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PHARMACOLOGICAL EVALUATION OF HEPATOPROTECTIVE ACTIVITY OF AQUEOUS AND ALCOHOLIC EXTRACTS OF AEGLE MARMELOS ROOT

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ABSTRACT

The aqueous and alcoholic extract of given herbal powder showed the presence of alkaloids, carbohydrates, phenols, flavonoids, glycosides, saponins and terpens. The aqueous and alcoholic extract of given herbal powder at two dose levels 200 mg/kg and 400 mg/kg body weight administered orally for five days to the CCl_4 challenged rats produced significant reversal of biochemical changes in liver and serum intoxicated by CCl_4 treatment. The aqueous and alcoholic extracts prevented wide range of tissue injury in CCl_4 challenged rats as evidenced by significant reduction in GOT, GPT, ALP, Total bilirubin and LPO levels. The aqueous and alcoholic extracts treated animals produced significant increase in the levels of total protein, GPx, GST, GRD, SOD and catalase in animals challenged with CCl_4.The present study has made an attempt to demonstrate the hepatoprotective activity of aqueous and alcoholic extracts of herbal powder containing in protecting the CCl_4 induced hepatocellular damage. As the aqueous and alcoholic extracts possess various active constituents a need arise for further phytochemical and biochemical studies for identifying the constituent responsible for hepatoprotective property, thereby revealing the hepatoprotective role of aqueous and alcoholic extracts of in the given herbal powder.

KEYWORDS: Hepatoprotective, alkaloids, carbohydrates, phenols, flavonoids, glycosides, saponins and terpens.

INTRODUCTION

From olden days, traditional systems of medicine are of global importance. Even today in many developing countries, majority of population relies greatly on traditional practioners and medicinal plants to meet primary health care needs, despite of availability of modern medicines. These herbal phytomedicines have become popular for historical and cultural reasons. Currently many people in developed countries have began to turn to alternative herbal medicines.

Scientific evaluation of medicinal herbs for their medicinal value and application is on rise. Safety and efficacy of data of many herbs, their extracts and active ingredients, and the preparations containing them was well established. However the market of herbal drugs is poorly regulated and assurance of safety, efficacy, and quality of such herbal products has now become a key issue in industrialized and in developing countries.

The universal role of plants in the treatment of disease is exemplified by their employment in all major systems of medicine irrespective of the underlying philosophical premise, as examples we have western medicine with origins in Mesopotamia and Egypt. The unani (Islamic) and ayurvedic (Hindu) systems centred in Western Asia and the Indian subcontinent and those of the orient (China, Japan, Tibet etc). The plant kingdom undoubtedly still holds many species of plants containing substances of medicinal value which have yet to be discovered large number of plans are constantly being screened for their possible pharmacological effects.

Liver as organ

Liver is the largest gland in the body, situated in the upper part of the abdominal cavity occupying the greater part of the right hypochondrial region and weighing between 1 and 2.3 kilogram. Its upper part and anterior surfaces is irregular. Liver is enclosed in a thin capsule and incompletely covered by a layer of peritoneum. Folds of peritonium form supporting ligaments attaching the liver to the interior surface of diaphragm. The liver is described as having four lobes, the most obvious are the large right lobes and smaller wedge shaped, left lobe.

Liver diseases and medicinal plants

Liver diseases may be classified as

1. Acute or chronic hepatitis (inflammatory liver disease)
2. Hepatisis (non inflammatory liver disease) and
3. Cirrhosis (degenerative disorder resulting in fibrosis of the liver)

Liver diseases are mainly caused by

1. Chemical agents (certain antibiotics, peroxidised oil, aflatoxin carbon tetrachloride, chlorinated hydrocarbon, etc)
2. Excess consumption of alcohol
3. Infection and autoimmune disorder

Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages in liver. Enhanced lipid peroxidation produced during the liver microsomal metabolism of ethanol may result in hepatitis and cirrhosis.

90% of the acute hepatitis is due to viruses B, A, C, D (delta agents) E and G. Hepatitis B infection often results in chronic liver diseases and cirrhosis of liver.

PLANT PROFILE

T AXONOMICAL CLASSIFICATION

Kingdom : Plantae

Phylum : Spermatophyta

Sub phylum : Angiospermae

Order : Sapindales

Family : Rutaceae.

Subfamily : Aurantioideae.

Genus : Aegle

Species : Aegle marmelos.

Botanical name : Aegle marmelos

Aegle marmelos belonging to family Rutaceae, is commonly known as Bael in indigenous systems of medicine and has been regarded to possess various medicinal properties. The bael is one of the sacred trees of the Hindus. Leaves are offered in prayers to Shiva and Parvathi since ancient t times.

Synonyms: *Bilva, Bael, Maredu*

Vernacular names

English: Bengal quince, Beal fruit, Golden apple, Indian quince, Stone apple **Tami l :** Aluvigam, Iyalbudi, Kuvilam, Mavilangai, Vilwam, Villuvam.

Telugu: Bilvamu, Maluramu, Maredu, Sailushamu, Sandiliyamu, Sriphalamu.

Hindi: Bel, Bili, Sirphal, and Bela,

Sanskrit: Adhararutha, Asholam, Atimangaliya, Bilva.

Bengal: Bael, Bel

Gujarat: Billi

Kannada: Bela, Bilva

Malayalam: Koovalam, Vilwam

Orissa: Belo.

Botanical Description

Aegle marmelos is a slow growing medium sized tree, up to 12-15 m tall with short trunk, thick, soft, flaking bark, and spreading, sometimes spiny branches, the lower ones drooping. Young suckers bear many stiff, straight spines. The deciduous, alternate leaves, borne singly or in group, are composed of 3 to 5 oval, pointed and shallowly toothed leaflets, 4-10 cm long, 2-5cm wide, the terminal one with a long petiole.

Soil type

Bael is said to do best on rich, well-drained soil, but it has grown well and fruited on the oolitic limestone of southern Florida. It also grows well in swampy, alkaline or stony soils having pH range from 5 to 8. In India it has the reputation of thriving where other fruit trees cannot survive.

Origin and distribution

The bael tree has its origin from Eastern Ghats and Central India. It is native to India and is found growing wild in Sub-Himalayan tracts from Jhelum eastwards to West Bengal, in central and south India. Bael is found growing along foothills of Himalayas, Bihar, Chhattisgarh, Uttaranchal, Jharkhand and Madhya Pradesh.. It is also grown in some Egyptian gardens in Surinam and Trinidad.

Documented species distribution

Native range: India

Exotic range: Bangladesh, Egypt, Malaysia, Myanmar, Pakistan, Sri Lanka, Thailand.

Chemical Constituents

Extensive investigations have been carried out on different parts of Aegle marmelos and as a consequence, varied classes of compound viz., alkaloids, coumarins, terpenoids, fatty acids and aminoacids have been isolated from its different parts. Aegle marmelos leaves contained γ-sitosterol, aegelin, lupeol, rutin, marmesinin, β-sitosterol, flavone, glycoside, Oisopentenyl halfordiol, marmeline and phenylethyl cinnamamides.

The detailed investigations on isolated compound classes are as under,

Alkaloids

The alkaloids comprise the largest single class of secondary plant substances. New alkaloids from the leaves of A. marmelos were reported viz. halfordino, ethylcinnamamide and marmeline. Recently, series of phenylethyl cinnamides, which included new compounds named anhydromarmeline, aegelinosides A and B were isolated from Aegle marmelos leaves as α-glucosidase inhibitors.

