

# Formulation and Evaluation of Poly Herbal Formulation against CCl<sub>4</sub> Induced Hepatotoxicity

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## ABSTRACT

Aegle marmelos L, Eclipta alba L. and Phyllanthus amarus are well known herbs as hepatoprotective agents. The preventive effects of polyherbal formulation containing the above herbs were evaluated against carbon tetrachloride (CCl<sub>4</sub>) induced hepatotoxicity in rats. CCl<sub>4</sub> induced fatty degeneration and vacuole formation and significantly increased the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in rat's plasma. Treatment with the **herbal** formulation significantly decreased the levels of AST and ALT in plasma. Also histopathological studies showed that the formulation reduced the incidence of liver lesions induced by CCl<sub>4</sub>. The study proved that the formulation prepared from *A. marmelos*, *P. amarus* and *E. alba* may be used in the treatment of CCl<sub>4</sub> induced hepatotoxicity.

**Keywords:** Poly Herbal Formulation, *A. marmelos*, *P. amarus* and *E. alba* Carbon Tetra Chloride, Hepatotoxicity.

## 1. INTRODUCTION

Liver and biliary diseases affect people of all ages and walks of life. At present, an estimated 5.5 millions of people (approximately 2 to 3 percent of adults) have chronic liver disease or cirrhosis, and more than 20 million (approximately 12 percent of adults) have gallbladder disease. The current burden of liver and biliary diseases calls for greater efforts in their prevention and control. Progress in controlling liver and biliary diseases depends largely on advances in understanding of these diseases through biomedical research. There is a need to develop poly herbal formulations to treat liver

diseases to limit the side effects and toxicity that the available treatments presently<sup>[1]</sup>. In traditional system of medicine, the drugs are primarily dispensed as water decoctions or ethanolic extracts, fresh plant parts, juices, or crude powder. Also, medicinal plant parts should be authenticated and may be free from microbial contamination. Aegle marmelos L (Rutaceae), Eclipta alba (L.) Hassk. (Asteraceae) and Phyllanthus amarus (Euphorbiaceae) are well known herbs as hepatoprotective agents. Carbon tetrachloride consistently causes liver toxicity, resulting in fatty degeneration, cellular necrosis, fibrosis and cirrhosis and induces hepatocellular carcinomas through various routes of administration (oral, inhalation, and parenteral exposure). Toxic effects of carbon tetrachloride in animals were also reported<sup>[2]</sup>. Mechanistic studies suggest following events in the carcinogenicity of carbon tetrachloride 1)

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formation of trichloromethyl radical and trichloromethyl peroxy radical by CYP2E1, 2) lipid peroxidation by trichloromethyl peroxy radical, 3) loss of calcium homeostasis leading to activation of degradative enzymes and cytotoxicity and 4) regenerative and proliferative changes in the liver in response to hepatotoxicity. So, the initial phase involves the metabolism of CCl<sub>4</sub> by cytochrome P450 to the trichloromethyl radicals, which lead to membrane lipid peroxidation and finally to cell necrosis. The second phase of CCl<sub>4</sub>-induced hepatotoxicity involves the activation of Kupffer cells, which is accompanied by the production of proinflammatory mediators<sup>[3]</sup>. Antioxidants and radical scavengers protect liver cells from CCl<sub>4</sub>-induced damage by breaking the chain reaction of lipid peroxidation<sup>[4]</sup>. Many plants and natural compounds showed protective effects against CCl<sub>4</sub> induced liver toxicity<sup>[5-7]</sup> and in many cases the protection effects were due to antioxidant properties<sup>[8-12]</sup>. Hepatoprotective activity was assessed by estimation of different parameters viz. morphological, biochemical and histopathological parameters.

