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EVALUATION OF HEPATOPROTECTIVE AND ANTI-OXIDANT ACTIVITY OF ROOT EXTRACTS OF *AEGLE MARMELOS* IN CARBON TETRACHLORIDE INDUCED HEPATOTOXICITY WISTAR ALBINO RATS

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ABSTRACT: Herbal medicines are effective in the treatment of various ailments. Very often these drugs are unscientifically exploited and / or improperly used. Therefore 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. The present study has made an attempt to demonstrate the hepatoprotective activity of aqueous and alcoholic root extracts containing and in protecting the CCl_4 induced hepatocellular damage. The aqueous root extract of *Aegle marmelos* showed the presence of carbohydrates, phenols, flavonoids, glycosides, saponins and terpens, proteins and steroids. Significant amount of steroids were present. The alcoholic root extract of *Aegle marmelos* showed the presence of alkaloids, carbohydrates, phenols, flavonoids, glycosides, saponins and terpens, proteins and steroids. The aqueous and alcoholic extract of given root extracts 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.

INTRODUCTION: Ayurveda is the most ancient traditional system of medicine in India with sound philosophical, experiential and experimental basis which is used to treat the human disease [1].

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Due to increased side effects, high cost of new drugs, lack of curative treatment at root level and development of new diseases, people not only in developed countries but also in developing countries rely on herbal medicines [2]. India has 2.4% of world's area with 8% of global biodiversity; more than 1.5 million practitioners are using herbs or herbal formulations based on 25000 medicinally important plants, for the effective management and the treatment of human diseases [3]. Though scientific studies have been carried out by

scientists on many Indian botanicals, but still numerous drugs have entered the international market though the exploration of ethno-pharmacology and traditional medicine [4].

Medicinal plants play a key role in the human health care. About 80% of the world population relies on the use of traditional medicine which is predominantly based on plant materials [5]. The traditional medicine refers to a broad range of ancient natural health care practices including folk / tribal practices as well as Ayurveda, Siddha, and Unani. These medical practices originated from time immemorial and developed gradually, to a large extent, by relying or based on practical experiences without significant references to modern scientific principles. These practices incorporated ancient beliefs and were passed on from one generation to another by oral tradition and / or guarded literature. Although 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.

It is estimated that about 7,500 plants are used in local health traditions in, mostly, rural and tribal villages of India. Out of these, the real known or hitherto unknown to the main stream population. The classical systems of medicine such as Ayurveda, Siddha, Unani and Tibetan use about 1,200 plants [6]. A 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 dreaded diseases. Random screening of plants has not proved economically effective [7].

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.

Plant Profile: *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 times [8].

Synonyms: Bilva, Bael, Maredu.

Vernacular names:

- **English:** Bengal quince, Beal fruit, Golden apple, Indian.
- **Tamil:** 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, 4-10cm long, 2-5cm wide, the terminal one with a long petiole [9].

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.

Tree Management: The tree has no exacting cultural requirements, doing well with a minimum of fertilizer and irrigation. The spacing in orchards 6-9 m between trees. Seedlings begin to bael in 6 to 7 years, vegetatively propagated trees in 5 years. Full production is reached in 15 years. Normally, the fruit is harvested when yellowish-green and kept for 8 days while it loses its green tint. Then the stem readily separates from the fruit. A tree may yield as many as 800 fruits in a season.

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.

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.

MATERIALS AND METHODS:

Plant Collection and Identification: For the present investigation *Aegle marmelos* was collected from an area of Mahabubabad, District 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 masses 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 soxhlet 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 aqueous extracts 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: The experimental protocol was approved by the institutional Animal ethics committee (IAEC). Reference no: 1505/PO/a/14CPCSEA 2014.

Healthy wistar 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 °C) and humidity (60-65%) within 12 hours light 12 hr dark cycle. They were acclimatized 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 animal ethical committee studies. The animals were randomly distributed into seven groups with six animals in each group.

Preliminary Phytochemical Screening: The basic herbal powder is subjected to preliminary chemical screening for their presence or absence of active phytochemical constituents.

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 somato-motor 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 [10-11].

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 5 days and carbon tetrachloride (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 silymarin (200mg/kg body weight) 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 5th 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.

The following Bio-chemical assays were carried out in serum like Serum glutamic oxaloacetate transaminase (SGOT), Serum glutamic pyruvate transaminase (SGPT), alkaline phsohatase (ALP), Total bilirubin and Total protein. The supernatant liver homogenate was used for the assay of the following enzymes and non-enzymes. All the enzyme assays were undertaken at particular nm using shimadzu spectrophotometer, UV-1601 model [12].

Assay of Glutamate Oxaloacetate Transaminase (GOT): The enzyme activity was assayed in serum and liver homogenate using a GOT test kit based on the Reitman and Frankel method [29].

Assay of Glutamate - Pyruvate Transaminase (GPT): The enzyme activity was assayed in serum and liver homogenate using a GPT test kit based on the Reitman and Frakel method [29].

Assay of Alkaline Phosphatase (ALP): Alkaline phosphatase was assayed serum by using a test kit based on kind and King's method [13-14].