Phenylpropenoids

These are naturally occurring phenolic compounds, which have an aromatic ring to which three carbon side chain is attached. Among the phenylpropenoids are included hydroxylcoumarins, phenylpropenes and lignans.

Terpenoids

The essential oil of A. marmelos (L.) Correa leaves were studied very much extensively in India by various workers since 1950. α-Phellandrene was found to be the common constituent of the essential oil from leaves, twigs and fruits. α-Phellandrene (56%) and p-cymene (17%) were reported from leaf oil. Limonene (82.4%) was reported as the main constituent from A. marmelos leaves and it was shown that limonene is characteristic marker for identification of A. marmelos oil samples.

Tannins

The maximum tannin content in bael fruit was recorded in the month of January. There is as much as 9% tannin in the pulp of wild fruits, less in cultivated type.

Tannin is also present in leaves as skimmianine, it is also named as 4, 7, 8-trimethoxyfuro, quinoline.

Carotenoids

Carotenoids are responsible for imparting pale colour to fruit. Marmelosin, skimmianine and umbelliferone are the therapeutically active principles of bael plant.

Minor constituents like flavonoids, glycosides, saponins, ascorbic acid,

2.0. SCOPE AND PLAN OF WORK

Herbal medicines are effective in the treatment of various ailments. Very often these drugs are unscientifically exploited and/or improperly used. Therefore these plant drugs deserve detailed studies in the light of modern science. The detailed investigation and documentation of plants used in local health traditions and pharmacological evaluation of these plants and their taxonomical relatives can lead to the development of invaluable plant drugs for many diseases.

Therefore based on the above facts, as no preclinical screening has been carried out regarding the hepatoprotective activity of the given herbal extract, the present study has been undertaken to evaluate the hepatoprotective activity of the given herbal extracts in CCl_4 intoxicated liver injury in rats.

MATERIALS AND METHODS

Plant materials

Plant collection and identification

For the present investigation Aegle marmelos was collected from an area of Mahabubabad, Warangal, (Telangana) surrounds.

Identification and authentication of roots of Aegle marmelos

The plant material was taxonomically identified by the Assistant Professor Dr.Md.Mustafa, Department of Botany, Kakatiya University, Warangal.

Drying and size reduction

The roots were carefully dried in shade for 15 days. Then roots were subjected to size reduction to make powder by using mechanical grinder. The crushed mass of roots were then carried out for the process of extraction.

Preparation of extract

The roots of Aegle marmelos was collected and coarsely powdered. The powder was then successfully extracted with ethanol and distilled water using soxhlat apparatus. After effective extraction the solvent was distilled off. The extract where dried using a rotary vaccum evaporator and stored in a desicator until its further use.

The ethanolic and aqueos extract of herbal powder thus obtained were used for the preliminary phytochemical screening and pharmacological studies. The extracts were administered to animals by dissolving in 0.6% carboxy methyl cellulose (CMC).

Experimental Animals

Healthy wister rats of either sex (200-225g) were used in the study. The animals were kept in polyacrylic cages and maintained under standard having conditions of temperature (24-27 degree Celsius) and humidity(60-65%) within 12 hours light 12h dark cycle. They were acclimiatized for 10 days. Food was provided in the form of dry pellets and water ad libitum. The experiments were performed based on animal ethics of guidelines of university animals ethics committee studies. The animals were randomly distributed into seven groups with six animals in each group.

THE EXPERIMENTAL PROTOCOL WAS APPROVED BY THE INSTITUTIONAL ANIMAL ETHICS COMMITTEE (IAEC)

Reference no: 1505/SURA/a/14CPCSEA 2024

Preliminary Phytochemical screening

The basic herbal powder is subjected to preliminary chemical screening for their presence or absence of active phytochemical constituents by the following methods.

1. Test for alkaloids

Treated with dilute Hydrochloric acid and filtered. The filtrate was treated with various alkaloidal agents.

a) Mayer's-test

Treated with Mayer's reagent. Appearance of cream colour indicates the presence of alkaloid.

b) Dragendorff's –test

When little amount of the sample was treated with the Dragendorff's reagent, the appearance of reddish brown precipitate indicates the presence of alkaloid.

c) Hager's –test

Treated with the Hager's reagent, the appearance of yellow colour precipitate indicates the presence of alkaloid.

d) Quinoline alkaloids test

Little amount of extract is added with glacial acetic acid gives reddish brown fumes and with concentrated sulphuric acid gives blue fluorescence in U.V. light.

2. Test for carbohydrates

Dissolve small quantities (300mg) of alcoholic and aqueous extracts separately in 4 ml of distilled water and filter. The filtrate may be subjected to (a) Molish's test to detect the presence of carbohydrates. Dissolve a small portion of the extract in water and treat with (b) Fehling's solution A and B, (c) Benedict's reagents and (d) Barfoed's reagents to detect and presence of different sugars.

3. Test for steroids

a) Libermann burchard test

When the extracts were treated with concentrated sulphuric acid, few drops of glacial acetic acid, followed by the addition of acetic anhydride, appearance of green colour indicates the presence of steroids.

4. Test for proteins

a) Biuret's -test

When the extracts were treated with copper sulphate solution, followed by the addition of sodium hydroxide solution, appearance of violet colour indicates the presence of proteins.

b) Millon's -test

When the extracts were treated with Millon's reagents, appearance of pink colour indicates the presence of proteins.

5. Test for tannins

a) When the extracts were treated with 10% lead acetate solution, appearance of white precipitate indicates the presence of tannins.

b) When the extracts were treated with aqueous bromine solution, appearance of white precipitate indicates the presence of tannins.

6. Test for phenols

a) When the extracts were treated with neutral ferric chloride solution, the appearance of violet colour indicates the presence of phenols.

b) When the extracts were treated with 10% sodium chloride solution the appearance of cream colour indicates the presence of phenols.

7. Test for flavonoids

a) 5ml of the extract solution was hydrolyzed with 10% v/v sulphuric acid and cooled. Then, it was extracted with diethylether and divided into three portions in three separate test tubes. 1 ml of diluted sodium carbonate, 1ml of 0.1 sodium hydroxide, and 1 ml of strong ammonia solution were added to the first,second and third test tubes respectively. In each test tube, development of yellow colour demonstrated the presence of flavonoids.

b) Shinoda's test: The extract was dissolved in alcohol, to that one piece of magnesium followed by conc. HCl were added dropwise and heated. Appearance of magenta colour shows the presence of falvonoids.

8. Test for gums and mucilage

The extracts were treated with 25ml of absolute alcohol, then solution was filtered. The filtrate was examined for its swelling properties.

9. Test for glycosides

When a pinch of the extract were dissolved in the Glacial acetic acid and few drops of ferric chloride solution was added, followed by the addition of concentrated sulphuric acid, formation of red ring at the junction of two liquids indicates the presence of glycosides.

10. Test for saponins

Foam test

1 ml of the extracts are diluted to 20ml with distilled water and shaken well in a test tube. The formation of foam in the upper part of the test tube indicates the presence of saponins.

11. Test for terpenes

When the extracts were treated with Tin and thionyl chloride, appearance of pink colour indicates the presence of terpenes.

3.0. PHARMACOLOGICAL STUDIES

Acute oral toxicity study

The procedure was followed by using OECD guidelines (Organization of Economic Cooperation and Development) 423 (Acute Toxic Class Method).

The acute toxic class method is a step wise procedure with 3 animals of a single sex per step. Depending on the mortality and / or moribund status of the animals, on the average 2-4 steps may be necessary to allow judgement on the acute toxicity of the test substance. This procedure results in the use of a minimal number of animals while allowing acceptable data based scientific conclusion.

