

***Aegle marmelos* leaves protect Liver against Toxic effects of Cyclophosphamide in mice**Sangita Singh¹, Swarn Lata^{1*}, Kavindra Nath Tiwari²¹ Department of Zoology, MMV, Banaras Hindu University, Varanasi-221005, India² Department of Botany, MMV, Banaras Hindu University, Varanasi-221005, India.Email: bioinfo.sangita@gmail.com, Swarn Lata: swarn_vns@rediffmail.com, swarnlatabhu12@gmail.com, K. N. Tiwari: kntiwariibhu@gmail.com

Abstract: The present study was conducted to evaluate the phytochemical screening of leaves of *A. marmelos* and the hepatoprotective activity of aqueous extract of *Aegle marmelos* (AEAM) against CPA-induced liver damage in mice. AEAM (400, 500, and 600 mg/kg bw, orally) and CPA (200 mg/kg bw, intraperitoneally) were administered to mice for 5 weeks, once a week. Silymarin (100 mg/kg bw) was given as reference standard. Serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase, alkaline phosphatase, acid phosphatase, bilirubin, cholesterol levels, and lipid peroxidation were significantly increased, accompanied by a significant decrease in the level of albumin in CPA-induced hepatotoxic group of mice compared to the control. However, significant amelioration in these parameters was found in AEAM treated groups of mice. CPA treatment markedly decreased the level of superoxide dismutase and catalase in the liver as well as white blood cells and red blood cells counts, which were significantly enhanced by AEAM treatment. Histopathological examinations have also confirmed the protective efficacy of AEAM. The phytochemical screening of the extract revealed the presence of alkaloids, saponins, tannins, flavonoids and phenols, which may have hepatoprotective role. Hence, the results of the present study indicated that AEAM may be effective as a hepatoprotectant in CPA-induced toxicity. [Singh S, Lata S, Tiwari KN. *Aegle marmelos* leaves protect Liver against Toxic effects of Cyclophosphamide in mice. *N Y Sci J* 2014;7(9):43-53]. (ISSN: 1554-0200). <http://www.sciencepub.net/newyork>. 10

Key Words: Cyclophosphamide, *Aegle marmelos*, hepatoprotective role.

1. Introduction

Cyclophosphamide (CPA) is a drug with a wide spectrum of clinical uses. It is a chemotherapeutic drug, belonging to the class of oxazaphosphorins, widely used in childhood, and adult malignancies as well as immunosuppressive agents for organ transplantation, systemic lupus erythematosus, multiple sclerosis, and other benign diseases. The cytotoxicity of CPA is mediated by alkylation of DNA at the N7 position of guanine and the formation of DNA-DNA cross-links, DNA-protein cross links and single strand break (Oh *et al.*, 2007).

The liver is an important organ responsible for metabolism, bile secretion, elimination of many substances, blood detoxification, synthesis and regulation of essential hormones. Liver diseases have become a worldwide problem and are associated with significant morbidity and mortality. The principal causative factors for liver diseases in developed countries are excessive alcohol consumption and virus-induced chronic liver diseases while in developing countries the most frequent causes are environmental toxins, parasitic disease, hepatitis B and C viruses, and hepatotoxic drugs, certain antibiotics, chemotherapeutic agents, high doses of paracetamol, carbon tetrachloride (CCL₄), thioacetamide (TAA) etc. Chronic liver cirrhosis and drug induced liver injury accounting the ninth leading cause of death in western and developing countries

(Saleem *et al.*, 2010). In the absence of reliable hepatoprotective drugs in modern medicine, a large number of herbal preparations have become increasingly popular for the treatment of liver disorders (Chatterjee, 2000).

Medicinal plants are the source of a large number of bioactive compounds, exploited for natural product-based drug development program for the treatment of many diseases. The protective role of plants is due to the presence of antioxidative constituents like phenolics, flavonoids, tannins, etc. which are able to delay or inhibit the oxidative stress (Lata *et al.*, 2014). A number of herbs show promising activity, including silymarin for liver cirrhosis. Silymarin, a flavonolignan from "milk thistle" *Silymarin marianum*, is widely used for hepatoprotection. Silymarin offers good protection in different toxic models of induced liver cirrhosis experiments by using laboratory animals.

Aegle marmelos (L.) Correa (Rutaceae), commonly known as bael, is a sacred tree for Hindu religion, native to northern India, but is found widely throughout the Indian peninsula and in Ceylon, Burma, Thailand and Indo-China. Leaves, fruits, stem and roots of this plant have been used in ethno medicines for several medicinal properties like as an astringent, antidiarrheal, antidyenteric, demulcent, antipyretic, anticourbutic, aphrodisiac and an antidote to snake venom (Khare, 2004). Essential oil isolated

from the leaves of the *A. marmelos* show antifungal activity. The leaves are astringent, laxative, expectorant and are useful in the treatment of ophthalmia, deafness, inflammation, cataract, diabetes, diarrhea, dysentery, heart palpitation and asthma. Fresh aqueous and alcoholic leaf extract of *A. marmelos* was reported to have cardio tonic effects in mammals. Hypoglycemic and antioxidant activity of *A. marmelos* leaves against alloxan induced diabetic rats have been found to be useful in the long term management of diabetes. Hepatoprotective activity of leaves of *A. marmelos* has also been evaluated with positive results (Vinodhini *et al.*, 2010). Both fruits and leaves of *A. marmelos* have radioprotective activity. *A. marmelos* fruit extract exhibits antihyperlipidaemic effect in streptozotocin-induced diabetic rats (Marzine and Gilbert, 2005).