## 2. MATERIALS AND METHODS <sup>[13-20]</sup>

Herbs such as *A. marmelos*, *P. amarus* and *E. alba* were collected from medicinal plant garden of Sree Vidyanikethan College of Pharmacy, A.Rangampet, Andhra Pradesh, India. These were duly authenticated by Dr. Yasodamma, Professor, Department of Botany, SV University, Tirupati, India.

### 2.1 Preparation of Plant Extract

The herbs (2 kg) were dried in shade, ground into coarse powder and macerated separately with 4 L of water-ethanol mixture (1:1). After 7 days of maceration, the extract was filtered and concentrated under vacuum using rotary vacuum evaporator. The residue obtained such as *P. amarus* extract (PAE, 19 % w/w), *A. marmelos* extract (AME, 19 % w/w) and *E. alba* extract (EAE, 19 % w/w) were kept in desiccators and used for the present study. The plant extracts were weighed and 20 gm of the each extract was dissolved in alcohol. These were cooled and concentrated to get 60 ml of alcoholic extract solution.

### 2.2 Preparation of Herbal Syrup

The syrup base was prepared as per Indian pharmacopoeia specifications. One gram of each extracts of *A. marmelos*, *P. amarus*, *E. alba* were dissolved in simple syrup base and the volume was made up to 100 ml. The materials used and quantity required for preparing the formulation is presented in Table (1).

### 2.3. Physical Evaluation of Formulated Herbal Syrup

Physical properties such as colour, odour, taste, specific gravity and pH were analyzed as per the standard procedures of Indian Pharmacopoeia. The pH was determined by using digital pH meter. One ml of poly herbal syrup was dissolved in 100 ml distilled water and stored for two hours. The measurement of pH of each formulation was done in triplicate and average values were calculated. The measurement of viscosity of the prepared poly herbal syrup was done by Brookfield DV-II+Pro viscometer.

### 2.4 Phytochemical Analysis

Preliminary phytochemical screening of the formulated syrup was carried out for the detection of various phytochemicals such as alkaloids, steroids, amino acids, tannins, carbohydrates, saponins, flavonoids, fixed oils and fats. Also the prepared extracts were subjected to thin layer chromatography (TLC). In this method, a thin layer of silica gel and slurry was coated on to the plates and is evenly distributed. The plates are then air dried. TLC Plates are activated (in hot air oven at 120°C for 15-20 minutes) after 5-10 minutes of their preparation. This served as stationary phase. The mobile phase used was Chloroform: Ethyl acetate: Toluene in the ratio 3.5: 6: 0.5.

### 2.5 In-vitro Anti-Microbial Evaluation<sup>[13,14]</sup>

Test for E.Coli: Take 1.3 gm of Macconkey agar and 100 ml of distilled water. Autoclave it for 15-20 min., pour it into a clean petri dish and allow it to solidify on cooling. Pour 1ml of prepared formulation and mix using rotating shaker and incubate at 35-37°C for 4 hours. Observe the microbial growth and formation of colonies.

Biochemical test: The colonies obtained on the Macconkey agar media are inoculated on peptone water

(5 ml) and the culture incubated for 24 hours at a temperature of 35-37°C. After the incubation period, 3 ml of peptone culture is pipetted into a test tube and equal volume of Kovac's reagent is added. Appearance of a pink ring is the indication of presence of E.Coli. Similarly parallel tests are conducted with pure stain.

Test for Staphylococcus aureus: Mix 1.3 gm of nutrient broth in 100 ml of distilled water, autoclave for 15-20 min. and pour in to a clean test tube. To this, pour 1 ml of test sample. This mixture is incubated at 35-37°C for 4 hours. 1 ml of the sample from this is plated on Baird-Parker agar media F, and incubated at 35-37°C for 48 hours. Black colonies of gram positive cocci, surrounded by clear zones indicate presence of S.aureus.

**Biochemical test:** Colonies obtained from Baird-Parker agar media are subjected to coagulase and DNAse test. In coagulate test, formation of coagulum after adding the Staphylococcus aureus culture and plasma (5-10 times diluted sample) and subsequent incubation for 2-4 hours, is the indication of the presence of coagulase (i.e., Staphylococcus aureus). With the DNase test, clearing is seen on the agar medium.

