence lipid peroxidation products, which may be in accordance to the oxidative injury. These injuries may cause changes in the membrane composition of liver cells. Poly unsaturated fatty acids are more prone to oxidation than saturated fatty acids and mono unsaturated fatty acids and increase susceptibility to oxidative injury by affecting antioxidant defense systems²⁶. The moderately higher carbohydrates, amino acids, fatty acids may have thus offered protection to liver cells.

Beta-carotene is an organic compound abundant in plants and is the precursor of Vitamin-A. Consuming foods rich in beta-carotene appears to protect the body from free radicals²⁷. Vitamin-C has the ability to donate electrons in a wide range of enzymatic and non-enzymatic reactions makes amino acid the main ROS-detoxifying compound in the aqueous phase. Vitamin C, as a powerful antioxidant, may help to fight cancer by protecting healthy cells from free radical damage and inhibiting the proliferation of cancerous cells. Ascorbic acid is one of the most studied and powerful antioxidants²⁸. Vitamin E is responsible for the protection of polyunsaturated fatty acids and which in turn protects the membrane qualities, (fluidity, phase separation and lipid domains). The lower level of vitamins such as α -tocopherol (Vitamin-E) and thiamine are more advantageous to the body, in the functions associated with cofactor such as a Catalase, an antioxidant and its deficiency is linked with increased production of reactive oxygen species²⁹. Tocopherol and Thiamine were found to be at trace levels in methanol extract of *Spinacia oleracea*.

Along with the vitamins, the mineral contents of *MESO* may also have helped reduce the elevated levels of ALT, AST and Bilirubin due to CCl₄ intoxication. It was evidenced that mineral aqueous solution containing sodium and potassium were capable of suppressing the expression of proinflammatory cytokines like tumor necrosis factor (TNF- α) and interleukin (IL-6). It was also reported to reduced the raised level of serum creatine, alanine transaminase (ALT) and aspartate amino transferase (AST) levels due to carbon tetrachloride (CCl₄) intoxication³⁰. Aminotransferases (AST) are concentrated mainly in the liver, and their leaching

into the circulation implicate that the integrity of the hepatocytes were disturbed³¹. Generally, bilirubin conjugates with glucuronic acid in the liver and is later excreted through bile. The presence of higher proportion of bilirubin in serum indicates that the process of conjugation is hampered, reflecting loss of hepatic function³².

Glutathione (GSH) is a tripeptide composed of three different amino acids, glutamate, cysteine and glycine that has numerous important functions within cells. A tripeptide glutathione (γ -glutamylcysteinylglycine) is an abundant compound in plant tissues. It has been found virtually in all cell compartments: cytosol, endoplasmic reticulum, vacuole and mitochondria where GSH executes multiple functions. GSH is the main storage form of sulfur, and it acts as a potent detoxifier of xenobiotics through GSH-conjugation, and can serve as a precursor of phytochelatin²⁵. Flavonoids are natural polyphenolic molecules common to most flowering plants. They include flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones³³. Although not considered vitamins and flavonoids have a number of nutritional functions have been described as biological response modifiers. Most of them act as antioxidants and some have anti-inflammatory properties. The antioxidants may act as free radical scavengers, reducing agents, chelating agents for transition metals, quenchers of singlet oxygen molecules, and activators of antioxidative defense enzyme systems to suppress radical damage in biological systems³⁴.

In this study, hepatotoxin CCl_4 was used to screen the efficiency of hepatoprotective agents of the plant extract. It was observed that, there was significant increase in the levels of ALT, AST and Bilirubin in the CCl_4 treated group. Generally

the oxidative stress and cell damages are correlated with increased level of thiobarbituric reactive substances (TBA) and decrease in packed cell volume (PCV) due to destruction of erythrocytes¹⁶. The packed cell volume (PCV) and haemoglobin concentration of CCl_4 control group was reportedly decreased significantly ($p < 0.05$), indicating that there might have been possible destruction of erythrocytes by CCl_4 . This observation agrees with the report that CCl_4 toxicity can lead to destruction of hepatocytes and erythrocytes¹⁶. At cell level metabolism, this xenobiotic chemical CCl_4 is rapidly transformed by cytochrome P450 2E1 into a trichloromethyl radical which is converted into a peroxy radical (ROO^*) in the presence of oxygen. Similarly, superoxide radical ($\text{SO}^{\cdot -}$) and hydroxyl radicals (OH^{\cdot}) are also produced as a part of normal metabolic processes. These radicals may interact with cellular macromolecules and initiate the peroxidative degradation of lipid membranes thus oxidative stress it leads to diverse pathological issues³⁵.