In this study, we have investigated the protective ability of aqueous extract of leaves of *A. marmelos* against CPA-induced hepatocellular damage and oxidative stress in mice *in vivo*.

2. Materials and methods

Drug and Chemicals

CPA and silymarin was obtained from Sigma Aldrich Ltd., New Delhi, India. The other chemicals and solvents used were of the highest purity of analytical grade.

Plant Material

The leaves of *A. marmelos* were collected from the campus of Banaras Hindu University, Varanasi, India during the months of September – December and was authenticated by Prof. N. K. Dubey, Department of Botany, Banaras Hindu University, Varanasi. A voucher specimen (Rutaceae/2014/1) has been kept in the herbarium for future reference.

Preparation of Aqueous Extract of *Aegle marmelos* (AEAM)

Fresh leaves were washed under running tap water, dried in shade at room temperature for a week and powdered mechanically. The powder (100 grams) was added in 400 ml deionized water under stirring at room temperature (25 ± 2 °C). The aqueous extraction was done following the method of Faremi *et al.* (2008) for aqueous extraction. After extraction, total filtrate was centrifuged at 5,000 rpm to separate the supernatant. The supernatant was concentrated to dryness in rotary vacuum evaporator at 45 °C. The dry extract was collected and stored in a refrigerator at 4°C for further use.

Phytochemical Screening

The aqueous extract was subjected for qualitative phytochemical analysis. For phytochemical analysis, the method of Evans (2009) was followed.

GC – MS Analysis

GC-MS technique was used in this study to identify the phytochemical present in the extract. The GC – MS analysis was carried out using a Clarus 500 Perkin – Elmer (Auto system XL) Gas Chromatograph coupled to a mass detector Turbo mass gold – Perkin Elmer Turbomass 5.1 spectrometer with an Elite – 1 (100% Dimethyl poly siloxane), 30m x 0.25 mm ID x 1µm of capillary column. The instrument was set to an initial temperature of 100 °C, and maintained at this temperature for 4 min. At the end of this period the oven temperature was increased up to 320 °C, at the rate of an increase of 5 °C/min and maintained for 13 min. Injection port temperature was ensured as 260 °C and Helium flow rate as 1 ml/min. The ionization voltage was 70 eV. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 40-600 (m/z).

Identification of Phytochemicals

Interpretation on Mass-Spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular weight and molecular formula of the components of the test materials were ascertained (Hossain *et al.*, 2011). Using computer searches on a NIST Ver.2.1 MS data library and comparing the spectrum obtained through GC – MS compounds present in the plant sample were identified.

Animal Model

All the experiments were performed in accordance with institutional practice and within the framework of revised animals (Committee for the Purpose of Control and Supervision of Experiments on Animals; CPCSEA) Act of 2007 of Govt. of India on animal welfare. The study was conducted on adult male Parke's strain mice (30 ± 3 g), which were obtained from Department of Zoology, Banaras Hindu University, Varanasi, India. The animals were fed with commercially available standard mice pellet feed and water was provided *ad libitum*. The mice were housed under conditions of controlled temperature (25 ± 2 °C) and acclimatized to a 12 h light/ 12 h dark cycle.

Experimental Design

Animals were divided into eight groups of six mice each and the treatment was done as shown below:

Group I:	Control mice received distilled water (intraperitoneally) once in a week for 5 weeks.
Group II:	AEAM (600 mg/kg bw, orally) once in a week for 5 weeks.
Group III:	Silymarin 100 mg/kg bw for 5 weeks (once in a week) orally.
Group IV:	Received CPA 200 mg/kg bw, for 5 weeks (once in a week) by intraperitoneal injection.
Group V-VII:	Received CPA (200 mg/kg bw, intraperitoneally) and AEAM 400, 500 and 600 mg/kg bw orally for 5 weeks (once in a week).
Group VIII:	Received CPA (200 mg/kg bw, intraperitoneally) and silymarin (well known standard hepatoprotective drug) 100 mg/kg bw orally for 5 weeks (once in a week).

Preparation of Tissues

At the end of the treatment period, the mice were sacrificed by cervical dislocation. The liver was removed, cleared of the adhering tissues, washed with ice-cold saline immediately, dried on tissue paper, weighed and frozen for the antioxidant assays. Blood was collected and serum was separated for analysis of biochemical parameters.

Biochemical Estimation

The blood was centrifuged at 2,500 rpm at 4 °C for 30 min to separate serum. The activities of serum glutamate oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), acid phosphatase (ACP), bilirubin, cholesterol and albumin were measured by using commercially available diagnostic kits.