The method uses defined doses (5, 50, 300, 2000 mg/kg body weight) and the results allow a substance to be ranked and classified according to the Globally Harmonized System (GHS) for the classification of chemical which cause acute toxicity.

Body weight of the rats before and after termination were noted and any changes in skin and fur, eyes and mucous membranes and also respiratory, circulatory, autonomic and central nervous system and somatomotor activity and behaviour pattern were observed, and also sign of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma were noted. The onset of toxicity and signs of toxicity were also noted, if any.

HEPATOPOROTECTIVE ACTIVITY

Study protocol

The animals were divided into 7 groups consisting of six rats in each group.

Group I : Animals were received single daily dose of normal saline on all 5 days (1ml/kg ,p.o.) and olive oil (1 ml/kg, s.c.) on days 2 and 3.

Group II: Animals were received single daily dose of normal saline (1 ml /kg p.o.) for days and carbon tetra chloride (2 ml/kg s.c) administered on days 2 and 3.

Group III: Animals were treated with 200 mg/kg, p.o. of aqueous extract of the herbal powder on all 5 days and carbon tetrachloride (2 ml/kg,s.c) administered on days 2 and 3.

Group IV: Animals were treated with 400 mg/kg, p.o. aqueous extract of the herbal powder on all 5 days and carbon tetrachloride solution (2ml/kg, s.c.) on days 2 and 3, 30 min after administration of extract.

Group V: Animals were treated with 200 mg/kg, p.o. of alcoholic extract of the herbal powder on all 5 days and carbon tetrachloride solution (2ml/kg, s.c.) on days 2 and 3, 30 min after administration of extract.

Group VI: Animals were treated with 400 mg/kg, p.o. of alcoholic extract of the herbal powder on all 5 days and carbon tetrachloride solution (2ml/kg, s.c.) on days 2 and 3, 30 min after administration of extract.

Group VII: Animals were treated with 1 ml/kg p.o. of liv-52 syrup on all 5 days and carbon tetrachloride solution (2 ml/kg, s.c) on days 2 and 3, 30 min after administration of extract.

All the animals were sacrificed on the 5^{th} day by cervical decapitation under light after anaesthesia for estimation of bio-chemical parameters .Liver was collected and wet weight of liver was noted as mg weight of liver/gm body weight.

Bio-chemical study

Blood collected from Jugular veins was allowed to clot for 30-40 min. Serum was separated by centrifuging at 3000rpm for 10 minutes.

Immediately after sacrifice, the liver was dissected out and washed in ice cold saline, and a homogenate was prepared in 0.05M sodium phosphate buffer pH 7.0. The homogenate was centrifuged at 3000rpm for 10 minutes and the supernatant was used for the assay of marker enzymes.

Assay of Glutathionate Peroxidase (GPx)

Glutathione peroxidase of liver homogenate was assayed according to the method Necheles et al.

Reagents

1. 0.1 M Phosphate buffer pH 7.4
2. 8 mM Reduced Glutathione (GSH).
3. 30mM Sodium azide.
4. 9mM Ethylene diamine tetra acetic acid (EDTA)
5. 18 mM Hydrogen peroxide.
6. 1mM 5,55" – Dithio –bis – 2 nitro benzoic acid (DTNB)
7. 10% Trichloroacetic acid.

Procedure

The final incubation mixture consisted of 0.2 ml liver homogenate, 1.0 ml of phosphate buffer, 0.5 ml; reduced glutathione, 0.5ml sodium azide, 0.5ml EDTA and 2.0 ml of distilled water. The solution was incubated at 37°C for 5minutes and the reaction was started by the addition of 1.0ml of hydrogen peroxide, 1.0ml samples were taken exactly at zero minute and one minute after the addition of hydrogen peroxide and to arrest the reaction, 2.0ml of 10 % TCA was added. Non enzymatic oxidation of glutathione was measured in a blank containing the above reagents with buffer substituted for the enzyme source. Under these conditions, the non enzymatic oxidation was minimal. The residual glutathione was then measured by the reaction with 1ml of DTNB at 412 nm.

The activity of glutathione peroxidase was expressed as n moles of GSH oxidized/min/mg protein in liver homogenate.

Assay of glutathione -s- transferases

Glutathione –s transferases of liver homogenate was assayed by the method of Habig et.al.

Reagent

1. 0.3 M phosphate buffer pH 6.5
2. 30mM 1- Chloro – 2,4- dinitrobezene (CDNB)
3. 30 mM Reduced Glutathione

Procedure

To 1.0 ml of phosphate buffer, 0.1 ml of 1- chloro – 2,4- dinitrobeneze, 1.7ml of water and 0.1 ml of liver homogenate was added. After 5 minutes incubation at 37°C, 0.1ml of reduced glutathione was added and the change in optical density was measured immediately for 3 minutes at 30 sec interval. Complete assay mixture without enzyme was used as control. The optical density was measured at 340 nm.

Activity of Glutathione S- Transferases was expressed as n moles of CDNB conjugate formed /min/ mg/ protein in liver homogenate.

Assay of glutathione reductase

Glutathione reductase activity of liver homogenate was measured by the method of Dubler. Et al.

Reagents

1. 50mM Sodium phosphate buffer, pH 7.5
2. 10mM Ethylene diamine tetra acetic acid (EDTA).
3. 0.67mM Glutathione Oxidized (GSSG)
4. Nicotinamide Adenine Dinucleotide phosphate (NADPH)

Procedure

The reaction mixture containing 50 mM phosphate buffer pH 7.5, 10 mM EDTA, 0.67 mM glutathione oxidized, and 0.1 mM NADPH was made up to 3.0 ml with water. The change in optical density was monitored after adding suitable diluted liver homogenate at 340 nm for 3 minutes at 30 seconds intervals.

The enzyme activity is expressed as n moles of GSSG utilized/ min/ mg/ protein in liver homogenate.

Assay of Superoxide Dismutase

Superoxide dismutase was assayed according to the method of Misra and fridovich.

Reagents

1. 0.1 M carbonate bicabonate buffer, pH 10.2 containing 5.7 mg EDTA / 100ml
2. 2.3 mM Epinephrine.

Procedure

To 0.05 ml of liver homogenate, 1.5 ml of buffer was added. The reaction was initiated by the addition of 0.4 ml epinephrine and change in optical density per minute was measured at 480nm. One unit of superoxide dismutase activity is the amount of protein required to give 50 % inhibition of epinephrine oxidation.

Assay of catalase

Catalase of liver homogenate was assayed according to the method of Bergmeyer et al.

Reagents

1. 50 mM phosphate buffer pH 7.0
2. 30 mM Hydrogen peroxide in phosphate buffer.

Procedure

To 1.2 ml of phosphate buffer 0.1 ml of the liver homogenate was added. The enzyme reaction was started by the addition of 1.0 ml of hydrogen peroxide solution. The decrease in absorbance was measured at 240 nm for 3 minutes at 15 second intervals.

The enzyme activity was expressed as n moles of hydrogen peroxide decomposed/min/mg protein in liver homogenate.

Estimation of Lipid Peroxide

Lipid peroxide concentration of liver homogenate was determined by thiobarbituric acid reaction as described by Ohkawa et al.

Reagents

1. 20 % Acetic acid
2. 8.1 % Sodium dodecyl sulphate (SDS)
3. 0.8 % Thiobarbituric acid (TBA)
4. N- Butanol - Pyridine mixture (15: 1 v/v)
5. Standard solution.

