

¹ Hepatoprotective Activity of Phyllanthus Amarus Seeds Extracts ² in CCl₄ Treated Rats: In Vitro & In Vivo

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⁷ **Abstract**

⁸ Aim - To study the hepatoprotective activity of Phyllanthus amarus seeds extracts in CCl₄
⁹ treated rats. Methods - The crushed and dried seeds of Phyllanthus amarus were divided into
¹⁰ two parts; one part was extracted successively with petroleum ether, benzene, chloroform and
¹¹ finally with methanol by soxhlet apparatus and concentrated by rotary vacuum. The other
¹² part is extracted by cold maceration process for aqueous extraction. The extracts were used
¹³ for In-vitro and In-vivo studies to analyze the reparative activity of liver injury due to CCl₄ in
¹⁴ rats. Results - In-vitro models like Reducing power, Superoxide anion scavenging activity,
¹⁵ Hydroxyl radical scavenging activity and Nitric oxide radical scavenging activity were carried
¹⁶ out with methanolic extract of Phyllanthus amarus seeds for its antioxidant properties. A
¹⁷ protective activity could be demonstrated in the CCl₄ induced liver damage in rats. In-vivo
¹⁸ methanolic and aqueous extracts of the seeds of Phyllanthus amarus 250mg/kg were found to
¹⁹ have protective properties in rats with CCl₄ induced liver damage as judged from serum
²⁰ biochemical enzyme marker activities and histopathological studies..

²¹

²² **Index terms**— Hepatoprotective activity, Phyllanthus amarus seeds, CCl₄ and Methanolic extract

²³ **1 Introduction**

²⁴ The liver is an organ of paramount importance not only for its metabolism of various xenobiotics and environmental
²⁵ pollutants [1] but for its unique and considerable regenerative capacity, even a moderate cell injury is not reflected
²⁶ by measurable change in its metabolic functions. However, some of its functions are so sensitive that abnormalities
²⁷ start appearing depending upon the nature and degree of its initial damage [2] .

²⁸ Reactive Oxygen Species (such as H₂O₂ , O₂ 2-, and OH - , collectively known as ROS) play important
²⁹ physiological functions and can also cause extensive cellular damage. Cells are provided with efficient molecular
³⁰ strategies to control strictly the intra cellular ROS level and to maintain the balance between oxidant and
³¹ antioxidant molecules. Oxidative stress, resulting from an imbalance between the generation of ROS and the
³² antioxidant defense capacity of the cell [3] , effects major cellular components including lipids, proteins and DNA.
³³ This phenomenon is closely associated with a number of human disorders such as many degenerative diseases
³⁴ including cardiovascular disease, diabetes, cancer, neurodegenerative disorders [4,5] and with almost all liver
³⁵ pathologies [6][7][8] . All these conditions appear mostly related to chronic oxidative stress. However, the acute
³⁶ exposure to high levels of ROS seems to be responsible for the development of different damages such as during
³⁷ ischemia/reperfusion is acute hepatotoxic agent, which induces peroxidative degeneration of membrane lipids
³⁸ causing hypoperfusion of the membrane. Cytosolic enzyme like SGPT, SGOT and ALP.

³⁹ A number of medicinal plants are used in traditional system of medicinal for the management of liver disorders.
⁴⁰ Nature has given us a large number of medicinal plants, some of which are yet to be explored and validated for
⁴¹ their medicinal value. The 21st century has seen a paradigm shift toward therapeutic evaluation of herbal
⁴² products in liver diseases, carefully synergizing the strengths of traditional medicine with the modern concept of
⁴³ evidence based medical evaluation, standardization and randomized placebo controlled clinical trials to support

9 A) IN-VITRO ANTIOXIDANT ACTIVITY

44 clinical efficacy. Several herbs are known to possess antioxidant properties and may be useful as liver protective
45 agents [10] .

46 The herbs containing antioxidant principles are reported to be highly effective in preventing or curing the
47 liver toxicities due to above mentioned challenges. In the present study, the herb Phyllanthus amarus containing
48 polyphenolic compounds is selected to assess hepatoprotective activity [11] .

49 2 II.

50 3 Materials and Methods

51 4 a) Plant Material

52 The seeds of Phyllanthus amarus were collected from local gardens of Tirupathi. The plant was b) Preparation
53 of Extracts The crushed and dried seeds of Phyllanthus amarus were divided into two parts; one part was
54 extracted successively with petroleum ether, benzene, chloroform and finally with methanol by soxhlet extraction
55 and concentrated by rotary vacuum [12] . The other part is extracted by cold maceration process for aqueous
56 extraction [13] . The obtained extracts were dried by evaporation. The yield 30% w/w and 15.6% w/w were
57 stored in refrigerator and weighed quantities were suspended in tween 80 and 2% tragacanth solution respectively
58 for the experiment. The extracts were used for In-vitro and In-vivo studies to analyze the reparative activity of
59 liver injury due to CCl 4 treated rats.