Estimation of Antioxidants in Liver Tissue

For antioxidant assays, the tissue homogenate was prepared in ice cold phosphate buffer saline (PBS) (0.1 M, pH 7.4) and centrifuged at 5,000 rpm at 4 °C for 30 min. The supernatant was quantified for the assay of lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase (CAT) were performed with commercially available diagnostic kits (Sigma-Aldrich, New Delhi, India).

Hematological Studies

The blood was collected from heart and used for the estimation of red blood cells (RBC) and white blood cells (WBC) count using hemocytometer.

Histopathological Studies

The liver was blotted free of mucus. The tissues were washed in normal saline, cut into pieces of desired size and fixed in aqueous Bouin's solution for 12 h. After fixation, the tissues were cleaned, dehydrated through a gradual series of alcohol and then processed for paraffin embedding. Tissue sections of 5 µm in thickness were mounted on slides and stained with hematoxylin and eosine for photomicroscopic observations.

Statistical Analysis

The results were expressed as Mean ± SE (SEM), analyzed through one-way ANOVA, followed by the post hoc Dunnett's test for comparison of various treatments using the SPSS 16.0. Differences were considered statistically significant at $p < 0.05$.

3. Results

The Phytochemical Screening

The phytochemical screening of leaves extract of *A. marmelos* is shown in Table.1. Primary screening of AEAM demonstrated the presence of phenols, alkaloids, saponin, tannins and flavanoids in the extract.

Fifty eight compounds were identified in extract of *A. marmelos* by GC-MS analysis. The chromatogram obtained are shown in fig 1. The studies on the active principles in the leaves of *A. marmelos* extract by GC-MS analysis clearly showed the presence of different compounds. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (area %) were presented in Table-2. The prevailing compounds were citronellol (37.85%), limonene (19.08%), ceneol (16.97%), menthol (12.90%), limonene oxide (6.27%), piperitone (3.61%), limonene (2.86%), linolenic acid (2.32%), chromane (2.20%), surfynol (1.93%), phthalic acid (1.83%), ocimenol (1.72%), silane (1.65%), oleic acid (1.39%), campholonic acid (1.36%), sinapic acid (1.11%), ledol (1.01%), carvenone (1.00%), verbenol (1.00%) etc.

Effect of AEAM on Serum Enzymes

The animals treated with toxic doses of CPA had markedly elevated values of the serum SGOT, SGPT, LDH, ALP, ACP, bilirubin, cholesterol and significantly decreased ($p < 0.001$) level of albumin compared to control mice (Fig. 2). Serum enzymes values in the animals treated with different doses of AEAM (400, 500 and 600 mg/kg) were significantly ($p < 0.001$, $p < 0.01$) lower than those of CPA-treated group and recovered serum levels of albumin near to normal content. The effects of the AEAM were comparable to that of silymarin treated mice. CPA-treated with AEAM at 600 mg/kg bw showed the maximum decrease level of SGOT, SGPT, LDH, ALP, ACP, bilirubin, cholesterol and augmentation in the level of albumin as compared to other groups received different doses of AEAM.

Effect of AEAM on Lipid Peroxidation (LPO)

The level of LPO in CPA-treated mice was significantly higher ($p < 0.001$) than those in the control group (Fig 3). Different doses of AEAM (400, 500 and

600 mg/kg bw) administered groups significantly ($p < 0.001$) inhibit the level of LPO. The maximum inhibition at the level of LPO seen in the group treated with 600 mg/kg of AEAM. The outcome suggests that AEAM at 600 mg/kg dose has shown a maximum decrease in the level of LPO when compared to 400 and 500 mg/kg bw.

Effect of AEAM on Superoxide Dismutase (SOD)

The level of antioxidant enzyme SOD was significantly decreased ($p < 0.001$) in CPA-treated groups as compared to the control group (Fig 3). CPA + silymarin and different doses of AEAM received groups significantly ($p < 0.001$) increase the level of SOD. The outcome suggests that all the doses of AEAM increase the level of SOD, but AEAM at 600 mg/kg was observed to be more effective.

Effect of AEAM on Catalase (CAT)

The level of CAT was significantly decreased ($p < 0.001$) in CPA-treated groups when compared to the control group. CPA + silymarin and different doses of AEAM (400, 500 and 600 mg/kg) received groups mice significantly ($p < 0.001$) increase the level of CAT. A slight increase was found after the treatment with 400 mg/kg bw dose when compared with the CPA-treated group. However administration of doses at 500 and 600 mg/kg bw produced significant increase in CAT levels (Fig 3).

Effect of AEAM on Hematological Parameters

The effect of AEAM on WBC and RBC of CPA-treated animals are given in Fig.4. Initially, there was a significant decrease ($p < 0.001$) in the WBC and RBC of CPA-treated mice but later WBC and RBC was found to be significantly higher ($p < 0.001$) in CPA + AEAM treated group. The outcome suggests that AEAM dose 600 mg/kg is more effective than the rest of the doses

of AEAM. The results indicate that the administration of AEAM could stimulate the hemopoietic system.