Assay of Total Bilirubin: Total bilirubin of the serum was assayed by using a test kit based on the Malloy and Evelyn method [15-16].

Assay of Total Protein: Total protein of both the serum and liver homogenate was assayed by using a test kit based on Biuret method [17].

Assay of Glutathionate Peroxidase (GPx): Glutathione peroxidase of liver homogenate was assayed according to the method Necheles *et al*.

Procedure: The final incubation mixture consisted of 0.2ml liver homogenate, 1.0ml of phosphate buffer, 0.5ml; reduced glutathione, 0.5ml sodium azide, 0.5ml EDTA and 2.0ml of distilled water. The solution was incubated at 37 °C for 5 minutes and the reaction was started by the addition of 1.0 ml 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 [18].

Assay of Glutathione- s- Transferases: Glutathione –s transferases of liver homogenate was assayed by the method of Habig *et al*.

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 [19].

Assay of Glutathione Reductase: Glutathione reductase activity of liver homogenate was measured by the method of Dubler *et al*.

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 [20].

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

Procedure: To 0.05 ml of liver homogenate, 1.5 ml of buffer was added. The reaction was initiated by the addition of 0.4ml 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 [21].

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

Procedure: To 1.2ml of phosphate buffer 0.1ml of the liver homogenate was added. The enzyme reaction was started by the addition of 1.0ml 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 [22].

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

Procedure: To 0.2ml of liver homogenate, 1.5ml of 20% acetic acid. 0.2ml of sodium dodecyl sulphate and 1.5ml of thiobarbituric acid were added. The mixture was made up to 4.0ml with distilled water and then heated for 30 min 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 for 10 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 [23].

Vitamin E: Vitamin E is determined by the method of Desai.

Procedure: To 1.5ml of liver homogenate, 1.5ml ethanol and 1.5ml xylene are added, they are centrifuged. 1.0ml xylene layer is separated out and mixed with dipyridyl reagent, mixed well. Aliquot 1.5ml was taken and reading at 460 nm, add 0.33ml $Fecl_3$ regent. Reading was taken 520 nm exactly after 1.5 mins. Vitamin E concentration was expressed as n moles / g wet tissue in liver homogenate.

Histopathological Studies: Small pieces of liver tissues were collected in 10% formalin solution for preparation of sections by using of microtome. The histopathological studies were carried out by the method described by the Kanai L Mukherjee [24].

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.

RESULTS:

Preliminary Phytochemical Screening: Table 1 depicts the results of preliminary phytochemical screening of the aqueous and alcoholic root extracts of the *Aegle marmelos* (L) Correa. The aqueous extract showed the presence of carbohydrates, steroids, terpenoids, flavonoids, saponins, phenol, glycosides, and proteins and reduced sugar. Alkaloids, gums and mucilages and tannins were absent. The alcohol extract showed the presence of alkaloids, carbohydrates, phenols, steroids, terpenoids, tannins, proteins, flavonoids and reduced sugar. Saponins, glycosides, gums and mucilages were absent.

Acute Oral Toxicity Study: The acute oral toxicity study was done according to the OECD guideline 423 (Acute toxic class method) and the results are shown in **Table 1**. 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 were no significant changes in body weight before and after termination of the experiment and no signs of toxicity were observed. The experiment was terminated on 14th day. The experiments were repeated again with the same dose level, 2000 mg / kg p.o. of aqueous and alcoholic extract of the given extracts 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).

TABLE 1: ACUTE ORAL TOXICITY STUDY

Group	Dose	Weight of animal (g)		Signs of toxicity	Onset of Toxicity	Reversible/ irreversible	Duration of observation
		Before test	After Test (on 4th day)				
T1	2000mg/kg	160	158	No	Nil	Nil	3 days
T2	2000mg/kg	168	164	No	Nil	Nil	3 days
T3	2000mg/kg	164	160	No	Nil	Nil	3 days
T4	2000mg/kg	145	144	No	Nil	Nil	3 days
T5	2000mg/kg	148	145	No	Nil	Nil	3 days
T6	2000mg/kg	142	142	No	Nil	Nil	3 days

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. There was a significant decrease in liver wet weight of animals treated with 200mg/kg and 400mg/kg of aqueous and alcoholic extracts ($p < 0.001$) respectively, when compared with group II. The dose of 400mg/kg of aqueous extract showed significant decrease in liver wet weight when compared with groups III, V, VI and significant increase in liver wet weight when compared with group VII.

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. A dose dependent reduction of ($p < 0.001$) GOT and GPT levels were observed in animals treated aqueous (200mg/kg and 400 mg/kg) and alcoholic extracts (200mg/kg and 400 mg/kg) when compared to group II. Silymarin (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.

TABLE 2: WEIGHT OF LIVER (MG/G BODY WEIGHT)

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

Note: 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 3: GLUTAMATE OXALOCETATE TRANSAMINASE (GOT/AST)

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

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

TABLE 4: GLUTAMATE PYRUVATE TRANSAMINASE (GPT/ALT)

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

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

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). Treatment with aqueous (200mg/kg and 400mg/kg) and alcoholic (200 mg/kg and 400mg/kg) extracts showed a significant ($p < 0.001$) reduction in ALP level when compared to group II animals. The Silymarin (1ml/kg) treated group also showed a significant decline of ALP ($P < 0.001$) when compared to group II animals.