60 5 c) Experimental Animals

61 Male albino rats weighing 130-160g were obtained from the animal house of Nizam Institute of Pharmacy,
62 Hyderabad and housed in Polycarbonate cages. The rats had free access to standard pellet chow and water ad
63 libitum throughout the experiment with the exception of some experiments (see below) in which the animals were
64 deprived of food, but not water, for 18-24 hr. before the experiments were performed. After procurement, all the
65 animals were divided into different groups and were left for one week for acclimatization to experimentation room
66 and were maintained on standard conditions (23 °C, 60%-70% relatively humidity and 12 hr. photo period).
67 There were five animals in each group for observational screening. All experimental protocols described below
68 were approved by the ethical board.

69 6 d) Acute Oral Toxicity Studies

70 The acute oral toxicity study is determined according to the guidelines of Organization for Economic Co-operation
71 & Development (OECD) following the up & down method (OECD guideline No. 423). Based on the method,
72 a limit test was performed to categorize the toxicity class of the compound and then main test was performed
73 on three female rats to estimate the exact LD50. The animals were fasted overnight with free access to water,
74 weighed and a single dose of the test substance was administered. Animals were observed individually during
75 first 30 min, periodically during 48 h with special attention given during first 4 h (short-term toxicity) and daily,
76 thereafter for total of 14 days (short-term toxicity). LD50 was found to be greater than 2500 mg/kg, in limit
77 test. The test substance could be classified in the hazard classification as Class 5 -2000 mg/kg <LD50 <5000
78 mg/kg in the globally harmonized system (GSH). LD50 of test drug was found to be 2500mg/kg from main test
79 [14] .

80 7 e) Hepatotoxins

81 It is emphasized that hepatotoxin that cause acute hepatitis should have close resemblance with the viral hepatitis,
82 clinically, biochemically and histopathologically. Certain drugs are also responsible for many hepatic diseases,
83 such as chronic hepatitis, fatty liver, cirrhosis and certain vascular lesions of liver. In many instances drug induced
84 hepatitis is distinguishable from viral hepatitis chemical injured viral hepatitis for experimental studies should
85 be severe enough to cause cell death or to modify hepatic functions. The mechanism of acute hepatic injury
86 depends upon the chemical compound and the species of animal used. We have studied the hepatoprotective
87 activity against CCl 4 induced hepatotoxicity.

88 8 III. Methods for the Hepatoprotective Evaluation ear 2012 Y

89 CCl 4 is one of the most powerful hepatotoxin in terms of severity of injury it causes toxic necrosis leading to
90 biochemical changes having clinical features similar to those of acute viral hepatitis [] , liver injury was produced
91 by administration of CCl 4 mixed with tween 80. Animals were given single doses of CCl 4 2 mg/kg, i.p per day
92 throughout the experimental setup. Control animals received an equal volume of tween 80.

93 9 a) In-vitro antioxidant activity

94 In-vitro models were carried out to evaluate antioxidant activity are Reducing power, Superoxide anion scavenging
95 activity, Hydroxyl radical scavenging activity and Nitric oxide radical scavenging activity.

96 10 Reducing power

97 The reducing power of methanolic and aqueous extracts of *Phyllanthus amarus* seeds were determined according
98 to the method of Oyaizu [] Procedure: Extracts of *Phyllanthus amarus* seeds were mixed in 1ml of distilled water
99 so as to get 20 μ g, 40 μ g, 60 μ g, 80 μ g, and 100 μ g concentration. This was mixed with phosphate buffer (2.5ml,
100 0.2M, pH 6.6) and potassium ferricyanide (2.5ml, 1%). The mixture was incubated at 50°C for 20 minutes. A
101 portion (2.5ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm
102 for 10 minutes.

103 The upper layer of the solution (2.5ml) was mixed with distilled water (2.5ml) and FeCl 3 (0.5ml, 0.1%), and
104 the absorbance (OD) was measured at 700nm. Increased absorbance of the reaction mixture indicates increased
105 in reducing power.

106 The percent reducing power was calculated by using the formula: % increase in absorbance = Control OD-Test
107 OD Control OD × 100

108 11 Superoxide anion scavenging activity

109 Oxygen is essential for the survival of aerobic cells, but it has long been known to be toxic to them 16 1.

110 12 2.

111 when supplied at concentration greater than those in normal air. The biochemical mechanisms responsible for
112 oxygen toxicity include lipid peroxidation and the generation of H₂O₂ + the superoxide radical, O₂ + . This
113 superoxide radical can inhibit or propagate the process of lipid peroxidation. Measurement of superoxide anion
114 scavenging activity of *Phyllanthus amarus* seeds was done by using the method explained by Nishimiki [] and
115 modified by Ilhami et al., Procedure: About 1ml of nitroblue tetrazolium (NBT) solution (156 μ M NBT in 100mM
116 phosphate buffer, pH 7.4), and 0.1ml of sample solution of methanolic extract of *Phyllanthus amarus* seeds and
117 standard in water was mixed. The reaction was started by adding 100 μ l of phenazine methosulphate (PMS)
118 solution (60 μ M PMS in 100mM phosphate buffer, pH7.4) to the mixture. The reaction mixture was incubated
119 at 25°C for 5 minutes, and the absorbance at 560nm was measured against blank.