Effect of AEAM on Histopathological Study

Histopathological examination of the liver sections of the control group showed normal architecture of the liver with distinct hepatic cells (Fig. 5a). The AEAM alone and silymarin treated mice showed a normal hepatic architecture with normal hepatocytes (Fig. 5b, c). The liver section of CPA intoxicated group showed inflammation in central vein and picnotic nuclei (Fig. 5d). Whereas the mice treated with AEAM at doses of 400, 500 and 600 mg/kg bw showed recovery from CPA-induced liver damage as evidenced from normal hepatocytes. The higher dose of 600 mg/kg bw showed significant attenuation of inflammation of liver central vein, indicating a marked protective activity similar to that observed in silymarin treated mice liver sections (Fig. 5h) and the effect was found to be dose dependent (Fig. 5e-g).

Table 1: Chemical analysis for phytoconstituents in the crude aqueous leaf extract of *A. marmelos*.

S. No.	Examination	Test performed	Result
1.	Alkaloids	Mayer's test	+
2.	Saponin	Foam test	+
3.	Carbohydrate	Fehling's test	-
4.	Protein and Amino Acids	Biuret test	-
5.	Phenolic	Folin-Ciocalteu's method	+
6.	Tannins	Folin and Ciocalteu method	+
7.	Flavanoids	Shinoda test	+
8.	Glycosides	Borntrager's test	-

(+) Presence, (-) Absence

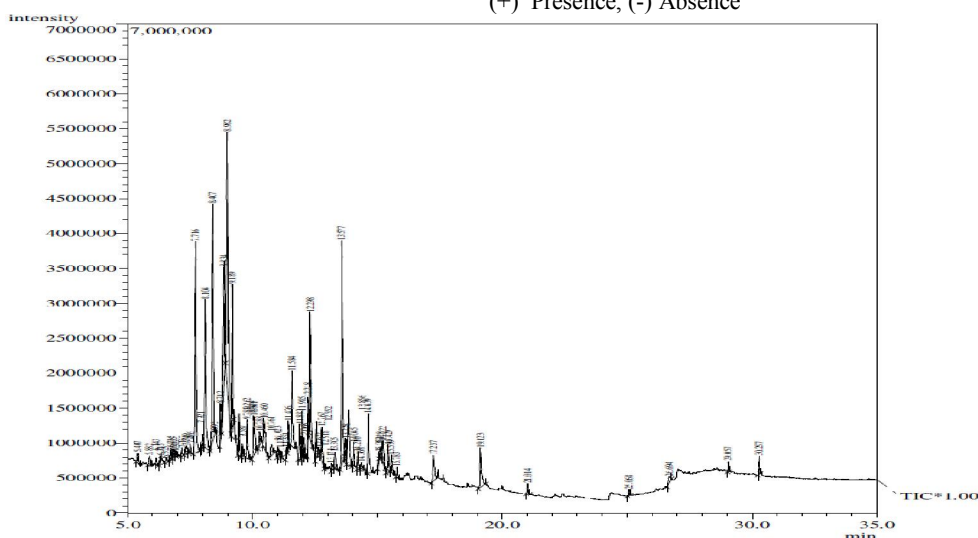


Fig 1. GC-MS analysis of leaf extract of *A. marmelos*.