TABLE 5: ALKALINE PHOSPHATASE (ALP)

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

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

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. Aqueous (200mg/kg and 400mg/kg) and alcoholic (200mg/kg and 400mg/kg) extracts of given herbal extracts treated rats showed significant decrease ($p < 0.001$) of total bilirubin when to CCl_4 treated rats. The Silymarin (1mg/kg) treated animals (group VII) also showed significant ($P < 0.001$) decrease of total bilirubin level when compared to group II animals.

TABLE 6: TOTAL BILIRUBIN (mg/dl)

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

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

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). Treatment with aqueous (200

mg/kg and 400mg/kg) and alcoholic (200mg/kg and 400mg/kg) extracts showed a significant ($P < 0.001$) increase of protein level, when compared to (group II) animals. The Silymarin (1ml/kg) treated animals (group VII) also showed significant ($p < 0.001$) increase in protein level when compared to CCl_4 challenged (group II) rats.

TABLE 7: TOTAL PROTEIN

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

Note: P values – ^{a} < 0.001 vs group I, ^{b}< 0.001 vs group II. Values are mean SE of 6 animals in each group.

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). The aqueous extract (200mg/kg and 400mg/kg dose levels) significantly increased ($p < 0.001$) the GPx levels, when compared to group II. However alcoholic extract (200mg/kg and 400mg/kg) showed less significant increase ($p < 0.05$, $p < 0.001$ respectively) in GPx levels when compared to group II animals. Siltmarin (1ml/kg) treated animals also showed significant ($p < 0.001$) increase of GPx level in the liver homogenate compared with group II animals.

TABLE 8: GLUTATHIONE PEROXIDASE (GPX) (n MOLES OF GSH OXIDISED/min/mg PROTEIN)

Group	I	II	III	IV	V	VI	VII
Liver	314.11	190.85	238.44	270.44**	214.38	269.21*	295.12***
	6.06	4.941	10.063^{b}	5.069^{b}	7.896^{b}	6.839^{b}	5.796^{b}

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

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. Treatment with aqueous extract of the given herbal extracts at 200 mg/kg and 400mg/kg dose levels showed significant increase ($p < 0.001$) in GST level when compared to CCl_4 treated group. Alcoholic extracts (200mg/kg and 400mg/kg) also showed significant ($p < 0.01$, $p < 0.001$ respectively), increase of GST level in liver homogenate. Silymarin (1ml/kg) treated animals also showed significant ($p < 0.001$) increase of GST level when compared to group to group II.

TABLE 9: GLUTATHIONE-S-TRANSFERASE (GST) (n MOLES OF CDNB CONJUGATE FORMED/min/mg PROTEIN)

Group	I	II	III	IV	V	VI	VII
Liver	314.11	190.85	238.44	270.44**	214.38	269.21*	295.12***
	6.06	4.941	10.063^{b}	5.069^{b}	7.896^{b}	6.839^{b}	5.796^{b}

Note: 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.

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). The aqueous (200mg/kg and 400 mg/kg) and alcoholic (200mg/kg and 400mg/kg) extracts showed significant increase ($p < 0.0001$) in GRD level, when compared to group II animals. Silymarin (1 ml/kg) treated group also showed significant ($p < 0.001$) increase of GRD level when compared to group II.

TABLE 10: GLUTATHIONE REDUCTASE (GRD) (n MOLES OF GSSG UTILIZED /min/mg PROTEIN)

Group	I	II	III	IV	V	VI	VII
Liver	25.56	14.38	16.16	18.54**	15.99	18.29*	20.36***
	0.348	0.335	0.187^{b}	0.120^{b}	0.353^{b}	0.239^{b}	0.295^{b}

Note: P values – ^{a} < 0.001 vs group I, ^{b}< 0.001 vs group II. Values are mean SE of 6 animals in each group.

Superoxide Dismutase (SOD): Liver Superoxide dismutase level was significantly reduced ($p < 0.001$) in CCl_4 treated animals when compared with normal animals. The aqueous (200mg/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 200mg/kg did not show a significant increase in SOD level when compared with CCl_4 treated animals, but the 400mg/kg dose alcoholic extract treated animals showed significant ($p < 0.001$) increase of SOD level when compared with group II. Silymarin (1ml/kg) treated animals also showed significant ($p < 0.001$) increase of SOD level when compared to group II.

TABLE 11: SUPEROXIDE DISMUTASE (SOD) (kat / g PROTEIN)

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

Note: 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.

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

TABLE 12: CATALASE (CAT) (n MOLES OF H_2O_2 DECOMPOSED / min / mg PROTEIN)

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

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

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). Treatment with aqueous (200mg/kg and 400mg/kg) and alcoholic (200mg/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 Silymarin (1ml/kg) treated group also showed a significant ($p < 0.001$) decline in the LPO level when compared to group II animals.