120 Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. %
121 inhibition of OD was calculated by using the formula mentioned earlier.

122 associated with several diseases oxygen reacts with the excess nitric oxide to generate nitrite and peroxy nitrite
123 anions, which acts as free radicals. This forms the basis of this experiment.

124 Procedure: Nitric oxide (NO) radical were generated from sodium nitroprusside solution of physiological pH
125 [20] . Sodium nitroprusside (1ml of 10mM) were mixed with 1ml of methanolic extract of *Phyllanthus amarus*
126 seeds of different concentration (20-100 μ g/ml) in phosphate buffer (pH 7.4). The mixture of incubated at 25°C
127 for 150 min. To 1ml of incubated solution, 1ml Griess's reagent (1% sulphanilamide, 2% o-phosphoric acid and
128 0.1% naphthyl ethylene diamine dihydrochloride) was added. Absorbance was read at 546nm. % inhibition of
129 OD was calculated by using the formula mentioned earlier.

130 13 IV. Results

131 In vitro: It was observed that methanolic extract demonstrated dose dependent increase in the reducing property.
132 25mcg sodium metabisulphite (Std.) showed 73.09% reducing property. Methanolic extract at 100mcg had more
133 reducing property than 25mcg ear 2012 Y

134 14 Hydroxyl radical scavenging activity

135 In biochemical systems, superoxide radical and H₂O₂ react together to form the hydroxyl radical which can
136 attack and destroy almost all known biochemical [18] Phenylhydrazine when added to erythrocyte hosts cause
137 peroxidation of endogenous lipids and alteration of membrane fluidity. This peroxidation damage to erythrocytes
138 is probably initiated by active oxygen species like O₂ -

139 ? , OH ? and H₂O₂ which are generated in solution from auto-oxidation of phenyl hydrazine. This forms
140 the basis of this experiment.

141 Procedure: Hydroxyl radical generation by phenyl hydrazine has been measured by the 2deoxyribose
142 degradation, assay of Halliwell and Gutteridge [] In 50mM phosphate buffer (pH 7.4), 1mM deoxyribose and
143 0.4ml of methanolic extract of *Phyllanthus amarus* seeds and standard were taken. 0.2ml phosphate buffer was
144 added to make reaction solution 1.6ml. after 10 min incubation 0.4ml of 0.2mM phenyl hydrazine was added.
145 Incubation was terminated after 1hr and 4 hrs and 1ml each of 2.8% TCA and 1% (w/v) thiobarbituric acid
146 were added to the reaction mixture and heated for 10 minutes in a boiling water bath. The tubes were cooled
147 and absorbance was taken at 532nm. Decreased in absorbance is indicating the hydroxyl free radical scavenging
148 activity. The % reduction in the OD is calculated.

149 15 Nitric oxide radical scavenging activity

150 Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons etc.
151 involved in the regulation of various physiological processes. Excess concentration of NO is

21 DISCUSSION

152 16 b) In-vivo antioxidant activity

153 The Wister rats were divided into 5 groups of 6 individuals each [21] for 5days study. Table ?? 1

154 17 Group

155 Group I received Tween 80 1ml/kg I.P., on 2 nd and 3 rd day. Group II, III, IV and V received CCl 4 2mg/kg
156 I.P., on 2 nd , 3 rd , days. The Group III, IV, V, received Liv 52 100mg/kg p.o., Methanolic Extract 250mg/kg
157 p.o., Aqueous Extract 250mg/kg p.o., before 30min of toxicant respectively. Animals were sacrificed on the 5 th
158 day under mild ether anesthesia.

159 The blood samples were collected from retro orbital plexus for evaluating the serum biochemical parameters
160 and liver was dissected out, blotted off blood, washed with saline and stored in 10% formalin and preceded for
161 histopathology to evaluate the details of hepatic architecture in each group microscopically.

162 18 c) Statistical analysis

163 The statistical analysis was carried out by oneway analysis of variance (ANOVA). The values are represented
164 as mean \pm SE. Comparison of mean values of different groups treated with different dose levels of extracts and
165 positive controls were estimated by Tukey's Multiple Comparison Test. P < 0.05 was considered significant.
166 sodium metabisulphate i.e. 88.88%.

167 There was percentage increase in the superoxide anion scavenging activity. Methanolic extract showed lesser
168 activity than standard i.e. 70.64%.

169 It was observed that methanolic extract demonstrated dose dependant percentage increase in the hydroxyl
170 radical scavenging activity in case of 1 hr. incubation period i.e. 64.20% But, showed higher in case of 4 hrs
171 incubation period i.e. 55. 55% It was observed that that methanolic extract demonstrated dose dependant
172 percentage increase in the nitric acid radical scavenging activity. 25mcg sodium metabisulphate (Std.) showed
173 70.52% activity. Methanolic extract had more nitric oxide radical scavenging activity at 100 mcg than compared
174 to 25 mcg sodium metabisulphate. i.e. 71.78%.