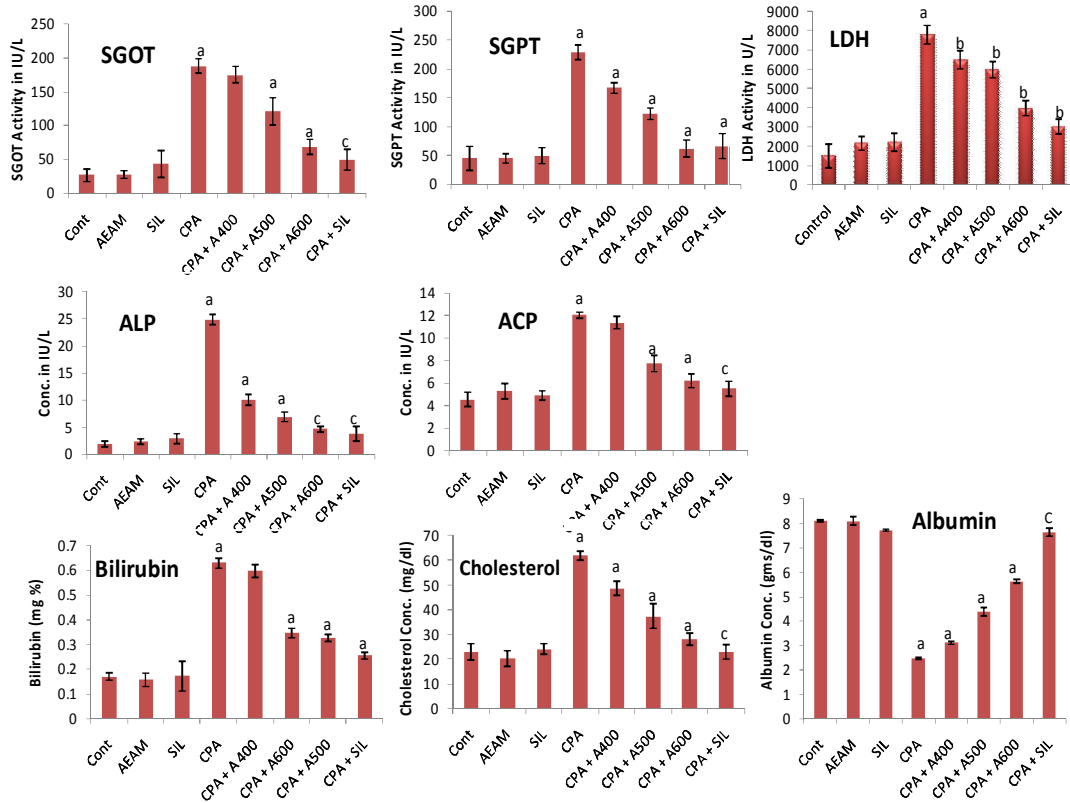


Fig. 2

Fig. 2. Protective effect of aqueous extract of *A. marmelos* on CPA-induced hepatotoxicity in the serum enzymes. Each bar represents the Mean \pm SE, n=6; ^ap<0.001, ^bp<0.01, ^cP<0.05 indicate statistically significantly different from control group.

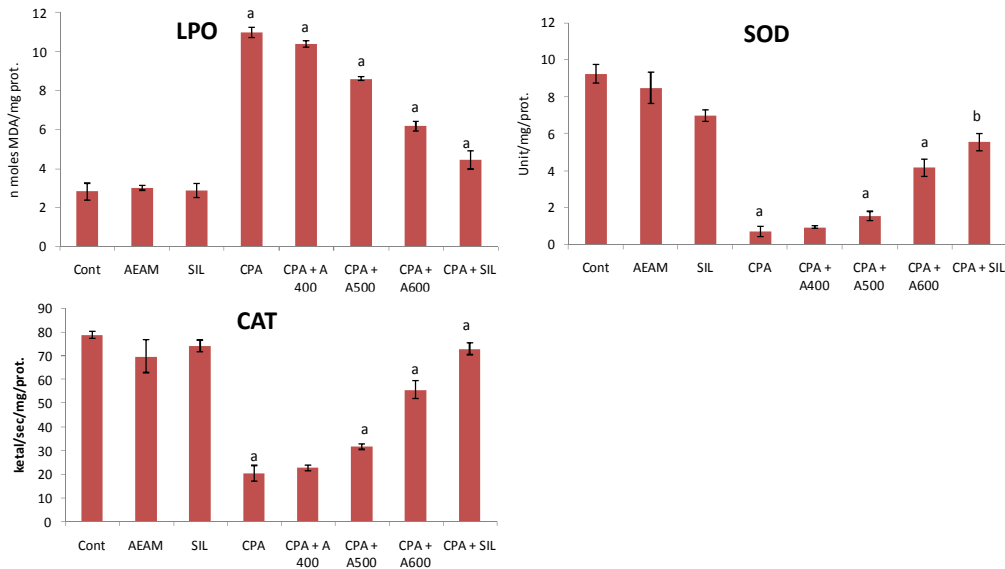


Fig. 3

Fig. 3. Protective effect of aqueous extract of *A. marmelos* on CPA-induced hepatotoxicity in the liver tissue. Each bar represents the Mean \pm SE, n=6; ^ap<0.001, ^bp<0.01, ^cP<0.05 indicate statistically significantly different from control group.

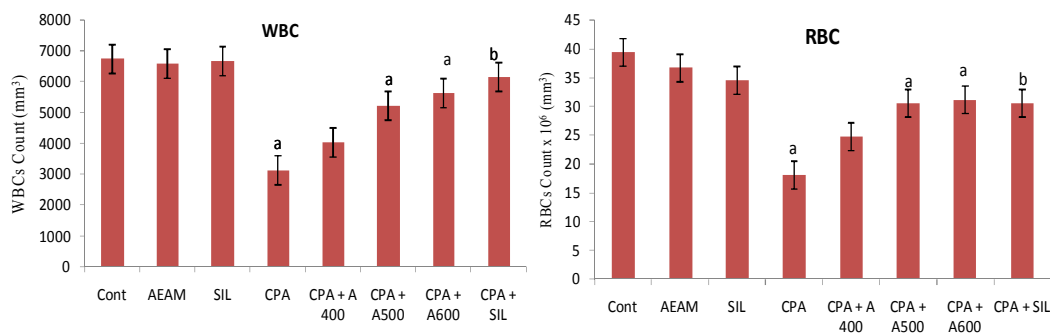


Fig. 4. Protective effect of aqueous extract of *A. marmelos* on CPA-induced hepatotoxicity in the hematology. Each bar represents the Mean \pm SE, n=6; ^ap<0.001, ^bp<0.05 indicate statistically significantly different from control group.