However, the elevated levels of ALT, AST and Bilirubin due to CCl_4 intoxication were moderately reduced ($p < 0.05$) with *MESO* treatment when compared with CCl_4 exposed control rats. Maximum activity was found with 300 $\mu\text{g}/\text{ml}$ dose of *MESO*. The blood parameters such as erythrocytes, white blood cells and packed cell volumes were also decreased ($p < 0.05$) significantly. The lowered blood parameters were improved to near normal levels with 300 $\mu\text{g}/\text{ml}$ dose of *MESO*. This may be due to improved levels of SOD, GSH, Catalase enzymes and phytochemicals that are present already in the plant part as per preliminary screening and these may offer antioxidant activity such as phenolics,

flavonoids Vit. E, Vit.C, β- Carotene and Thiamine³⁶.

Table 1: Biochemical Factors of methanol extract of *Spinacia oleracea* (MESO) (g/100g)

Factors	<i>Spinacia oleracea</i>
Carbohydrates (g/100g)	40.5
Protein (g/100g)	18.4
Fat (g/100g)	1.20
Total amino acid (g/100g)	12.5

Values are expressed in Mean ± SD of six individuals

Table 2: Vitamins and Minerals Factors of methanol extract of *Spinacia oleracea* (MESO)

Factors	<i>Spinacia oleracea</i>
β- Carotene (µg/100g)	30.2
Ascorbic acid (mg/100g)	355
Thiamine (µg/100g)	0.21
Total Ash (g/100g)	4.2
K (mg/100g)	412
Na (mg/100g)	511
Fe (mg/100g)	9.7
Ca (mg/100g)	345

Values are expressed in Mean ± SD of six individuals

Table 3: Antioxidants activity, total phenolics and flavonoids contents of methanol extract of *Spinacia oleracea*

Factors	<i>Spinacia oleracea</i> (MESO)
AOA% (mg/mL)	67
SOD (µgm / minute / mg protein)	214
Catalase (µ/mol/min/g)	56.9
GSH (mg/g)	56
Total phenolics Content (mg GAE/g dry extract)	26.47
Total flavonoids content (mg QE/g dry extract)	15.7

GAE = Gallic acid equivalent; QE = Quercetin equivalent

Table 4: Effect of methanol extract of *S. oleracea* (L.) root (MESO) on ALT, GOT and Bilirubin in CCl₄-induced hepatotoxicity in rats

Groups	ALT (SGPT) (IU/L)	AST (SGOT) (IU/L)	Bilirubin (mg/dL)
Group-I (normal control)	39.5 ±1.11	359 ±1.01	0.48 ±1.01
Group-II (negative control)	101 ±1.11**	1288 ±1.02**	0.94 ±1.01**
Group-III with CCl ₄ and 100µgm/ml	77 ±1.7 ^a	781 ±1.94 ^a	0.76 ±0.12 ^a
Group-IV with CCl ₄ and 200µgm/ml	48 ±1.7 ^a	422 ±2.09 ^a	0.54 ±0.15 ^a
Group-V with CCl ₄ and 300µgm/ml	40 ±1.2 ^{ab}	345 ±2.09 ^{ab*}	0.49 ±0.20 ^{ab}

Each value represents the mean ± SD (n = 6); superscript a = significantly different (p<0.05) Vs normal control; ab= significantly lower (p<0.05) Vs negative control; ** = significantly higher (p<0.05) Vs normal control; * = significantly lower (p<0.001) Vs normal control.

Table 5: Effect of methanol extract of *Spinacia oleracea* (MESO) on enzymatic and non-enzymatic antioxidants in CCl₄-induced hepatotoxicity in rats