TABLE 13: LIPID PEROXIDE (LPO) (n MOLES OF mda / mg PROTEIN)

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

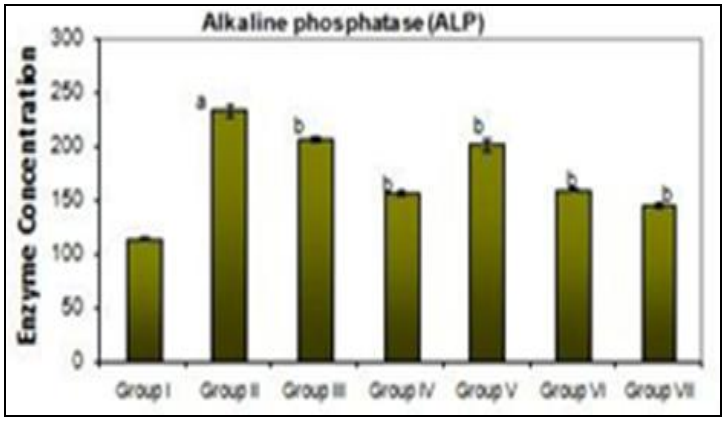
Note: P values – [a] < 0.001 vs group I, [b]< 0.001, [c]< 0.01 vs group II. Values are mean SE of 6 animals in each group.

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). The aqueous (200mg/kg and 400 mg/kg) and alcoholic (200mg/kg and 400mg/kg) extracts showed significant increase ($p < 0.001$) of Vitamin E level when compared to group II animals. Silymarin (1ml/kg) treated group also showed significant ($p < 0.001$) increase in Vitamin E level when compared to group II.

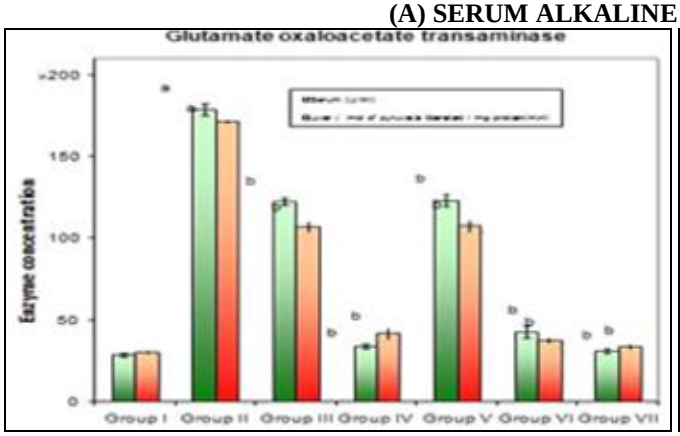
TABLE 14: VITAMIN E (n MOLES / g WET TISSUE)

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

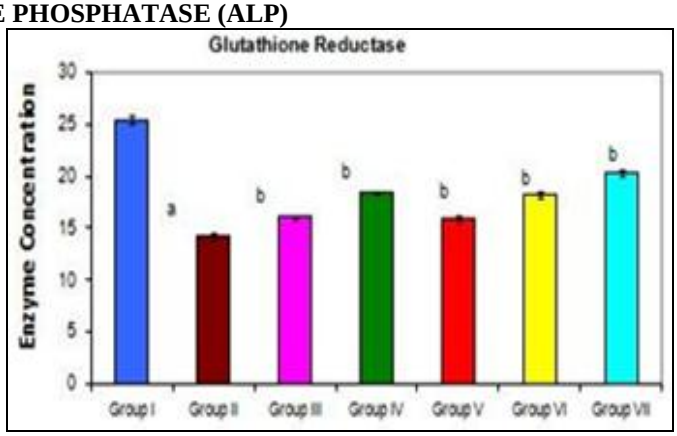
Note: P values – [a] < 0.001 *vs* group I, [b]< 0.001 vs group II. Values are mean SE of 6 animals in each group.



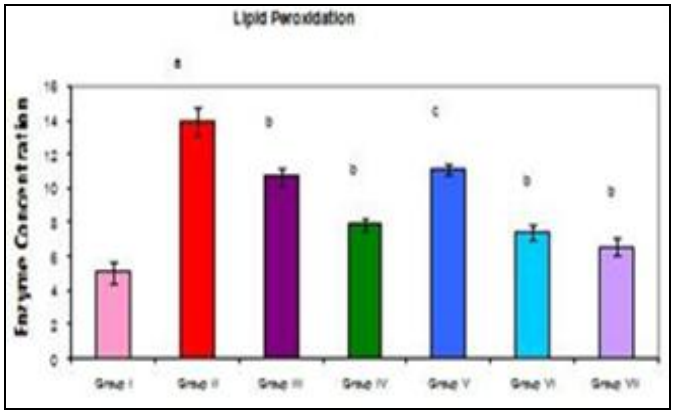
(A) SERUM ALKALINE PHOSPHATASE (ALP)