Test for aerobic bacteria, yeasts and mould: Mix 1.3 gm of nutrient broth and 100 ml of distilled water, autoclave it 15 pas for 15-20 min and pour in to a clean test tube. To this, pour 1 ml of test sample. This mixture is incubated at 35-37°C for 4 hours, 1 ml of sample is plated on casein-soyabean digest agar, and incubated at 35-37°C for 4 hours.

Biochemical test: Streptomycin is added to the mixture incubated at 40-42°C for hours. From this mixture 1 ml of sample is plated on corn meal agar media, and 1ml of sample is plated on Sabouraud's dextrose agar (SDA) media. Both the corn meal agar media sample and the SDA media sample are incubated at 35-37°C for 4 days.

#### **Experimental Rats**

Male Wistar rats of either sex were used for the study. They were maintained in a well-ventilated room with 12:12 hour light:dark cycle in polypropylene cages. Standard pellet feed (Hindustan Lever Limited., Bangalore) and water ad

libitum was provided to the animals. Rats were acclimatized to laboratory conditions one week prior to initiation of experiments. Ethics committee clearance was obtained from IAEC (Institutional Animal Ethics Committee) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) (Reg. No. IAEC/930/a/06/CPCSEA).

**2.6. Acute toxicity studies: Acute toxicity studies were carried out using "Up and Down" method and the maximum non lethal dose were found to be 2000 mg/kg body weight.**

#### **2.7 Hepatoprotective Activity<sup>[15-20]</sup>**

Experimental procedure: Hepatoprotective activity was tested using Male Wistar rats (150-180 gm). The animals were grouped into 6 groups of 5 animals each. Normal Group A: Serve as normal control receiving 4 % gum acacia 1 ml/kg body weight orally for 4 days with 2 ml olive oil given subcutaneously on 2<sup>nd</sup> and 3<sup>rd</sup> day. Positive Control Group B: Serve as toxicant receiving 4 % gum acacia 1 ml/kg body weight orally for 4 days with 1:1 CCl<sub>4</sub> in olive oil 2 ml/kg body weight subcutaneously on 2<sup>nd</sup> and 3<sup>rd</sup> day. Test standard Group C: Serve as standard receiving poly herbal syrup 1 ml/kg body weight orally for 4 days with 1:1 CCl<sub>4</sub> in olive oil 2 ml/kg body weight subcutaneously on 2<sup>nd</sup> and 3<sup>rd</sup> day. Test Group D: Receiving (HA-I) syrup (1ml/kg body weight) orally for 4 days with 1:1 CCl<sub>4</sub> in olive oil 2 ml/kg body weight subcutaneously on 2<sup>nd</sup> and 3<sup>rd</sup> day. Test Group E: Receiving polyherbal formulation prepared using aqueous extract of all 3 crude drugs syrup at 500 mg/kg body weight for 4 days with 1:1 CCl<sub>4</sub> in olive oil 2ml/kg body weight subcutaneously on 2<sup>nd</sup> and 3<sup>rd</sup> day. Rats were anaesthetized with anaesthetic ether on 5<sup>th</sup> day and blood was collected from retro-orbital plexus and then sacrificed by cervical dislocation. The liver was carefully isolated and preserved in 10 % formalin.

The serum was separated by centrifugation and used for estimation of different bio-chemical parameters such as Serum Glutamate Oxaloacetate Transaminase (SGOT) (IU/L), Serum Glutamate, Pyruvate Transaminase (SGPT) (IU/L), Serum, Alkaline Phosphatase (SALP)

(IU/L), and Serum Bilirubin (mg/ml) levels. The weight of each liver was recorded and then subjected to histopathological studies (17). This includes the histological changes in the liver architecture such as arrangement hepatic lobules, inflammatory cell infiltration and fatty changes.