Procedure

To 0.2 ml of liver homogenate, 1.5 ml of 20% acetic acid. 0.2 ml of sodium dodecyl sulphate and 1.5 ml of thiobarbituric acid were added. The mixture was made up to 4.0 ml with distilled water and then heated for 30min at 95°C in a water bath. After cooling 4.0ml n-butanol pyridine mixture was added and shaken well. After centrifugation at 4000 rpm for10 min with the organic layer was taken and its absorbance was read at 532 nm.

The Lipid peroxide concentration was expressed as n moles of MDA / mg protein in liver homogenate.

Vitamin E

Vitamin E is determined by the method of Desai.

Reagents

1. Ethanol
2. Xylene
3. 2-2' Dipyridyl reagent
4. $Fecl_3$ reagent
5. α- tocopherol

Procedure

To 1.5ml of liver homogenate, 1.5 ml ethanol and 1.5 ml xylene are added, they are centrifuged. 1.0ml xylene layer is separated out and mixed with dipyridyl reagent, mixed well. Aliquot 1.5 ml was taken and reading at 460 nm, add 0.33ml $fecl_3$ regent. Reading was taken 520 nm exactly after 1.5mins.

Vitamin E concentration was expressed as n moles /g wet tissue in liver homogenate.

STATISTICAL ANALYSIS

The statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Dunnet's 't' test, p values < 0.05 were considered as significant.

4.0. RESULTS

Preliminary phytochemical screening Table I depicts the results of preliminary phytochemical screening of the aqueous and alcoholic extracts of the given herbal powder.

The aqueous extract showed the presence of carbohydrates, steroids, terpenoids, flavonoids, saponins, phenol, glycosides, proteins and reduced sugar. Alkaloids, gums and mucilages and tannins were absent. Alcohol extract

showed the presence of alkaloids, carbohydrates, phenols, steroids, terpenoids, tannins, glycosides, proteins, flavonoids and reduced sugar. Saponins and glycosides were absent.

Acute oral toxicity study

The acute oral toxicity study was done according to the OECD guidyline 423 (Acute toxic class method) and the results are shown in Table 2.

A starting dose of 2000 mg/kg body weight / p.o. of aqueous and alcoholic extracts were administered to 3 male rats/ group, respectively and observed for three days. There was no significant changes in body weight before and after termination of the experiment and no signs of toxicity was observed. The experiment was terminated on14th day. The experiments were repeated again with the same dose level, 2000 mg/kg p.o. of aqueous and alcoholic extract of the given herbal powder for 3 days more. No significant changes were observed from the first set of experiment. LD_{50} cur of mg/kg body weight was observed as X (Unclassified) and Globally Harmonised System (GHS) classes also comes under X (Unclassified).

4.1. Hepatoprotective study

Weight of liver CCl_4 treated animals (group II) showed a significant increase ($p<0.001$) in wet weight of the liver compared to control (Table 3). There was a significant decrease in liver wet weight of animals treated with 200 mg/kg and 400 mg/kg of aqueous and alcoholic extracts ($p<0.001$) respectively, when compared with group II (Fig. 1).

Glutamate oxaloacetate transaminase (GOT) and Glutamate pyruvate transaminase (GPT)

GOT and GPT levels of serum and liver homogenate were significantly increased ($p<0.001$) in group II challenged with $CC1_{4,}$ when compared to control (Table 4 and 5). A dose dependent reduction of ($p < 0.001$) GOT and GPT levels were observed in animals treated aqueous (200 mg/kg and 400 mg/kg) and alcoholic extracts (200 mg/kg and 400 mg / kg) when compared to group II. Liv – 52 (group VII) produced a significant reduction (p.<0. 001) at the dose of 1 ml/ kg body weight /p.o. in both indices when compared to group II (Fig . 2 & 3).

Serum Alkaline Phosphatase (ALP)

The serum ALP level was significantly increased ($p< 0.001$) in CCl_4 –challenged rats (group II) when compared to control rats (group 1) (Table 6.). Treatment with aqueous (200 mg /kg and 400 mg /kg) and alcoholic (200 mg/kg and 400 mg/kg) extracts showed a significant ($p<0.001$) reduction in ALP level when compared to group II animals. The Liv-52 (1 ml/kg) treated group also showed a significant decline of ALP ($P<0.001$) when compared to group II animals. (Fig 4).

Total bilirubin

There was a significant increase ($p<0.001$) in the level of total serum bilirubin in CCl_4 treated animals (group II), when compared to group I (Table 7). Aqueous (200 mg /kg and 400 mg /kg) and alcoholic (200 mg/kg and 400 mg /kg) extracts of given herbal extracts treated rats showed significant decrease (p.<0.001) of total bilirubin when to CCl_4 treated rats. The Liv – 52 (1 mg /kg) treated animals (group VII) also showed significant ($P<0.001$) decrease of total bilirubin level when compared to group II animals (Fig. 5).

Total Protein

A significant ($p<0.001$) reduction in the total protein of serum and liver was observed in CCl_4 treated rats (group II) when compared to control (group I) (Table 8). Treatment with aqueous (200 mg/kg and 400 mg/kg) and alcoholic (200 mg/kg and 400 mg/kg) extracts showed a significant ($P<0.001$) increase of protein level, when compared to (group II) animals. The Liv – 52 (1 ml/ kg) treated animals (group VII) also showed significant ($p<0.001$) increase in protein level when compared to CCl_4 challenged (group II) rats (Fig. 6).

4.2. Glutathione Perxidase (GPx)

Liver Glutathione peroxidase activity was significantly ($p<0.001$) reduced in CCl_4 treated animals (group II) when compared to control (group I) (Table 9). The aqueous extract (200 mg/kg and 400 mg/kg dose levels) significantly increased ($p<0.001$) the GPx levels, when compared to group II. However alcoholic extract (200 mg/kg and 400 mg /kg) showed less significant increase ($p<0.05$, $p<0.001$ respectively) in GPx levels when compared to group II animals. Liv – 52 (1 ml/kg) treated animals also showed significant ($p<0.001$) increase of GPx level in the liver homogenate compared with group II animals (Fig. 7).

Glutathione – S- transferase (GST)

Liver Glutathione – S transferase level was significantly reduced ($p<0.001$) in CCl_4 treated animals when compared with normal animals (Table 10). Treatment with aqueous extract of the given herbal extracts at 200 mg/kg and 400 mg/kg dose levels showed significant increase ($p<0.001$)in GST level when compared to CCl_4 treated group. Alcoholic extracts (200 mg /kg and 400 mg /kg) also showed significant ($p<0.01$, $p<0.001$ respectively), increase of GST level in liver homogenate. Liv- 52 (1 ml/kg) treated animals also showed significant ($p<0.001$) increase of GST level when compared to group to group II (Fig. 8).

Glutathione reductase (GRD)

Liver GRD activity was significantly ($p<0.001$) reduced in CCl_4 treated animals (group II), when compared to control (group I) (Table 11). The aqueous (200 mg/kg and 400 mg/kg) and alcoholic (200 mg/kg and 400mg/kg) extracts showed significant increase ($p<0.0001$) in GRD level, when compared to group II animals. Liv – 52 (1 ml/kg) treated group also showed significant ($p< 0.001$) increase of GRD level when compared to group II (Fig. 9).

Superoxide dismutase (SOD)

Liver Superoxide dismutase level was significantly reduced ($p<0.001$) in CCl_4 treated animals when compared with normal animals (Table 12). The aqueous (200 mg/kg and 400 mg/kg) extracts showed significant increase ($p<0.01$ and $p<0.001$ respectively) in SOD levels, when compared to group II. The alcoholic extract at 200 mg/kg did not show a significant increase in SOD level when compared with CCl_4 treated animals, but the 400 mg/kg dose alcoholic extract treated animals showed significant ($p<0.001$) increase of SOD level when compared with group II. Liv-52 (1 ml/kg) treated animals also showed significant ($p<0.001$) increase of SOD level when compared to group II (Fig.10).