Groups	GPx µgm /min/ mg protei n	SOD IU/min/ mg protein	CAT µ/mol/min/ mg protein	Protein in g/L	GSH (µgm /mg protei n)	Ascorbic Acid (Vit.C- mmol/ mg tissue)	Tocopherol (Vit.E - mmol/m g tissue)
Group-I (normal control)	5.01 ±1.02	7.88 ±1.05	90.5 ±1.05	6.7 ±2.01	4.55 ±0.11	1.63 ±1.01	1.24 ±1.01
Group-II (Negative control)	2.08 ±1.02*	4.1 ±1.01*	42 ±1.04*	5.3 ±1.01*	1.42 ±1.05*	1.15 ±1.01 ^{a*}	0.52 ±1.02 ^{a*}
Group-III (CCl ₄ and 100µgm/ml)	3.8 ±0.58*	6.4 ±2.51*	66.5 ±1.57*	6.2 ±0.29 ^{a*}	2.97 ±0.44*	1.27 ±0.22 ^{a*}	0.79 ±0.14 ^{a*}
Group-IV (CCl ₄ and 200µgm/ml (MESO))	4.25 ±0.25*	6.92 ±2.64*	74.7 ±1.01*	6.35 ±0.34 ^{a*}	4.25 ±0.41 ^{a*}	1.39 ±0.16 ^{a*}	0.87 ±0.12 ^{a*}
Group-V (CCl ₄ and 300µgm/ml (MESO))	5.31 ±0.31*	7.42 ±2.72*	82.7 ±1.47*	6.45 ±0.50 ^{a*}	4.38 ±0.46 ^{a*}	1.52 ±0.19 ^{a*}	1.02 ±0.16 ^{a*}

Each value represents the mean ± SD (n = 6); superscript a = significantly different (p<0.05) Vs other experimental (MESO) groups; * = significantly lower (p<0.05) Vs normal control; ** = significantly higher (p<0.001) Vs normal control;

Table 6: Effect of methanol extract of *S. oleracea* L (MESO) on the blood parameters of the CCl₄ induced Hepatotoxic Liver of the Rat

Parameters	WBC (*10 ³ µL)	RBC (*10 ⁶ µL)	Hb (g/dL)	HCT (PCV) (%)
Group-I (Normal control)	22.1±1.02	8.7±1.01	14.2±0.22	47±1.12
Group-II (Negative control) with CCl ₄	12.2±1.02*	5.7±1.01*	8.2±0.22*	30±1.01*
Group-III with CCl ₄ and 100µgm/ml (MESO)	16.7±0.34*	7.9±0.49*	12.7±0.56*	39.5±1.41*
Group-IV with CCl ₄ and 200µgm/ml (MESO)	17.9±0.57*	8.3±0.41*	13.5±0.39*	42.2±1.21*
Group-V with CCl ₄ and 300µgm/ml (MESO)	21.5±0.63*	8.6±0.45*	13.9±0.51*	44.9±1.36*

Each value represents the mean ± SD (n = 6); Significance p < 0.05 vs. Control; * = significantly lower

($p < 0.05$) Vs normal control; **= significantly higher ($p < 0.001$) Vs normal control;

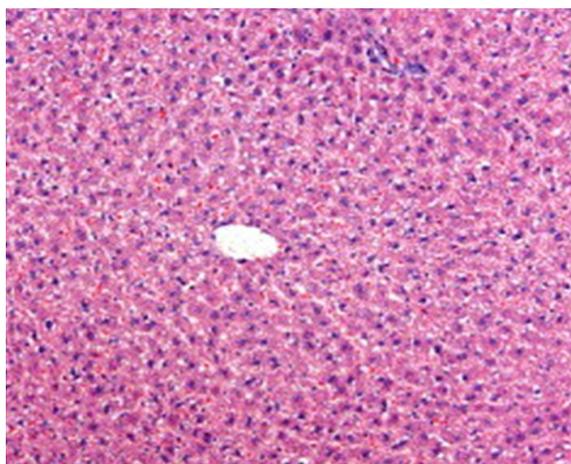


Fig 1: Light Micrograph of a Haematoxylin and Eosin stained liver of a control rat showing normal hepatocytes arrangement in fairly radial position with respect to central vein. HE, 200