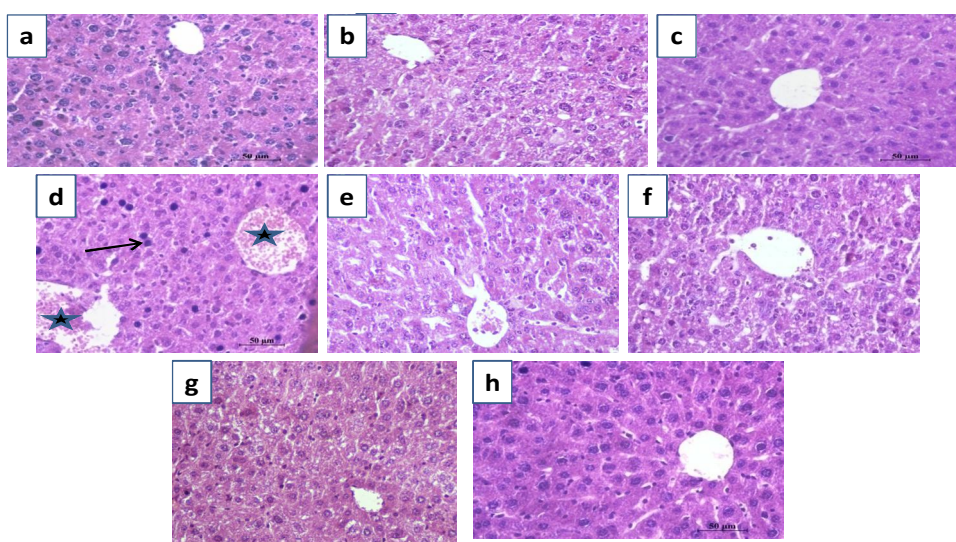


Fig. 5

Fig.5. Representative photomicrographs of liver sections (400X, H & E). The liver section from control animals showed regular cellular architecture with distinct hepatic cells and a central vein (a). In the AEAM alone and silymarin treated liver showed normal hepatic cells (b & c). The liver sections from the toxic-induced mice showed inflammation in central vein (Star), picnotic nuclei (Arrow) (d). The treatment of animals with CPA + AEAM at 400, 500, and 600 mg/kg revealed a better preservation of the liver architecture (e-g). CPA + silymarin treated liver showed normal hepatic cells (h).

Table 2: Phytochemicals identified in the leaf extract of *A. marmelos* based on GC-MS analysis.

No.	Retention Time (RT)	Molecular Formula	Molecular Weight	Area (%)	Name of Compounds
1	5.407	C ₈ H ₁₄ O ₂	146	0.46	Acetic Acid
2	5.882	C ₅ H ₁₂ N ₂ O ₂	132	0.47	Ornithine
3	6.143	C ₁₀ H ₁₈ O ₂	170	0.29	Linalooloxide
4	6.313	C ₁₀ H ₁₈	138	0.20	Citronellene
5	6.525	C ₁₁ H ₂₀ O ₂	184	0.20	Formic Acid,Pinane
6	6.735	C ₁₀ H ₁₆ O	152	0.48	Citral
7	6.835	C ₁₀ H ₁₆ O	152	0.48	Citral
8	7.349	C ₁₀ H ₁₈ O	154	0.46	Cineol
9	7.716	C ₁₀ H ₁₈ O ₂	170	8.55	Cineol
10	8.104	C ₁₀ H ₁₆ O	152	6.27	Limonene Oxide
11	8.712	C ₁₀ H ₁₆ O	152	1.00	Verbenol
12	8.834	C ₁₀ H ₁₈	138	7.32	Citronellol
13	8.982	C ₁₀ H ₁₈ O ₂	156	12.90	Menthol