175 In vivo: There was increased level of SGPT in CCl4 treated group 312.420U/L. The SGPT level was restored
176 to 77.84 U/L by 250mg/kg methanolic extract of the seeds which was near to effect of 100mg/kg Liv.52 i.e.
177 65.395 U/L.

178 SGOT levels increased significantly in CCl4 treated group i.e. 318.412 U/L. methanolic extract of the seeds
179 reduced the elevated level of SGOT to 72.69 U/L, which was very near to 100mg/kg Liv.52 i.e. 71.212 U/L.

180 In case of bilirubin, methanolic extract reduced the level of bilirubin by 4.892mg/dl to 1.20mg/dl.

181 There was increase in ALP level observed in CCl4 treated group (235.86 IU/L). ALP level was restored to
182 97.44 IU/L by methanolic extract of the seeds which was near to effect of 100mg/kg Liv.52 i.e.

183 19 IU/L.

184 There was no significant rise in total cholesterol and triglycerides levels in CCl4 treated group. Significant effect
185 was observed with in methanolic extract and aqueous extract was comparable with 100mg/kg Liv.52.

186 Liver section of control rat showed normal hepatocytes and normal architecture (Figure 1A). Liver sections
187 from CCl4 treated rats demonstrated the destruction of architectural pattern, nodule formation in the lobular
188 zone, inflamed periportal zone, and moderate inflammation of portal area (Figure 1B). Liver sections from Liv
189 52 treated rats showed regeneration of normal hepatocytes (Figure 1C). Liver sections from a methanolic extract
190 of Phyllanthus amarus seeds treated rat showing normal lobular architecture (Figure 1D). Liver section from an
191 aqueous extract of Phyllanthus amarus seeds treated rat showing normal lobular architecture no necrosis or fatty
192 changes or any inflammatory reaction can be seen. (Figure 1E). These histopathological findings demonstrate a
193 hepatoprotective effect of the extracts against CCl4mediated liver damage.

194 20 VI.

195 21 Discussion

196 The purpose of this study was to explore the hepatoprotective effect of extracts of Phyllanthus amarus seeds in
197 the hepatic damage caused by CCl 4 .

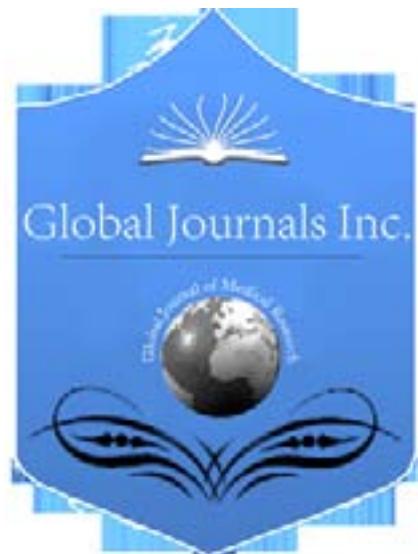
198 Administration of CCl 4 to normal rats increased serum levels of AST, ALT, ALP, and bilirubin. The enzymes
199 leaking out from damaged liver cells into circulating blood represent the damage to hepatic cells. It is well
200 established that the toxic metabolite of CCl 4 , a free radical CCl 3 is responsible for damage to liver cells.
201 Invitro models like Reducing power, Superoxide anion scavenging activity, Hydroxyl radical scavenging activity
202 and Nitric oxide radical scavenging activity were carried out with methanolic extract of Phyllanthus amarus seeds
203 for its antioxidant properties. A protective activity could be demonstrated in the CCl 4 induced liver damage in
204 rats. In-vivo methanolic and aqueous extracts of the seeds of Phyllanthus amarus 250mg/kg were found to have
205 protective properties in rats with CCl 4 induced liver damage and caused statistically significant decrease in all
206 the above parameters.

207 Liver section of control rat showed normal hepatocytes and normal architecture (Figure 1A). Liver sections
208 from CCl4 treated rats demonstrated the destruction of architectural pattern, nodule formation in the lobular

209 zone, inflamed periportal zone, and moderate inflammation of portal area (Figure 1B). Liver sections from Liv
210 52 treated rats showed regeneration of normal hepatocytes (Figure 1C). Liver sections from a methanolic extract
211 of *Phyllanthus amarus* seeds treated rat showing normal lobular architecture (Figure 1D).