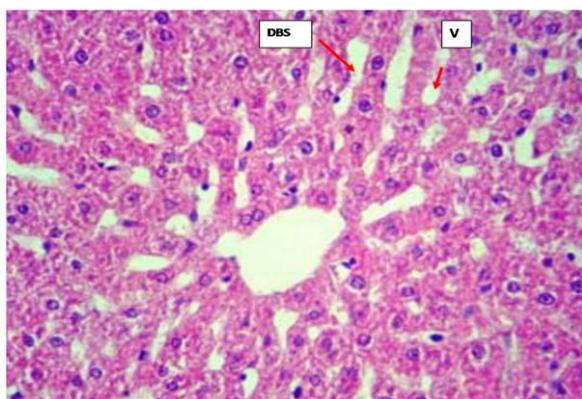
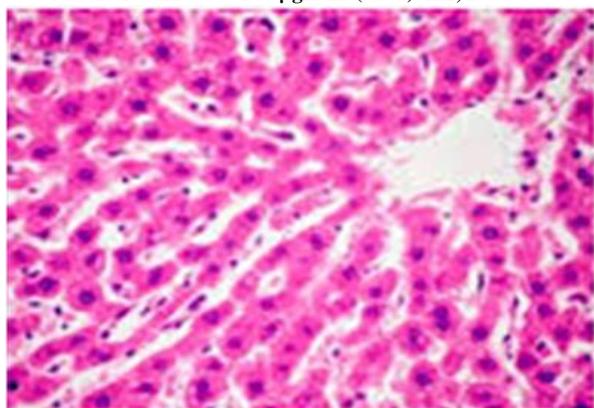
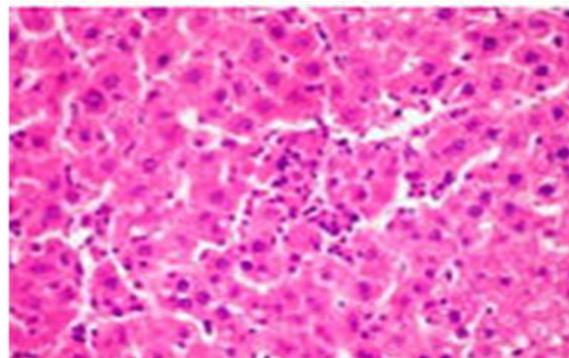


Fig 2: Light Micrograph of a Haematoxylin and Eosin stained liver of a rat exposed to CCl_4 for 14 days showing dilation of hepatic cords (v), blood sinusoids (DBS) and single cell necrosis ($\times 400$). HE, 200

a) Image showing well disturbed hepatic cords and no restorative effects at $100 \mu\text{g}/\text{ml}$ ($\times 300$). HE, 200



b) Image showing evidence of regeneration but to minimal level. Still necrosed area is observed at $200 \mu\text{g}/\text{ml}$ ($\times 300$). HE, 200



c) Image showing regeneration of hepatic architecture largely yet dilated vacuoles are seen in hepatic cells at $300 \mu\text{g}/\text{ml}$ ($\times 300$). HE, 200

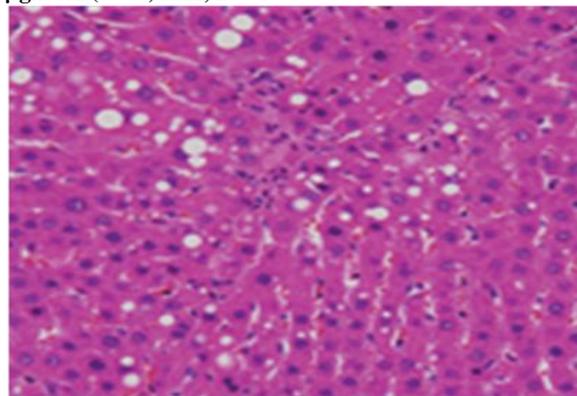


Fig 3: Light Micrograph of a Haematoxylin and Eosin stained liver section of the rat treated with CCl_4 supplemented with methanol extract of *Spinacia oleracea*

5. CONCLUSION

In this study, male wistar rats were exposed to CCl_4 and in the “in vivo” study, a significant hepatic damage was reported due to oxidative stress. As a result there was rise in the liver marker enzyme activities of ALT, AST and Bilirubin in comparison with normal control rats. The blood parameters such as total WBC, RBC, Hb, Packed cell volume and hepatic architecture were proved to be under disturbance. This may be an indication of cellular leakage and loss of functional integrity of hepatocytes cell membrane. The phytochemical analysis revealed the high content of phenolics and flavonoids in *Spinacia oleracea*; vitamins and minerals also in a considerable quantity; upon exposure to *MESO* it was observed and recorded that the reversal of hepatotoxic effects and hepato-remediation did occur. Hence based on the preliminary findings on antioxidant and hepatoprotective activities here

reported, it may be concluded that, methanol extract of *Spinacia oleracea* (**MESO**) possess significant protection against CCl₄-induced hepatotoxicity in the *in vivo* studies. This study also delivers the scope to continue study, identify and characterize the active principle(s) and the mechanism involved in the field of Hepatoprotection and Chemoprevention of Hepatotoxicity offered by the antioxidants fraternity of *Spinacia oleracea*.

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Conflict of Interest

The authors declare that they have no conflict of interest.