14	9.189	C ₁₀ H ₁₆ O ₂	168	3.61	Piperitone
15	9.262	C ₁₅ H ₂₆ O	222	0.34	Viridifloral
16	9.781	C ₁₀ H ₁₆ O ₂	168	1.36	Campholonic Acid
17	10.061	C ₁₀ H ₁₈ O	154	1.72	Ocimenol
18	10.761	C ₇ H ₁₄ O ₂	130	0.69	Heptanoic Acid
19	11.139	C ₁₅ H ₂₆ O	222	0.13	Globulol
20	11.584	C ₁₀ H ₁₆ O ₂	168	2.86	Limonene
21	11.882	C ₁₄ H ₂₄ O	208	1.12	Chromane
22	11.985	C ₈ H ₁₄ O ₂	152	1.00	Carvenone
23	12.068	C ₁₅ H ₂₆ O ₂	238	0.58	Cedrane
24	12.552	C ₁₀ H ₁₆ O ₂	168	1.09	Limonene
25	12.672	C ₂₉ H ₃₈ O ₈	514	0.23	Anisic Acid
26	12.799	C ₁₀ H ₁₈ O	154	8.42	Cineol
27	12.918	C ₁₅ H ₂₆ O	222	0.34	Veridiflorol
28	13.171	C ₁₅ H ₂₆ O	222	0.30	Ledol
29	13.305	C ₂₉ H ₃₈ O ₈	514	0.67	Roridin
30	13.693	C ₁₀ H ₁₂ O ₂	164	0.35	Eugenol
31	13.728	C ₂₀ H ₄₂ O	298	0.47	Eicosanol
32	14.059	C ₁₀ H ₁₆ O	153	9.95	Limonene
33	14.639	C ₁₆ H ₂₂ O ₄	278	1.83	Phthalic Acid
34	15.019	C ₁₀ H ₂₀ O ₂	172	28.40	Citronellol
35	15.052	C ₁₉ H ₃₀ O ₃	306	0.28	Anavar
36	15.429	C ₁₁ H ₁₂ O ₅	224	1.11	Sinapic Acid
37	15.559	C ₁₄ H ₂₈ O ₂	228	0.69	Myristic Acid,
38	15.783	C ₁₈ H ₃₈ O	270	0.27	Alfol
39	16.828	C ₁₉ H ₃₂ O ₂	204	2.15	Linolenic Acid
40	17.237	C ₁₈ H ₃₄ O ₂	282	1.39	Oleic Acid
41	17.250	C ₁₁ H ₂₀ O	168	0.14	Pinanol
42	17.412	C ₃₀ H ₃₄ O ₁₃	602	0.79	Picrotoxin
43	17.664	C ₁₃ H ₂₂ Si	206	1.65	Silane
44	18.161	C ₁₀ H ₂₀ O ₂	172	2.13	Citronellol
45	18.467	C ₁₀ H ₁₄ O ₂	166	0.02	Cerulignol
46	20.128	C ₁₄ H ₂₄ O	208	1.08	Chromane
47	20.275	C ₁₁ H ₁₆ NO ₂	208	0.49	Pilocarpine
48	20.468	C ₁₅ H ₂₆ O	222	0.18	Viridifloro
49	21.155	C ₁₀ H ₁₈ O	154	0.41	Caranal
50	21.411	C ₁₅ H ₂₆ O	222	1.01	Ledol
51	21.525	C ₂₀ H ₂₆ O	378	0.35	Carinol
52	21.792	C ₁₀ H ₁₈ O ₂	170	1.93	Surfynol
53	23.741	C ₂₀ H ₄₂ O	298	0.75	Eicosanol
54	24.428	C ₁₅ H ₂₄ O	220	0.26	Isospathulenol
55	24.540	C ₁₇ H ₂₈ O ₄	296	0.12	Nerolidol-Epoxyacetate
56	26.017	C ₁₅ H ₂₄ O	220	0.20	Spathulenol
57	30.852	C ₂₀ H ₃₆ O ₂	308	0.17	Linoleic Acid
58	34.125	C ₁₄ H ₂₃ N	205	0.75	Octylaniline

4. Discussion

The liver is one of the vital organs in human body, responsible for detoxification of toxic chemicals and drugs. Thus, it is the target organ for all toxic chemicals and drugs. A number of drugs and toxic industrial chemicals have been reported to cause severe hepatic injuries in human, which are sometimes difficult to manage by medical therapies. CPA is a well-known chemotherapeutic drug that is used in cancer treatment. The side effect of this drug is fatal hepatic damages in humans and experimental animals. Thus, it can be treated as a hepatotoxic agent (Shokrzadeh *et al.*, 2014).

The therapeutic and toxic effect of CPA is the requirement of bioactivation by hepatic microsomal cytochrome P450 mixed function oxidase system (Lindley *et al.*, 2002). Metabolic activation through the predominant pathway yields 4-hydroxycyclophosphamide (HCP) that exist in equilibrium with aldophosphamide, which degrades by β -elimination to form phosphoramidate mustard (PM) and an equimolar amount of the toxic byproduct, acrolein. Phosphoramidate mustard brings about interstrand cross-links between opposite DNA strands and hampers the replication and transcription process that characterize the clinical activity of CPA (Paolo *et al.*, 2005). Hence, the therapeutic effect of CPA is

attributed to PM, while acrolein is associated with unwanted side effects. Bioconversion of CPA to these metabolites leads to the formation of high levels of Reactive Oxygen Species (ROS), which result in decreased antioxidant capacity (Stankiewicz *et al.*, 2002).

Hepatic cells participate in a variety of metabolic activities and contain several enzymes. Serum transaminases (SGOT and SGPT) are the most universally important markers for hepatic tissue injury (Lata *et al.*, 2014). Serum transaminases are the cytoplasmic enzymes involved in amino acid metabolisms (Wang *et al.*, 2012). Liver marker enzymes are localized in the cytosol of hepatic cells and thus are extruded into the serum when cells are damaged or necrotic. Therefore, determination of serum transaminases has great clinical and diagnostic significance. In the present study, CPA-induced cytotoxicity is manifested by increased level of serum transaminases (SGOT and SGPT). This indicated the presence of necrotic cells that resulted leakage of these enzymes to serum. Our results are parallel to that of the previously reported by Shanmugarajan *et al.* (2008) that, CPA administration induced significant increase in serum transaminases. Combined therapy with AEAM prevented CPA-induced toxic consequences and restored the serum transaminases towards control. The reduction in levels of serum enzymes by extract is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by CPA (Habibi *et al.*, 2014).