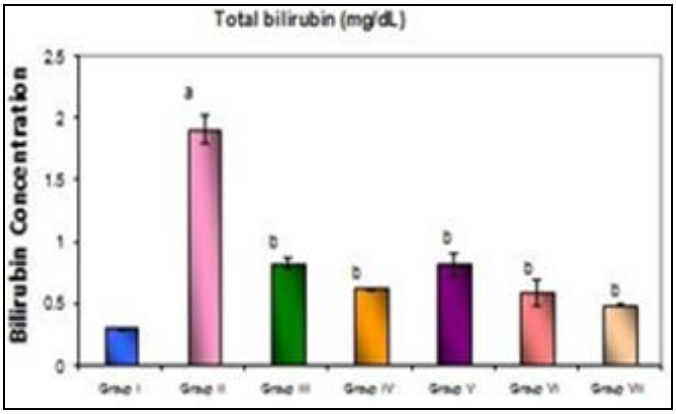
(B) Glutamate Oxalocetate Transaminase (GOT/AST)



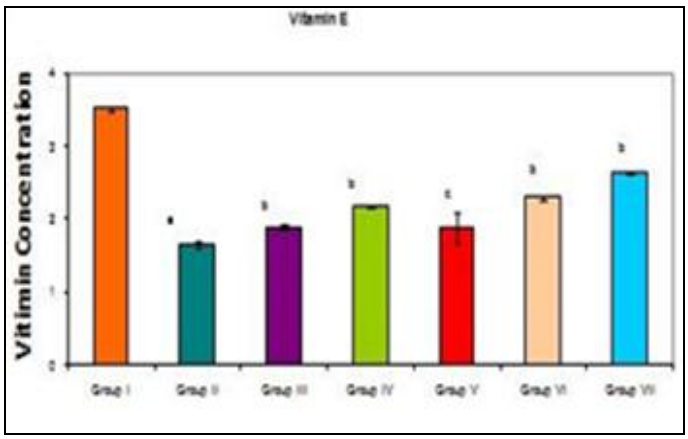
(C) Glutathione reductase (GRD)



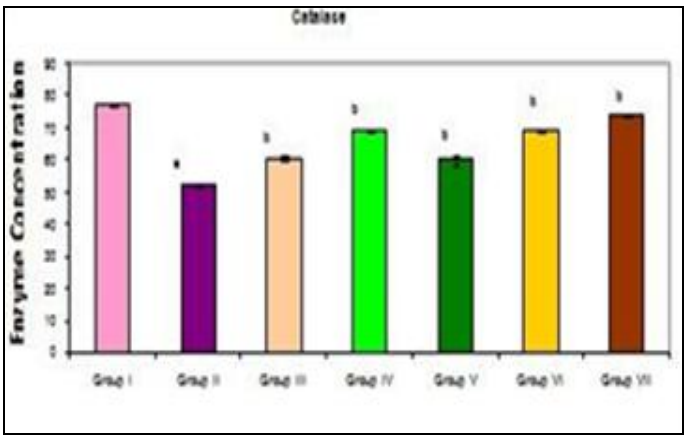
(D) Lipid Peroxidation (LPO)



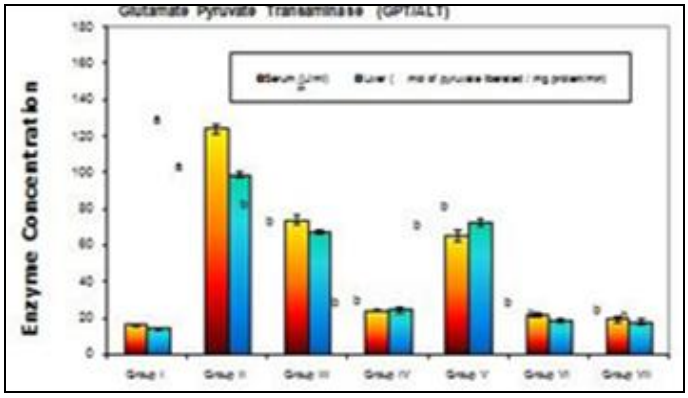
(E) Total bilirubin (mg/dl)



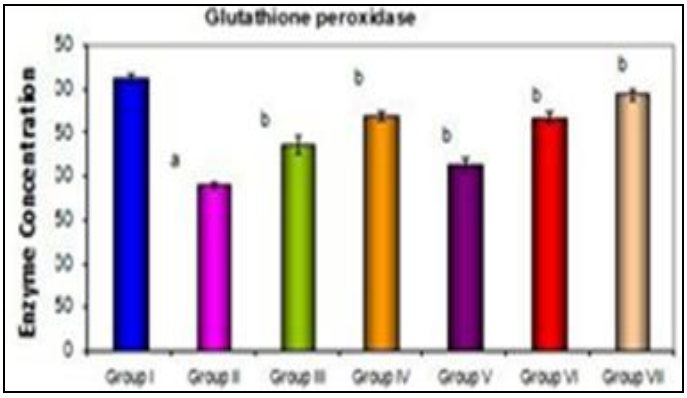
(F) Vitamin E



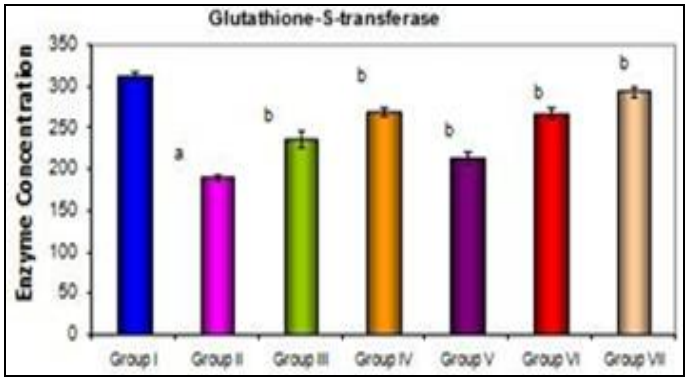
(G) Catalase (CAT)