Catalase (CAT)

Liver Catalase activity was significantly ($p<0.001$) reduced in CCl_4 treated animals (group II), when compared to control (group I) (Table 13). The aqueous (200 mg/kg and 400 mg/kg) and alcoholic (200 mg/kg and 400 mg/kg) extracts significantly increased ($p<0.001$) the CAT level when compared to group II animals. Liv- 52 (1 ml/kg) treated group also showed significant ($p<0.001$) increase of catalase level when compared to group II (Fig. 11).

4.3. Lipid Peroxidation (LPO)

The lipid peroxide of liver homogenate was significantly increased ($p<0.001$) in CCl_4 challenged rats (group II) when compared to control rats (group I) (Table 14). Treatment with aqueous (200 mg/kg and 400 mg/kg) and alcoholic (200 mg/kg and 400mg/kg) extracts showed significant ($p<0.01$, $p<0.001$) decrease in LPO level when compared with CCl_4 treated (group II) animals. The Liv – 52 (1 ml/kg) treated group also showed a significant ($p<0.001$) decline in the LPO level when compared to group II animals (Fig. 12).

Vitamin E

Vitamin E activity of liver was significantly ($p<0.001$) reduced in CCl_4 treated animals (groups II), when compared to control animals (group I) (Table 15). The aqueous (200 mg/kg and 400 mg/kg) and alcoholic (200 mg/kg and 400 mg/kg) extracts showed significant increase ($p<0.001$) of Vitamin E level when compared to group II animals. Liv 52 (1ml/kg) treated group also showed significant ($p<0.001$) increase in Vitamin E level when compared to group II. (Fig.13).

Table 1: Preliminary phytochemical Screening.

SI. No.	Constituents	Test	Present /Absent	
			Aqueous Extract	Alcoholic Extract
1.	Alkaloids	a. Mayer's reagent b. Dragendorffs reagent c. Hagner's reagent d. Quinoline alkaloids	Absent Absent Absent Absent	Present Present Present Present
2.	Carbohydrates	a. Molisch's reagent b. Fehling's solution A and B c. Benedict's reagent d. Barfoed's reagent	Present Present Present Present	Present Present Present Present
3.	Steroids	a. Libermann's burchard test b. Millon's reagent	Present Present	Present Present
4.	Protein	a. Biuret test b. Millon's reagent	Present Present	Present Present
5.	Tannins	a. 10% Lead acetate solution b.10% Sodium chloride c. Aqueous bromine solution	Absent Absent Absent	Present Present Present
6.	Phenols	a. Ferric chloride b. 10% Sodium chloride	Present Present	Present Present
7.	Flavonoids	a. Amly alcohol + Sodium acetate + Ferric chloride b. Con. H_2SO_4 c. Magnesium turning test	Present Present Present	Present Present Present
8.	Gums and Mucilage	Swelling test	Absent	Absent
9.	Glycosides	Glacial acetic acid + Ferric chloride + Con. Sulphuric acid	Present	Absent
10.	Saponins	Foam test	Present	Absent
11.	Terpins	Tin + Thionyl chloride	Present	Present

Table 3: Weight of liver (mg/g body weight).

Group	I	II	III	IV	V	VI	VII
Liver weight	33.15 ±0.671	46.69 ±0.376[a]	41.53 ±0.442[b]	36.98 ±0.461[b]	38.17 ±0.497[b]	37.24 ±0.519[b]	35.41 ±0.269[b]

P values – [a] < 0.001 vs group I, [b]< 0.001 vs group II.

Values are mean ± SE of 6 animals in each group.

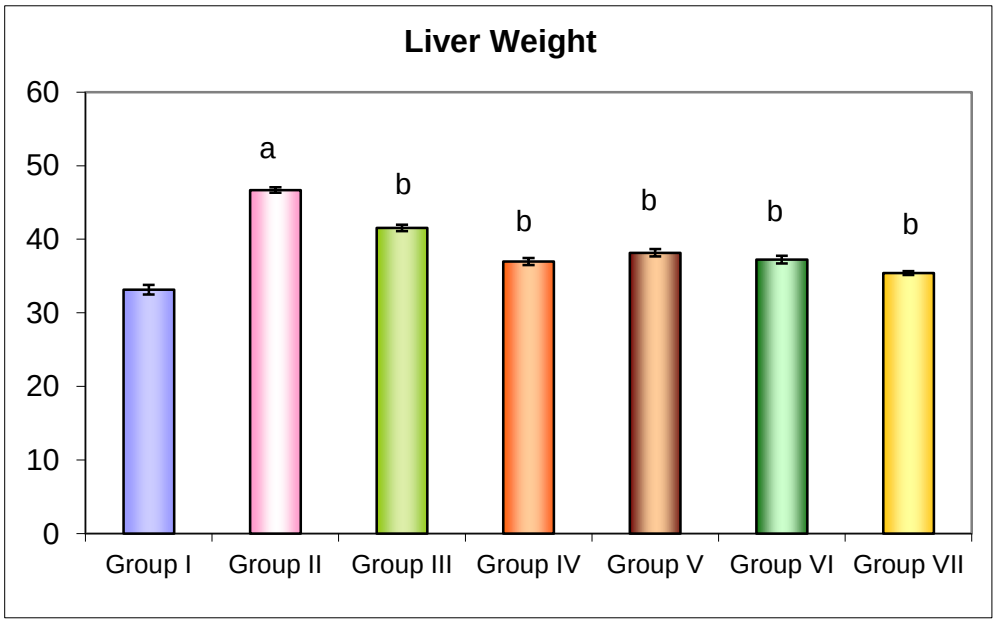

Fig. 1.

Table 4: Glutamate Oxalocetate Transaminase (GOT/AST)

Group	I	II	III	IV	V	VI	VII
Serum (U/ml)	28.83 ± 0.87	179.16 ±3.64[a]	122.83 ±2.23[b]	34.00 ±1.53[b]	123.83 ±3.66[b]	43.20 ±3.66[b]	31.33 ±1.28[b]
Liver (µmol of pyruvate liberated/ mg protein/min)	30.66 ± 0.76	171.83 ±0.96[a]	107.33 ±2.82[b]	42.20 ±3.39[b]	107.83 ±3.18[b]	37.66 ±1.45[b]	34.00 ±1.29[b]

P values – [a] < 0.001 vs group I, [b]< 0.001 vs group II.
Values are mean ± SE of 6 animals in each group.

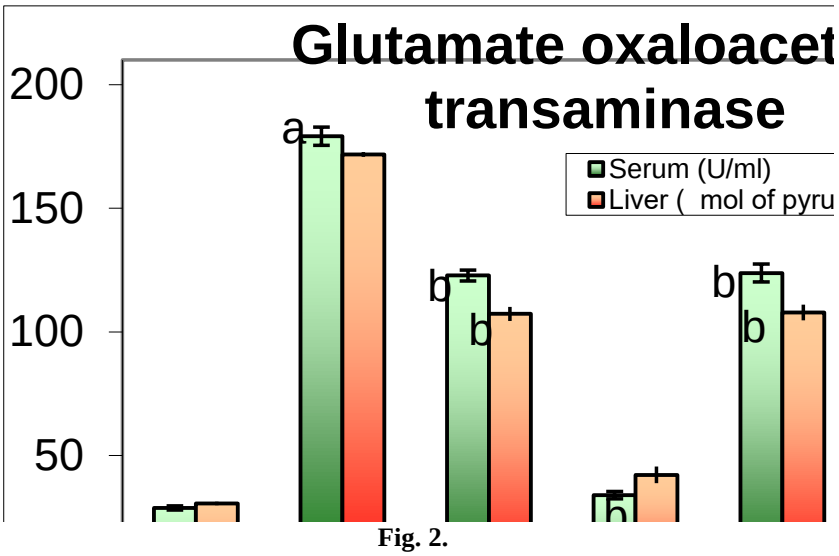

Fig. 2.