#### **Statistical Analysis**

One-Way analysis of variance (ANOVA) was applied for each parameter in order to estimate significant inter group differences.

### **3. RESULTS AND DISCUSSION**

The poly herbal formulation was prepared based on Table (1). The physical parameters such as colour, odour, taste, pH and viscosity were determined and presented in Table (2). Phytochemical analysis of the formulation was carried out using various chemical reagents and the results are summarized in Table (3). The poly herbal formulation was evaluated by thin layer chromatography and  $R_f$  value of the formulation was found to be 0.81.

The microbiological study of poly herbal formulation does not show any microbial growth for various microbiological assays. The formulation was found to be negative for the presence of *E.coli*, staphylococcus, yeast and mould (Fig. 1).

In acute toxicity study, the animals treated with prepared formulation at a higher dose of 2000 mg/kg did not manifest any significant abnormal signs, behavioral changes, body weight changes, or macroscopic findings at any time of observation. There was no mortality in the above-mentioned dose at the end of the 14 days of observation.

The results of carbon tetrachloride-induced hepatotoxicity of the formulation are presented in Table (4). The  $CCl_4$  only treated animals exhibited a significant increase ( $P < 0.001$ ) in the levels of SGOT, SGPT, alkaline phosphatase (ALP) and also total bilirubin when compared to the normal control group on 3<sup>rd</sup> day, indicating hepatocellular damage. The formulation at tested doses (group D and E) produced a significant reduction ( $P < 0.001$ ) in the  $CCl_4$ -induced elevated levels of SGOT, SGPT, alkaline phosphatase (ALP) and also total bilirubin when compared to the  $CCl_4$  only treated animals (group-B) after 3 days of treatment. Overall, the

formulated syrup at tested doses significantly reduced the levels of hepatic enzymes and total bilirubin in a dose dependent manner. After 3 days, the hepatic enzymes levels were almost restored to the normal after treating with formulation at the dose of 500 mg/kg [orally]. A standard drug, silymarin at dose 200 mg/kg (group-C) administered orally produced a significant reduction ( $p < 0.001$ ) compared to  $CCl_4$  only treated animals (group-B) on 3<sup>rd</sup> day and these protective effects almost close to the formulation 500 mg/kg, orally.

Histopathological examination of liver sections of control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein on 3<sup>rd</sup> day (Fig. 2a). Disarrangement of normal hepatic cells with centrilobular necrosis, vacuolization of cytoplasm and fatty degeneration were observed in  $CCl_4$  intoxicated rats (Fig. 2b). The liver sections of the group-E rats treated with formulation (250 and 500 mg/kg, orally) showed a sign of protection as it was evident by the moderate accumulation of fatty lobules, absence of necrosis and vacuoles (Fig. 2d and 2e). Almost similar sign of protection was shown in the liver sections of silymarin treated rats (Fig. 2c).

The Significance of differences among the group was assessed using one way analysis of variance (ANOVA). The test followed by Dunnett's test p values less than 0.05 were considered as significant. The poly herbal formulation exhibit a dose dependent significant ( $P < 0.01$  and  $P < 0.001$ ) reduction in various disorders of hepatotoxicity on comparison with the reference standard oral dose of silymarin 200mg/kg. There was also a significant reduction of the hepatotoxicity activity shows in poly herbal formulation treated groups.

#### **CONCLUSION**

The prepared poly herbal formulation has shown significant activity against the liver toxicity induced by  $CCl_4$  to the standard drug Sylimarin. The prepared poly herbal formulation showed no signs of foreign matter and microbial contamination. The present research might hopefully bring advancement in the treatment of liver diseases using herbs as well as in developing poly herbal formulations for the safe and effective management of liver diseases.