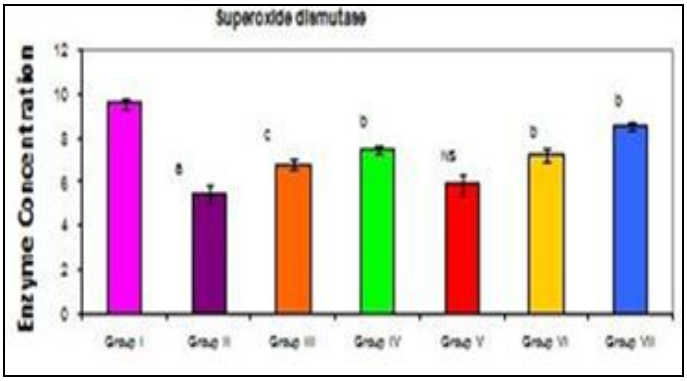
(H) Glutamate Pyruvate Transaminase (GPT)



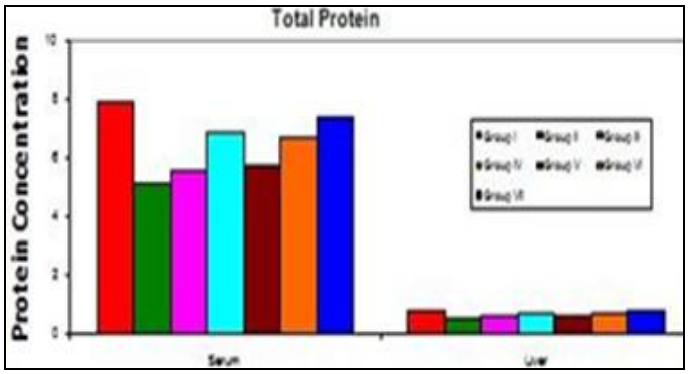
(I) Glutathione Perxidase (GPx)



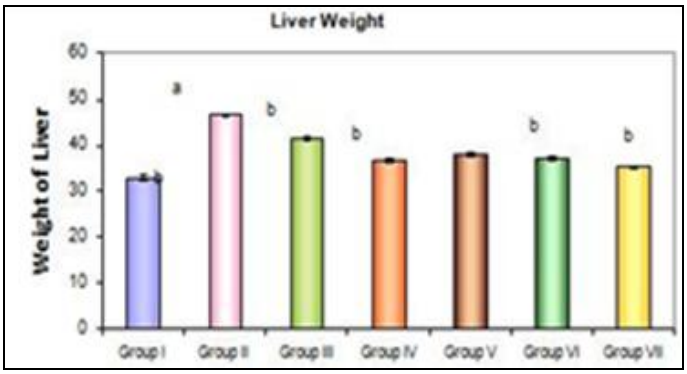
(J) Glutathione – S- transferase (GST)



(K) Superoxide dismutase (SOD)



(L) Total Protein



(M) Weight of liver

FIG. 1A-M: HEPATOPROTECTIVE STUDIES

Histopathological Studies: From the results of the histopathological studies, administration of aqueous extract and alcoholic root extracts of the *Aegle marmelos* reveals reduced cellular damage induced by CCl_4, the widely used hepatotoxicant. This result correlates well with the biochemical findings given above.

TABLE 15: HISTOPATHOLOGICAL STUDIES OF THE LIVER

S. No.	Groups	Observations
1.	Group I	Dilated central vein, normal hepatocytes.
2.	Group II	Perivenular inflammatory infiltration and hepatocytic fatty change, diffuse mild hepatocellular vacuolation
3.	Group III	Change central vein, mild fatty change
4.	Group IV	Dilated central vein. Mild sinusoidal dilation – No hepatocellular damage. (Near normal)
5.	Group V	Perilobular hepatocellular fatty change, (mild fatty change), Peripheral lobule
6.	Group VI	Sinusoidal dilation and peripheral hepatocytic fatty change
7.	Group VII	Normal central vein and mild hepatocytic fatty change

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 hepatoprotective 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 circulation 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 root extracts.