Table 5: Glutamate Pyruvate Transaminase (GPT/ALT).

Group	I	II	III	IV	V	VI	VII
Serum (U/ml)	16.66 ±0.61	124.33 ±2.16[a]	74.33 ±2.66[b]	24.66 ±0.71[b]	65.66 ±2.90[b]	22.33 ±0.80[b]	19.66 ±1.20[b]
Liver (µmol of pyruvate liberated/mg protein/min)	14.83 ±0.40	99.333 ±1.12[a]	67.5 ±1.12[b]	24.83 ±1.28[b]	73.33 ±2.16[b]	19.16 ±0.98[b]	18.5 ±1.18[b]

P values – [a] < 0.001 vs group I, [b]< 0.001 vs group II.
Values are mean ± SE of 6 animals in each group.

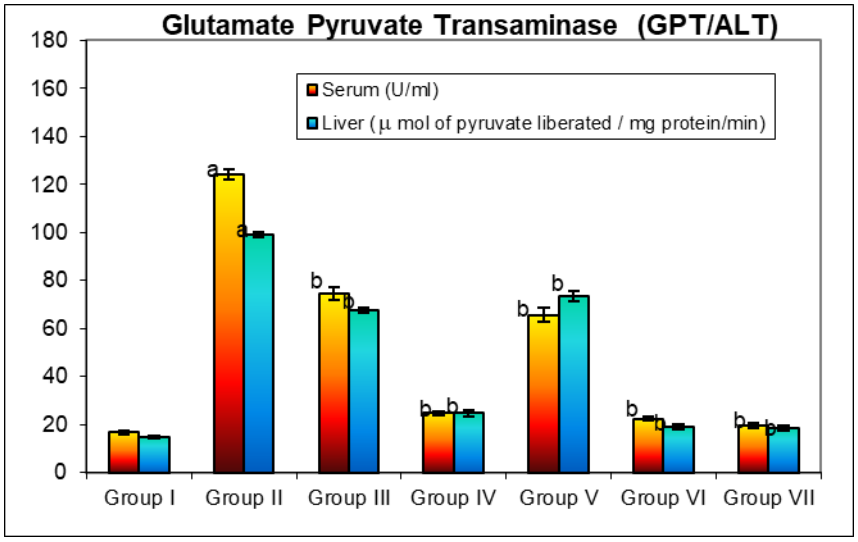

Fig. 3.

Table 6: Alkaline phosphatase (ALP).

Group	I	II	III	IV	V	VI	VII
Serum (KA units)	115.43 ±1.66	234.66 ±5.35[a]	208.21 ±3.01[b]	157.42 ±1.89[b]	202.54 ±4.92[b]	160.72 ±1.43[b]	146.74 ±2.36[b]

P values – [a] < 0.001 vs group I, [b]< 0.001 vs group II.

Values are mean ± SE of 6 animals in each group.

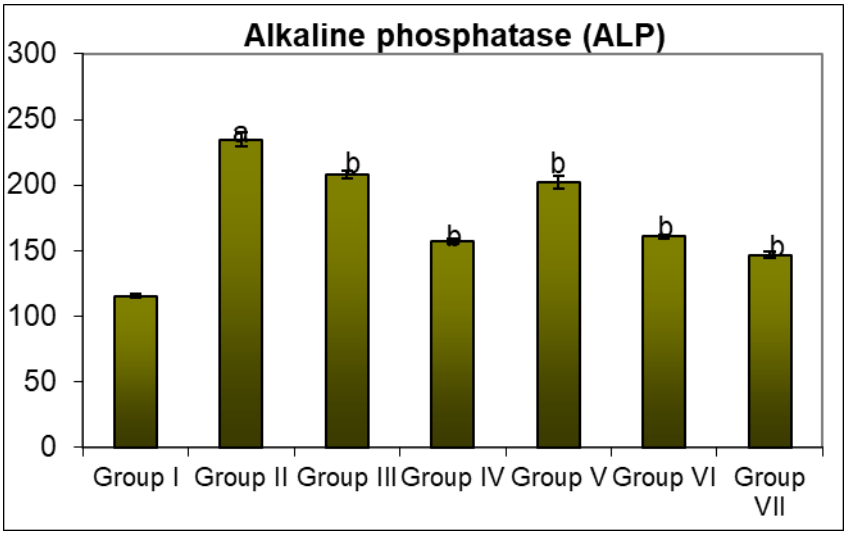

Fig. 4.

Table 7: Total bilirubin (mg/dL)

Group	I	II	III	IV	V	VI	VII
Serum	0.3073 ±0.01	1.9196 ±0.13[a]	0.832 ±0.04[b]	0.624 ±0.01[b]	0.820 ±0.10[b]	0.597 ±0.10[b]	0.493 ±0.01[b]

P values – [a] < 0.001 vs group I, [b]< 0.001 vs group II.

Values are mean ± SE of 6 animals in each group.

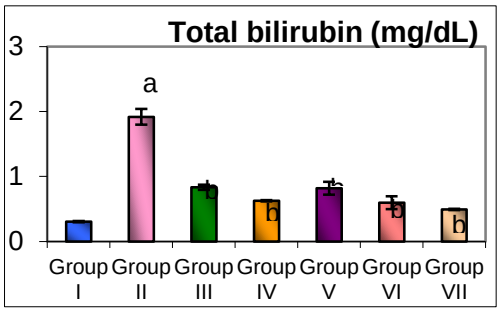

Fig. 5.

Table 8: Total Protein.

Group	I	II	III	IV	V	VI	VII
Serum (mg/dL)	7.854 ± 0.3	5.12 ±0.02[a]	5.506 ±0.02[b]	6.849 ±0.03[b]	5.736 ±0.04[b]	6.691 ±0.13[b]	7.376 ±0.050[b]
Liver (mg/g tissue)	0.7817 ±0.001	0.5164 ±0.004[a]	0.5748 ±0.003[b]	0.6529 ±0.002[b]	0.5963 ±0.015[b]	0.6963 ±0.004[b]	0.7290 ±0.004[b]

P values – [a] < 0.001 vs group I, [b]< 0.001 vs group II.

Values are mean ± SE of 6 animals in each group.

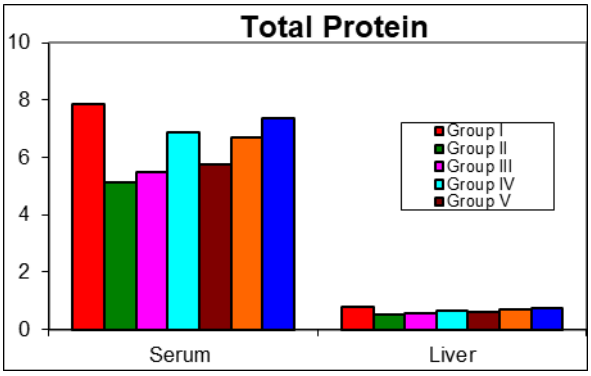

Fig. 6.

Table 9: Glutathione peroxidase (gpx) (n moles of gsh oxidised/min/mg protein).