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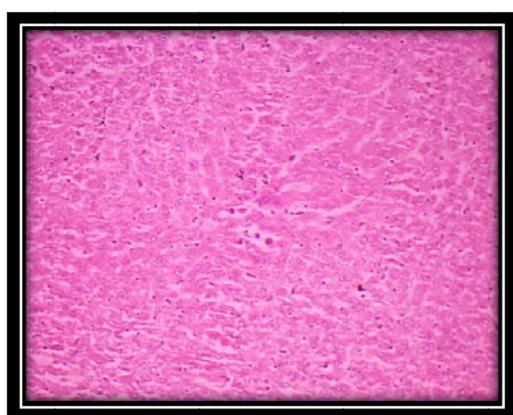


(a)

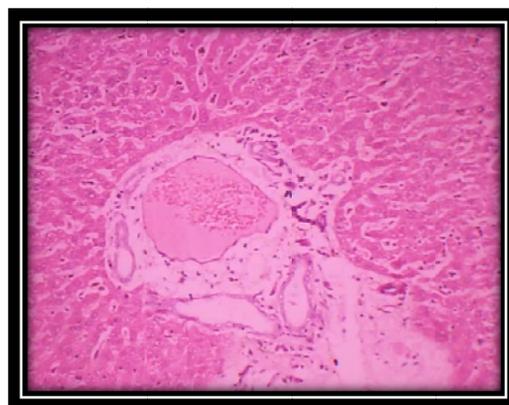


(b)

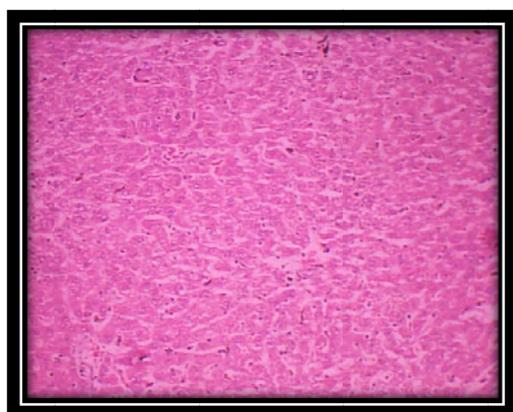
Figure 1. Microbial evaluation for (a) E.coli (b) yeast , mould and staphylococcus



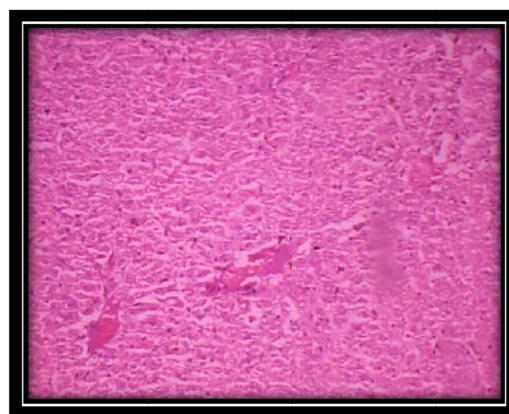
**(a) Normal control**



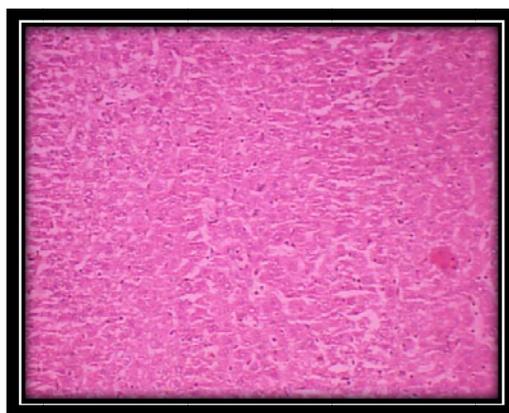
**(b) Negative control (CCl<sub>4</sub>)**