212 Liver section from an aqueous extract of *Phyllanthus amarus* seeds treated rat showing normal lobular
213 architecture no necrosis or fatty changes or any inflammatory reaction can be seen. (Figure 1E). These
214 histopathological findings demonstrate a hepatoprotective effect of the extracts against CCl4mediated liver
215 damage. The methanolic and aqueous extracts of *Phyllanthus amarus* seeds do have a protective capacity
216 both In-vitro and In-vivo models of CCl 4 mediated liver injury VII. Histopathological Studies in ccl4 Induced
217 Hepatotoxicity

218 Figure : 4a



1

Figure 1: Figure 1 :

219 1 2

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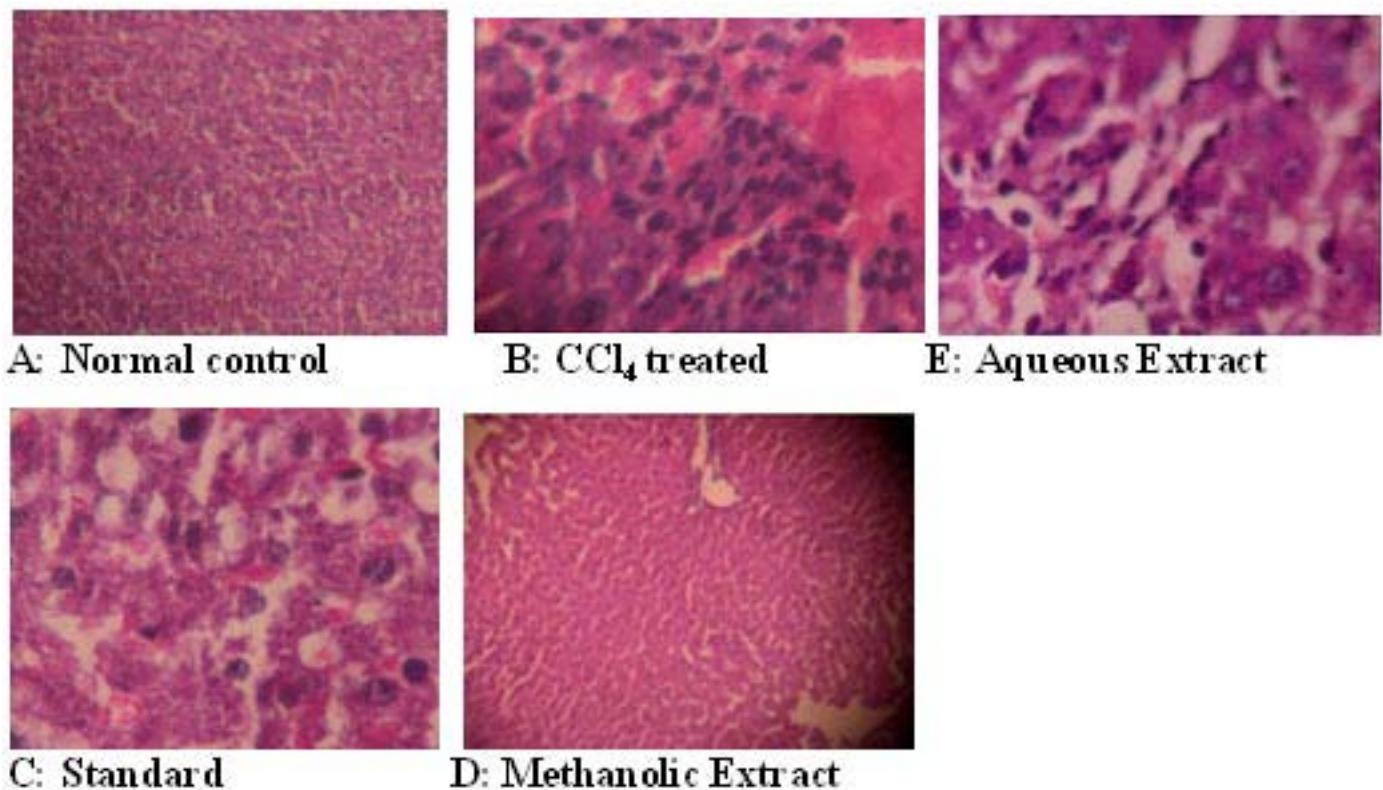