Group	I	II	III	IV	V	VI	VII
Liver	314.11 ±6.06	190.85 ±4.941	238.44 ±10.063[b]	270.44 ±5.069[b]	214.38 ±7.896[b]	269.21 ±6.839[b]	295.12 ±5.796[b]

P values – [a] < 0.001 vs group I, [b]< 0.001 vs group II.

Values are mean ± SE of 6 animals in each group.

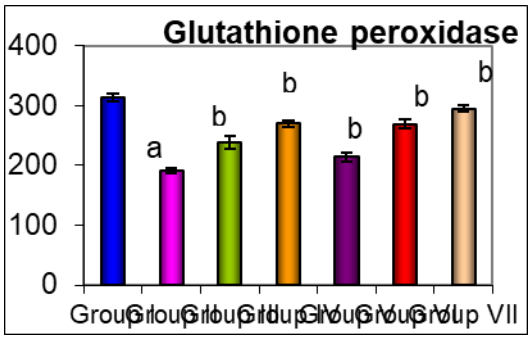

Fig. 7.

Table 10: Glutathione-S-transferase (GST) (n moles of CDNB conjugate formed/min/mg protein).

Group	I	II	III	IV	V	VI	VII
Liver	314.11 ±6.06	190.85 ±4.941	238.44 ±10.063[b]	270.44 ±5.069[b]	214.38 ±7.896[c]	269.21 ±6.839[b]	295.12 ±5.796[b]

P values – [a] < 0.001 vs group I, [b]< 0.001, [c]< 0.01 vs group II.

Values are mean ± SE of 6 animals in each group.

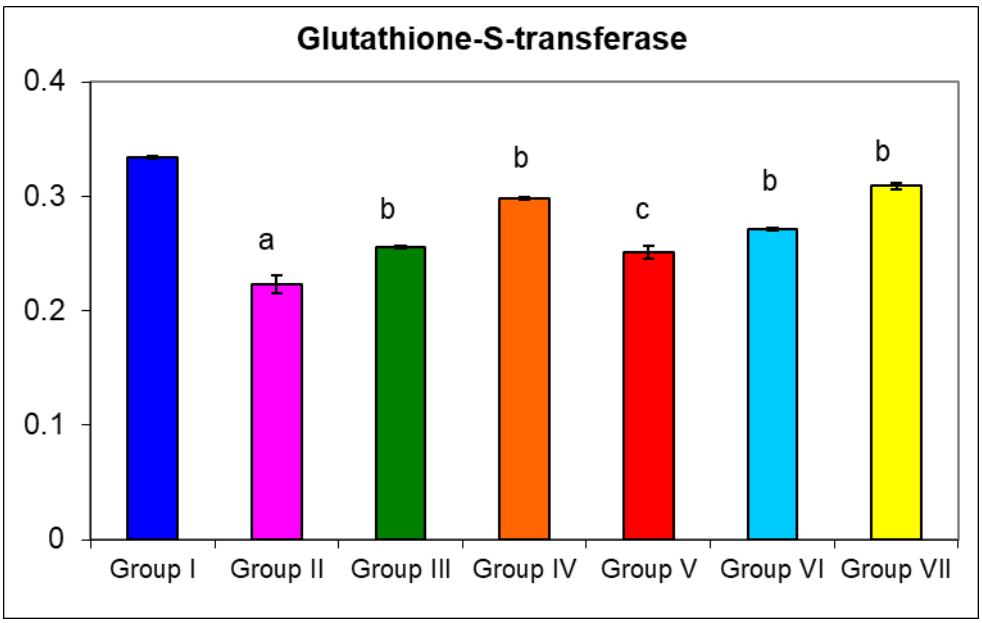

Fig. 8.

Table 11: Glutathione reductase (GRD) (n moles of GSSG utilised/min/mg protein).

Group	I	II	III	IV	V	VI	VII
Liver	25.56 ±0.348	14.38 ±0.335	16.16 ±0.187[b]	18.54 ±0.120[b]	15.99 ±0.353[b]	18.29 ±0.239[b]	20.36 ±0.295[b]

P values – [a] < 0.001 vs group I, [b]< 0.001 vs group II.

Values are mean ± SE of 6 animals in each group.

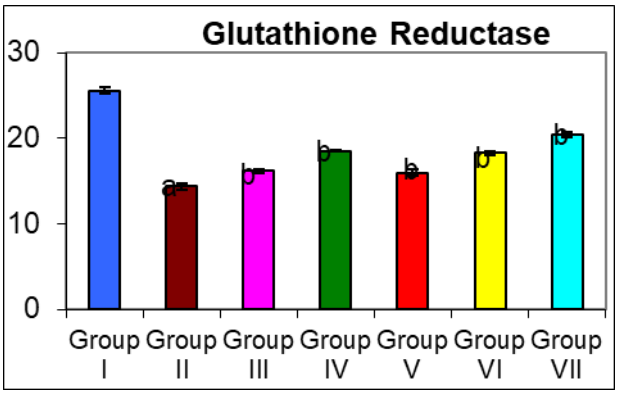

Fig. 9.

Table 12: Superoxide dismutase (SOD) (Kat/g protein).

Group	I	II	III	IV	V	VI	VII
Liver	9.62 ±0.261	5.43 ±0.399[a]	6.79 ±0.229[c]	7.50 ±0.227[b]	5.90 ±0.483[NS]	7.24 ±0.289[b]	8.56 ±0.156[b]

P values – [a] < 0.001 vs group I, [b]< 0.001, c< 0.01 vs group II, NS-non significant.

Values are mean ± SE of 6 animals in each group.

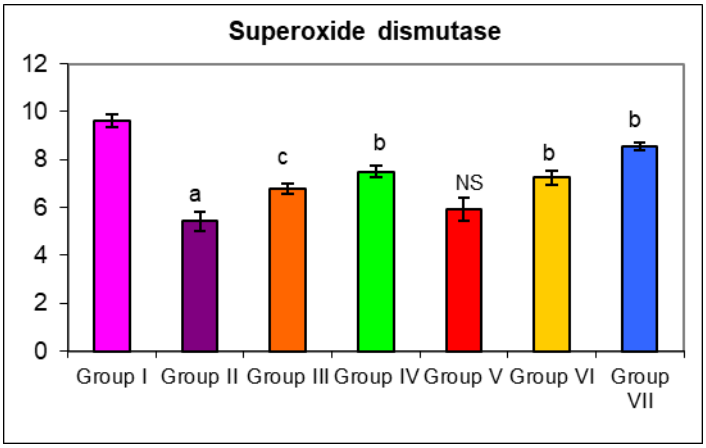

Fig. 10.

Table 13: Catalase (CAT) (n moles of H_2O_2 decomposed / min / mg protein).

Group	I	II	III	IV	V	VI	VII
Liver	77.51 ±0.257	52.22 ±0.272[a]	60.68 ±0.579[b]	69.69 ±0.410[b]	60.71 ±1.545[b]	69.37 ±0.452[b]	74.29 ±0.389[b]

P values – [a] < 0.001 vs group I, [b]< 0.001 vs group II

Values are mean ± SE of 6 animals in each group.

Table 14: Lipid peroxide (LPO) (n moles of MDA / mg protein).

Group	I	II	III	IV	V	VI	VII
Liver	5.11 ±0.595	13.99 ±0.875[a]	10.74 ±0.488[b]	7.91 ±0.430[b]	11.18 ±0.349[c]	7.49 ±0.432[b]	6.62 ±0.536[b]

P values – [a] < 0.001 vs group I, [b]< 0.001, [c]< 0.01 vs group II.