**(c) Standard (silymarin 200mg/Kg)**



**(d) Test (250mg/Kg)**



**(e) Test (500mg/Kg)**

**Figure 2. Histopathological studies of Liver**

**Table 1. Materials required for poly herbal syrup**

S. No	Ingredients	Quantity
1.	Aegle mameelos	1 part
2.	Phyllanthus amarus	1 part
3.	Eclipta alba	1 part
4.	Vanilla	0.5 ml
5.	Methyl paraben	0.5 mg
6.	Red colour	0.75 ml
7.	Simple syrup base	q.s.
<b>Total</b>		<b>100 ml</b>

**Table 2. Physical properties of formulation**

Parameter	Result
Colour	Reddish brown
Odour	Vanilla fragrance
Taste	sweet taste
Viscosity	40 mps
p <sup>H</sup>	3.98

**Table 3. Phytochemical evaluation**

Phytochemicals	Polyherbal syrup
Alkaloids	+
Steroids	+
Amino acids	-
Tannins	+
Carbohydrates	+
Saponins	+
Flavonoids	-
Fixed oils and fats	+

+ presence; - absence

**Table 4. CCl<sub>4</sub>-induced alteration of hepatic enzymes and serum bilirubin in rat liver**

Design of Treatment	Biochemical parameters			
	SGOT (U/ml)	SGPT (U/ml)	ALP (KA Units)	Bilirubin (mg/dl)
Group- A.: Normal control (saline)	52.12 ± 1.24	64.54 ± 1.286	18.57 ± 0.52	0.92 ± 0.03
Group- B: CCl <sub>4</sub> (1.25 ml/kg;oral)	192.24 ± 1.52 <sup>*c</sup>	158 ± 1.45 <sup>*c</sup>	49.57 ± 1.54 <sup>*c</sup>	3.16 ± 0.14 <sup>*c</sup>
Group-C: Standard (silymarin 200mg/kg)	62.14 ± 1.42	66.14 ± 0.62*	28.19 ± 1.86*	1.22 ± 0.2*
Group-D: PHF (250 mg/kg; p.o)	79.23 ± 1.36*	99.12 ± 1.45*	32.52 ± 1.15*	1.29 ± 0.04*
Group-E: PHF (500mg/kg; p.o)	67.29 ± 1.12*	70.58 ± 1.56*	28.38 ± 0.45*	1.10 ± 0.02*

Values are Mean ± SEM of 6 animals each in a group. <sup>\*c</sup>  $P < 0.001$ , when compared group A Vs group-B. <sup>\*</sup>  $P < 0.001$ , when compared group B Vs group, A,C,D,E. Statistical significance was done using one way Anova followed by Dunnet test. PHF- Poly Herbal Formulation.

## الآثار الوقائية للمستخلص العشبي (Polyherbal)

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### ملخص

النباتات الآتية *Aegle marmelos L*, *Eclipta alba L.* and *Phyllanthus amarus* هي نباتات معروفة لحماية الكبد. تم تقييم الآثار الوقائية للمستخلص العشبي polyherbal التي تحتوي على الأعشاب أعلاه مقارنة بالكلوريد الكربوني الرباعي (CCL4) ضد سمية الكبد في الفئران. CCL4 يسبب انحطاط في المواد الدهنية وتشكيل فجوة وزيادة كبيرة في مستويات الألانين ألانين (ALT) والألانين اسبارتاتي (AST) في بلازما الفئران المستخلص العشبي سبب انخفاضا بشكل ملحوظ في مستويات AST و ALT في البلازما. كما أظهرت الدراسات التشريحية المرضية أن المستخلص العشبي كان له اثر في تقليل حدوث آفات الكبد الناجم عن CCL4. أثبتت الدراسة أن المستخلص العشبي المعد من النباتات اعلاه يمكن أن تستخدم في علاج تسمم الكبد التي يسببها CCL4. الكلمات الدالة: المستخلص العشبي polyherbal، الكلوريد الكربوني، والألانين ألانين (ALT)، الألانين اسبارتاتي (AST).

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