Figure 2: Figure: 4b

Absorbance/ Groups

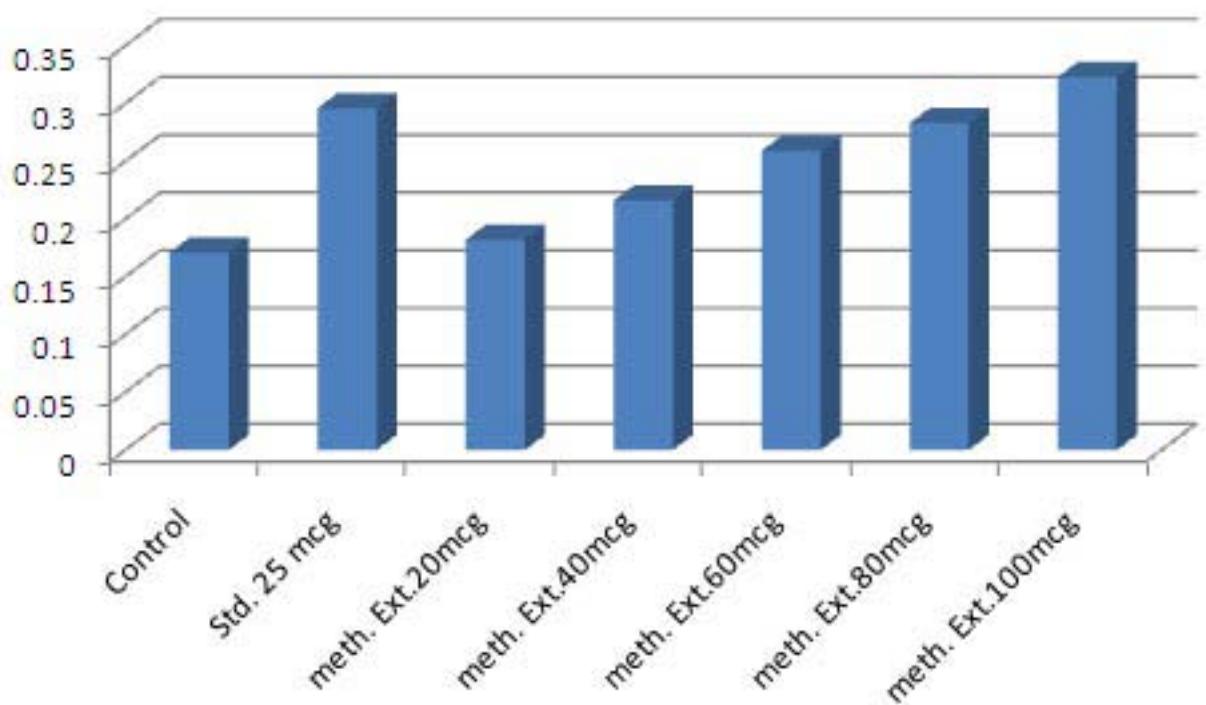


Figure 3:

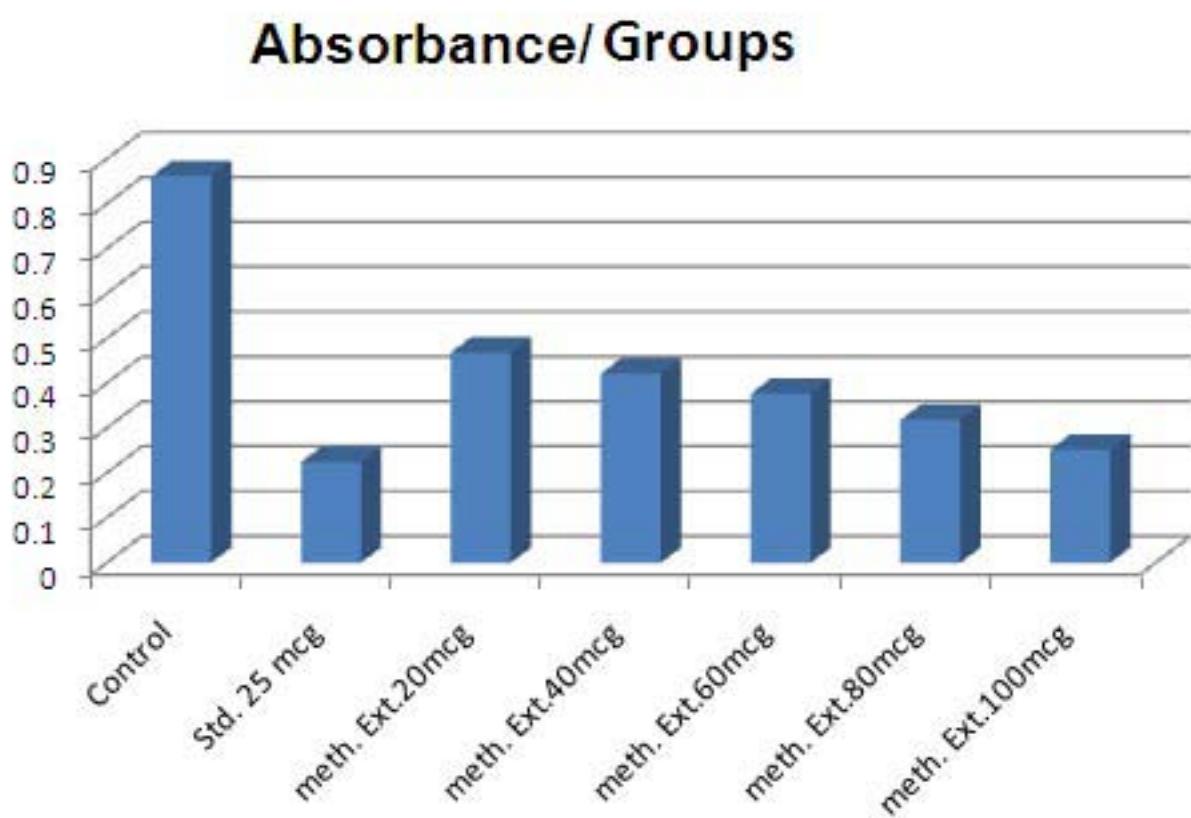


Figure 4:

Absorbance/Groups

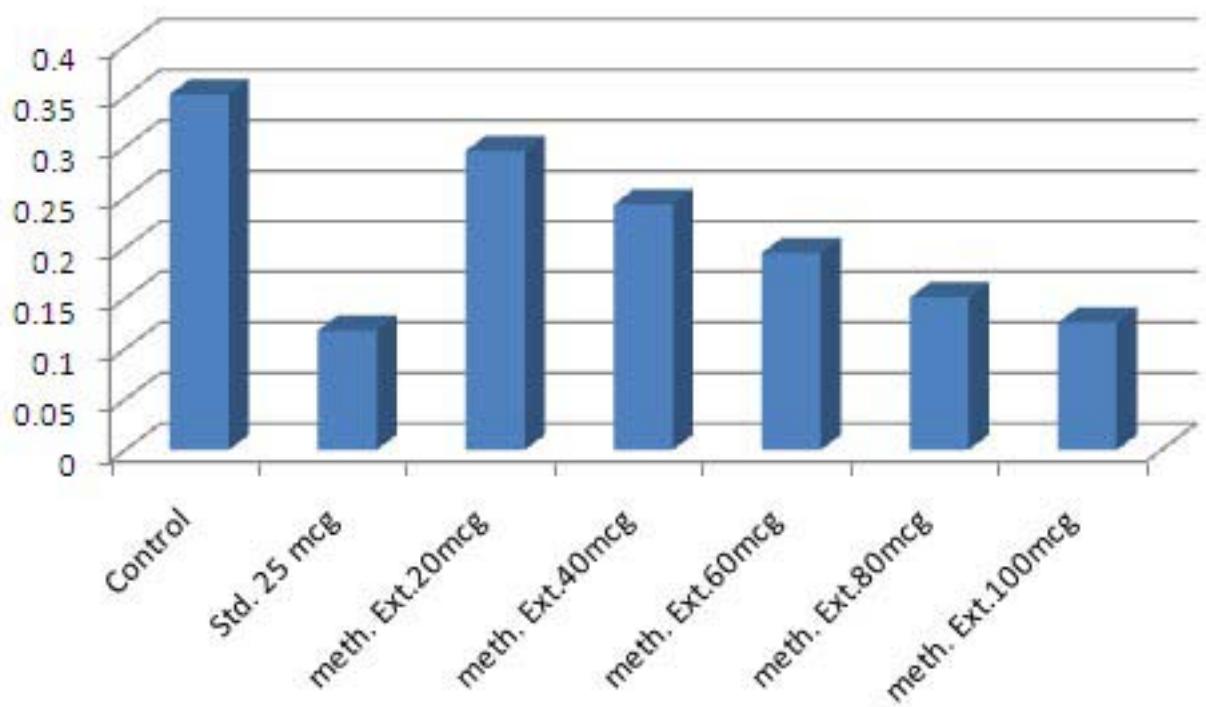


Figure 5:

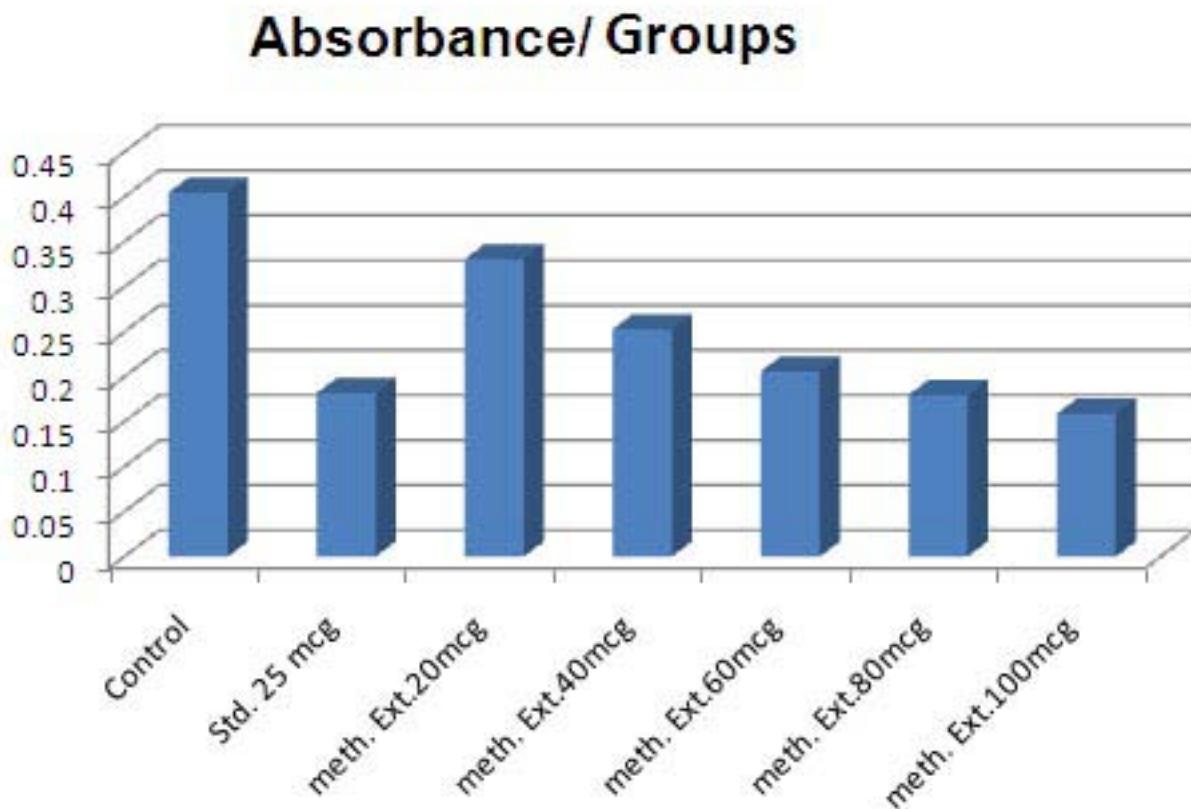


Figure 6:

2

Group	Absorbance Mean±SEM	% Increase
Control	0.171±0.001	—
Control + Std 25µg	0.296±0.002***	73.09
Control + methanolic extract 20µg	0.182±0.001*	6.43
Control + methanolic extract 40µg	0.216±0.002***	26.31
Control + methanolic extract 60µg	0.259±0.002***	51.46
Control + methanolic extract 80µg	0.283±0.002***	65.49
Control + methanolic extract 100µg	0.323±0.002***	88.88
Values are the mean ± S.E.M., n=3		
Significance***P<0.001 and * P<0.05 compared to control. Std: Sodium metabisulphite		

Figure 7: Table 2 :

3

Group	Absorbance Mean±SEM	% Increase
Control	0.862±0.020	—
Control + Std 25µg	0.225±0.001***	73.89
Control + methanolic extract 20µg	0.469±0.001***	45.59
Control + methanolic extract 40µg	0.422±0.002***	51.04
Control + methanolic extract 60µg	0.377±0.002***	56.26
Control + methanolic extract 80µg	0.319±0.002***	62.99
Control + methanolic extract 100µg	0.253±0.002***	70.64

Values are the mean ± S.E.M., n=3

Significance***P<0.001 compared to control. Std: Sodium metabisulphite

Figure 8: Table 3 :

4

Group	Absorbance Mean±SEM	% Inhibition	Absorbance Mean±SEM	% Inhibition
Control	0.352±0.0005	—	0.405±0.0020	—
Control + Std 25µg	0.118±0.0037***	66.47	0.182±0.0040***	55.06
Control + methanolic extract 20µg	0.296±0.0028***	15.90	0.331±0.003***	18.27
Control + methanolic extract 40µg	0.243±0.0020***	30.96	0.253±0.001***	28.14
Control + methanolic extract 60µg	0.195±0.0030***	46.59	0.206±0.002***	37.53
Control + methanolic extract 80µg	0.151±0.002***	57.10	0.180±0.003***	49.13
Control + methanolic extract 100µg	0.126±0.0026***	64.20	0.159±0.0041***	55.55

Values are the mean ± S.E.M., n=3

Significance***P<0.001 compared to control. Std: Sodium metabisulphite

Figure 9: Table 4 :

5

Group	Absorbance Mean±SEM	% Increase
Control	0.397±0.0015	—
Control + Std 25µg	0.117±0.0015***	70.52
Control + methanolic extract 20µg	0.242±0.0008***	39.04
Control + methanolic extract 40µg	0.218±0.0017***	45.08
Control + methanolic extract 60µg	0.186±0.0017***	53.14
Control + methanolic extract 80µg	0.142±0.0026***	64.23
Control + methanolic extract 100µg	0.112±0.0028***	71.78

Figure : 5

Figure 10: Table 5 :

6

Treatment	induced hepatotoxicity						
	SGPT U/L	SGOT U/L	ALP IU/L	Bilirubin (mg/dl)	Protein (g/dl)	Cholesterol (mg/dl)	Triglycerides (mg/dl)
Negative control	55.558	55.216	122.29	0.926	8.88	110.88	171.22
1ml/kg Tween 80	±	±	±	±	±	±	±
	3.331	5.617	6.486	0.029	0.34	10.771	7.198
Positive control	312.42	318.412	235.86	4.892	5.85	172.62	190.36
CCl ₄ treated	±	±	±	±	±	±	±
2ml/kg							
	14.275	13.543	8.207	0.451	0.40	10.522	7.516
CCl ₄ + Liv. 52	65.395	71.212	95.68	1.546	8.46	118.25	145.48
2ml/kg +	±	±	±	±	±	±	±
100mg/kg							

Figure 11: Table 6 :

220 [Ccl4 and Methanolic] , + Ccl4 , Methanolic .

221 [Ccl4 and Aqueous] , + Ccl4 , Aqueous .

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