Values are mean ± SE of 6 animals in each group.

Table 15: Vitamin E (n moles / g wet tissue).

Group	I	II	III	IV	V	VI	VII
Liver	3.51 ±0.029	1.65 ±0.042[a]	1.89 ±0.023[b]	2.19 ±0.017[b]	1.88 ±0.021[b]	2.29 ±0.026[b]	2.63 ±0.017[b]

P values – [a] < 0.001 vs group I, [b]< 0.001 vs group II.

Values are mean ± SE of 6 animals in each group.

4.4. RESULTS AND DISCUSSION

Acute toxicity studies revealed that both extracts are relatively nontoxic up to 2000 mg/kg/ body weight/ p.o indirectly pronouncing the safety profile of extracts. Chronic toxicity studies should be instituted to rule out the toxicity profiles, if any on long time treatment.

The present study has demonstrated the heparotective activity of aqueous and alcoholic extracts of the given herbal extract in CCl_4 induced liver injury in rats.

Damage to the structural integrity of liver is reflected by an increase in the levels of serum transminase because these are cytoplasmic in location and released into cirulation after cellular damage. It is generally accepted that hepatotoxicity of carbontetrachloride is attributes to trichlormethly free radical, and this free radical reacts rapidly with oxygen to form a trichloromethylperoxy radical, which may contribute to the hepatotoxicity and subsequent increase in hepatic enzymes. In this context we have observed a rise in the levels of GOT and GPT in carbon tetrachloride treated rats due to toxic compounds affecting liver and the reversal of the elevated enzymatic levels to normal after the treatment with both aqueous and alcoholic extract of given herbal mixture.

Alkaline phosphatase (ALP) is a membrane bound glycoprotein enzyme, with high concentration is sinusoids and endothelium. ALP reaches the liver mainly from bone. It is excreted into the bile so its elevation in serum occurs in hepatobiliary diseases. The aqueous and alcoholic extracts of the given herbal powder probably stabilize the hepatic plasma membrane from carbontetrachloride induced liver damage.The liver is known to play a significant role in the serumprotein synthesis, being the source of plasma albumin and fibrinogen and also the other important components like α, β and γ globulin.

The serum albumin level is low in hepatic diseases. The present results reveal that when animals pretreated with given herbal powder extracts prior to the challenge with CCI_4, the liver biosynthesis of protein continues to be unaffected.
The metabolic transformation of aminoacids occurs in liver by, transamination. Protein metabolism may be impaired due the escape of both non proteins and protein nitrogenous substances from injured liver cells as evidenced by raise in the serum enzyme levels of GOT, GPT and ALP. Extracts of herbal powder, may avert enzyme leakage in tissue response to CCl_4 poisoning leading to enhanced metabolic transformation of aminoacids in liver through synthesis and transmination.

Bilirubin, an endogenous organic anion binds reversibly to albumin is transported to the liver, and then conjugated with glucuronic acid and excreted in the bile. Hepatobiliary disease or hepatic injury is indicated when conjugated fraction of total bilirubin exceeds the upper limit of normal, even if the total serum bilirubin is normal or near normal.

Glutathione peroxidase (GPx) plays a pivotal role in H_2O_2 catabolism and the detoxification of endogenous metabolic peroxides and hydroperoxides, which catalyses GSH. GPx activity was significantly reduced in the CCl_4 treatment when compared to control. The reversal of the GPx activity to normal after pretreatment with the plant extracts exhibits the antioxidant activity of the extracts in scavenging/detoxifying the endogenous metabolic peroxides generated after CCl_4 injury in the tissues.

Many investigators have suggested that Glutathione-S-transferase (GST) offers protection against LPO by promoting the conjugation of toxic electrophiles with reduced glutathione (GSH).

GST plays a physiological role in initiating the detoxification of potential alkalating agents. Chemicals like chloroform, CCl_4 etc. alter the hepatic Glutathione-S-transferase activity.

GST level was significnatly reduced in CCl_4 treated animals and upward reversal was observed after the treatment with aqueous and alcoholic extracts of the plant. This may be attributed to a direct action of the extract on the hepatic GST activation, the mechanism of which is not known.

GRD implies that there is an attempt to protect the liver tissue from oxidative damage by regenerating GSH from its oxidized form (GSSG). The present study reveals the extract along with other protective mechanism also increase the auto protection of the liver function by the GR level.

Enzymatic antioxidants, superoxide dismutase, catalase, glutathione reductase synergestically defence against reactive oxygen species. The steady state level of superoxide, catalase and glutathione peroxidase are involved in removal of H_2O_2. Glutathione-S-transferase enhances the detoxificaiton of electrophilic and lipophilic compounds through conjugation with GSH and forming GSH conjugate.

In the present study the superoxide dismutase activity is significantly reduced in CCl_4 intoxicated rats. The SOD activity was brought to near normal after treatment with the extracts in CCl_4 intoxicated rats.

Decreased activity of catalase was observed in group II animals treated with CCl_4. Presumably a decrease in catalase activity could be attributed to cross linking and inactivation of the enzyme protein in the lipid peoxides. Decreased catalase activity is linked up to exhaustion of the enzyme as a result of oxidative stress caused by CCl_4. The catalase activity was restored to normal after treatment with extracts evidently show that antioxidative property of the extracts against OFR (Oxygen Free Radical).

Carbon tetrachloride treatment may elevate the level of malondialdehyde (MDA) a product of lipid peroxidation. Therefore an increase in the liver MDA level indicates an increase in the degree of lipid peroxidation, a well known biochemical mechanism of liver damage. In addition, the extensive lipid peroxidation results in membrane disorganization by peroxidizing the highly unsaturated fatty acids leading to a decrease in the membrane fluidity, which may be sufficient to cause cell death. A significant decrease in the levels of lipid peroxides in aqueous and alcoholics extracts of the herbal powder extracts pre-treated rats suggests that the extract may have the ability to protect the liver from free radical injury induced by carbon tetrachloride.

The level of Vitamin E was significantly depleted in carbon tetrachloride intoxicated rats. This depletion may be due to the excessive utilization of non-enzymatic antioxidants (vitamin E) involved in quenching the enormous free radicals produced during carbon tetrachloride intoxification. The herbal powder aqueous and alcoholic extracts pre treated rats showed an improvement in the levels of vitamin E. The levels of GSH also found to have increased in positive relation with vitamin E i.e., GSH may maintain vitamin E level either by direct reduction α-tocopheroxyl radical to vitamin E, hence the improvement in the level of vitamin E in oxidative stress induced by carbon tetrachloride.

The hepatoprotective nature of aqueous and alcoholic extracts of herbal powder containing (Aegle marmelos Corr root extracts) against CCl_4 induced hepatic oxidative stress may be attributed to the presence of phenolic compounds.

Further studies are required to isolate the active principles present in the herbal powder and evaluating its pharmacological activity which may give potential hepatoprotective drug molecule.

5.0. CONCLUSION

Hepatotoxicity is a chronic clinical syndrome, little is talked about in aspects of prevention and curation, but rather management, there is increased focus on herbal medicines in the search for appropriate hepatoprotective and hepatotoxic agents. Many medicinal plants are effective in hepatoprotective activity; acute toxicity study helps to improve acceptability and authenticity of the herbal plants. Herbal formulation consists of plant origin ingredients, which are individually used traditionally in the treatment of hepatotoxicity. Each plant act by different mechanism of action to treat disease.

On the basis of above results it could be concluded that extract of herbal powder formulation exerts significant hepatoprotective and anti-oxidant activities.

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