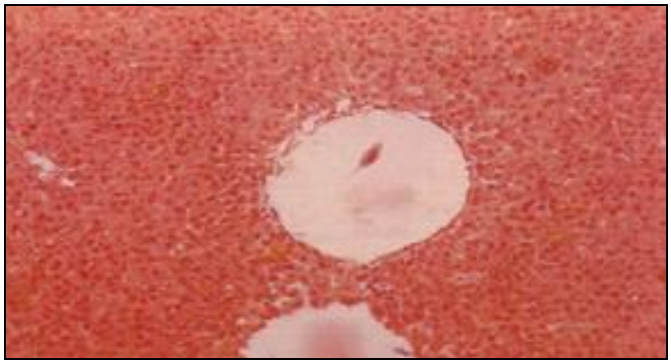


FIG. 2A: HISTOPATHOLOGIAL STUDY OF NORMAL ANIMAL (GROUP I)

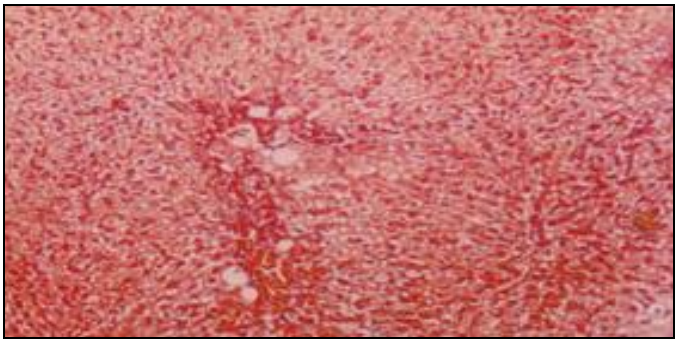


FIG. 2B: HISTOPATHOLOGIAL STUDY OF CCl_4 TOXIC ANIMAL (GROUP II)

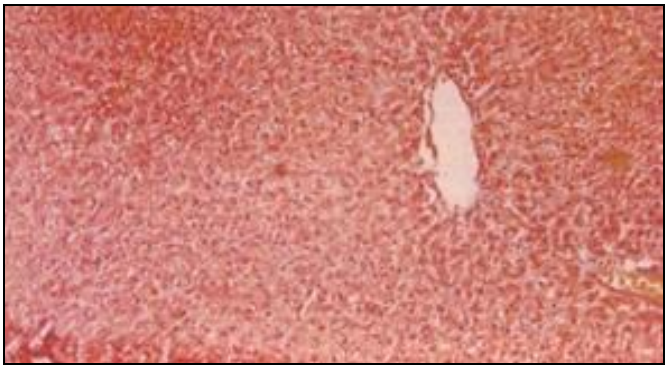


FIG. 2C: HISTOPATHOLOGIAL STUDY OF AQUEOUS EXTRACT 200mg/kg

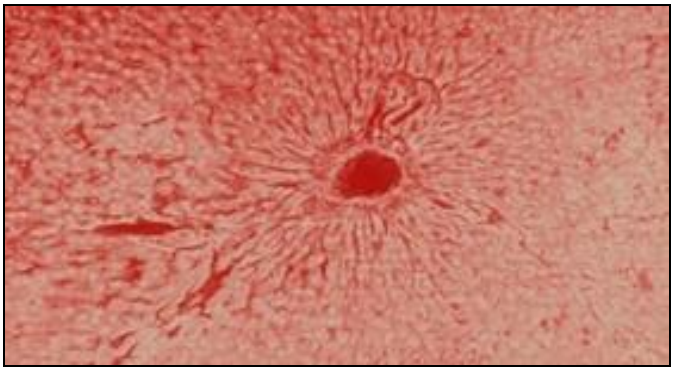


FIG. 2D: HISTOPATHOLOGIAL STUDY OF AQUEOUS EXTRACT 400 mg/kg AND CCl_4 TREATED ANIMAL (GROUP IV)

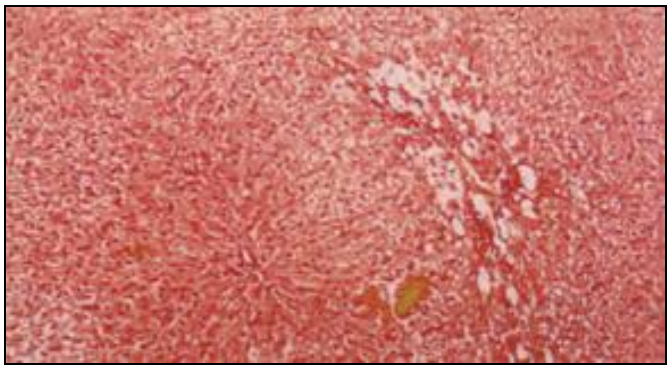


FIG. 2E: HISTOPATHOLOGIAL STUDY OF ALCOHOLIC EXTRACT 200 mg/kg AND CCl_4 TREATED ANIMAL (GROUP V)

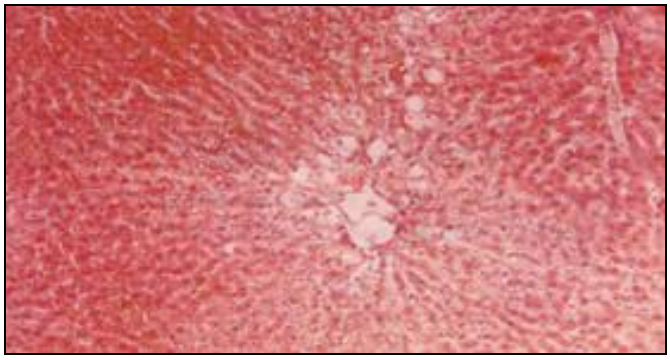


FIG. 2F: HISTOPATHOLOGIAL STUDY OF ALCOHOLIC EXTRACT 400 mg/kg AND CCl_4 TREATED ANIMAL (GROUP VI)

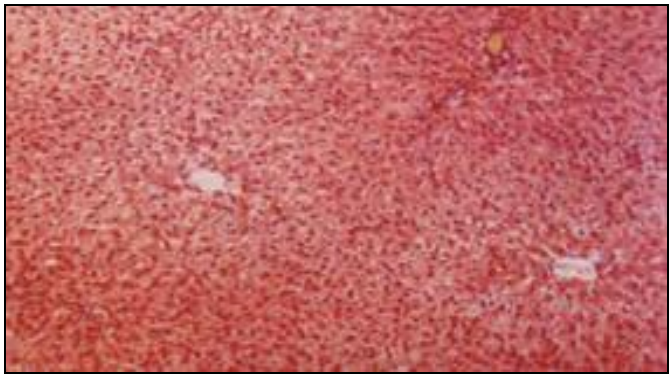


FIG. 2G: HISTOPATHOLOGIAL STUDY OF SILYMARIN (200mg)

Alkaline phosphatase (ALP) is a membrane bound glycoprotein enzyme, with high concentration in 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 root extracts probably stabilize the hepatic plasma membrane from carbon tetrachloride induced liver damage. The liver is known to play a significant role in the serum protein synthesis, being the source of plasma albumin and fibrinogen and also the other important components like, and globulin.

The serum albumin level is low in hepatic diseases. The present results reveal that when animals pre-treated with given aqueous and alcoholic root extracts prior to the challenge with CCI_4, the liver biosynthesis of protein continues to be unaffected. The metabolic transformation of amino acids 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 rise in the serum enzyme levels of GOT, GPT and ALP. Extracts of root powder, may avert enzyme leakage in tissue response to CCl_4 poisoning leading to enhanced metabolic transformation of amino acids 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 hydro-peroxides, 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 significantly 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 levels of superoxide, catalase and glutathione per-oxidase are involved in removal of H_2O_2. Glutathione-S-transferase enhances the detoxi-ficaiton 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 shows that anti-oxidative 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 per-oxidation, a well known biochemical mechanism of liver damage. In addition, the extensive lipid per-oxidation results in membrane disorganization by per-oxidizing 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 root 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 in toxification. The aqueous and alcoholic root 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 of 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.

Summary:

1. The aqueous root extract of *Aegle marmelos* showed the presence of carbohydrates, phenols, flavonoids, glycosides, saponins and terpens, proteins and steroids. Significant amount of steroids were present.

2. The alcoholic root extract of *Aegle marmelos* showed the presence of alkaloids, carbohydrates, phenols, flavonoids, glycosides, saponins and terpens, proteins and steroids. More significant amount of steroids, flavonoids, terpens were seen, moderate amount of phenols and alkaloids were seen.

3. The CCl_4 induced hepatotoxicity produced in rats leading to hepatic injury triggers the generation of toxic radicals which can be masked by using a correct antioxidant in adequate amount.

4. The presence of phenols, flavonoids, tannins, saponins and terpenoids in *Aegle marmelos* explain its role in hepato-protection by inhibiting the free radicals mediated damage, claimed that phenols, flavonoids, triterpens and tannin were antioxidant agent and may interfere with free radicals formation. The haemorrhage caused by CCl_4 in the liver was minimized by use of plant extract as flavonoids are known to be vasculo-protector.

5. The aqueous and alcoholic extract of given root extracts at two dose levels 200mg/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.

6. 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.

7. 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.

8. The present study has made an attempt to demonstrate the hepatoprotective activity of aqueous and alcoholic root extracts containing and in protecting the CCl_4 induced hepatocellular damage.

9. 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 root.

CONCLUSION: Using the animal model, we have shown that crude root powder of *Aegle marmelos* has potential to act against CCl_4 induced hepatic damage in albino rats. Moreover, the extent of hepatoprotective effect of the root powder is comparable to that of standard drug, Silymarin being used against hepato-toxicity, in general. This observation unambiguously suggests that the root powder of *Aegle marmelos* must contain lead compounds that may provide profound implications on designing de novo anti hepatic drugs.

Further working on identifying and elucidating the three-dimensional structures of a few lead compounds from the crude root powder may develop potential hepato-protective drugs. We strongly believe that the outcomes of the study will trigger exciting research on addressing liver diseases in a cost effective manner. It is quite evident from this work that *Aegle marmelos* is a divine tree which has taken an important place in Ayurveda, Unani, Siddha traditional system of medicine. The plant has various therapeutic applications due to its blessed presence of phyto-constituents. Almost all parts of the plant have been used for the treatment of various diseases.

Thus, upon conclusion, this work demonstrates the applications of the bioactive compounds of *A. marmelos* in a single roof, thereby paving way for the plant researchers to explore this plant for wider applications in the near future that might enable mankind to get maximum benefit from the Nature and Natural products.

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CONFLICT OF INTEREST: Nil.

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