Serum phosphatases (ALP and ACP) are involved in varieties of metabolic activities such as permeability, growth and cell differentiation, protein synthesis, absorption and transport of nutrients etc. The present results show that there is significant increase in serum phosphatases in CPA intoxicated mice. Elevated level of serum ALP may be due to increased synthesis in presence of increasing biliary pressure (Palanivel *et al.*, 2008). Administration of different doses of AEAM significantly attenuated the above parameter compared to CPA intoxicated mice. The above changes may be considered as an expression of the functional improvement of hepatocytes, which may be caused by an accelerated regeneration of parenchyma cells. Thus, the decreased level of serum phosphatases suggests the hepatoprotective potential of AEAM against CPA.

Lactate dehydrogenase (LDH) catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD⁺ (Pathak and Vinayak, 2005). According to the results obtained, after CPA administration, there is an increase in LDH in serum and liver showing increased rate of glycolysis with excess production of pyruvate than its utilization by Krebs cycle leading to lactate

synthesis. The mice treated with different doses of AEAM showed a significant decrease in LDH in dose dependent manner, which showed the restoration of the LDH to the near normal values.

Bilirubin is the breakdown product of haem in red blood cells in the liver. High levels will cause jaundice and are indicative of damage to the liver and bile duct ((Dubey and Mehta, 2014). Increase in total serum bilirubin concentration after CPA administration might be attributed to the failure of normal uptake, conjugation and excreted by the damaged hepatic parenchyma. AEAM and silymarin showed decreased level of serum bilirubin, which suggests that it may be used as protectant for jaundice.

Cholesterol is an essential structural component of mammalian cell membrane and it is required to establish proper membrane permeability and fluidity. In the present study, liver lipid profile such as cholesterol was significantly elevated in serum and this indicated deterioration in hepatic function due to the damage caused by CPA administration. The elevated level of cholesterol was significantly reduced in mice treated with AEAM as with silymarin.

Albumin is essential for maintaining the osmotic pressure needed for proper distribution of body fluids between blood vessels and body tissues. The level of albumin in serum with CPA administered mice was significantly decreased. Administration of the different doses of AEAM increased the level of albumin showing its protective role. Furthermore, the hepatoprotective effect of AEAM appeared to be as beneficial as that of silymarin

In normal condition, organs possess a powerful antioxidant defense system, such as SOD and CAT (Tobias and Philip, 2011). There are number of evidence that oxidative stress, produced by ROS, plays a vital role in the pathogenesis of CPA-induced hepatic tissue damage (Shanmugarajan *et al.*, 2008). The removal and neutralization of these noxious toxic metabolites are considered to be the vital initial steps in the prevention of CPA-related liver diseases.

In our study, we found that CPA administration significantly increased the hepatic LPO level and markedly decreased SOD and CAT levels. Similar findings have been reported by many investigators (Shanmugarajan *et al.*, 2008).

Lipid peroxidation (LPO) is one of the most important indicators of oxidative stress. Unsaturated fatty acids present in cellular membranes are a common target for ROS. Lipid components of the cell are especially susceptible to reactions with free radicals, resulting in LPO. Lipid peroxidation refers to the oxidative degradation of lipids. It is the action of abstraction of a hydrogen atom from the side chain of polyunsaturated fatty acids in the membrane. The present data revealed that CPA administration

produced a marked oxidative impact, as evidenced by the significant increase in LPO. Treatment with different doses of AEAM afforded better protection through decreased production of free radicals derivatives, as is evident from the decreased level of LPO in a dose dependent manner.

Superoxide is believed to be the cause of other ROS formations such as hydrogen peroxide and hydroxyl radicals. Therefore, superoxide scavenging capacity in the body is the first line of defense against oxidative stress. Superoxide anion has been suggested as a major cause of CPA toxicity (Rceci *et al.*, 2006). The decreased activity of SOD in the present study might be the reason of oxidative damage in CPA-treated animals. AEAM prevented the loss of SOD activity in the dose dependent manner, in CPA-treated mice. Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the hydrogen peroxide (H_2O_2) to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by ROS (Foyerand and Shigeoka, 2010). In the present investigation, it has been found that CPA-induced toxicity by damaging the antioxidant defense system of organs such as SOD and CAT in liver. However, treatment of mice, with AEAM showed a strong protective potential against oxidative stress caused by CPA.

The results of the present study showed that co-administration of varied doses of AEAM diminished CPA-induced oxidative stress by increasing antioxidant status. These results support the hypothesis that oxidative damage is neutralized when antioxidants such *Ficus hispida*, squalene and DL- α -lipoic acid are administered before or after the induction of oxidative stress (Shanmugarajan *et al.*, 2008).

The present study indicates that AEAM significantly enhanced the WBC and RBC count as compared to the CPA intoxicated mice. These observations assume great significance, as anemia is a common complication in cancer. The situation aggravates further during chemotherapy, since a majority of anti-neoplastic agents exert suppressive effects on erythropoiesis and thereby limiting the use of drugs (Sreelatha *et al.*, 2012).

The hepatoprotective effect of AEAM was confirmed by histopathological examination of the liver of controlled and treated animals. In the present study, the histological architecture of CPA treated liver section showed the degeneration of hepatocytes. Whereas the mice treated with AEAM at different doses showed recovery from CPA-induced liver damage in a dose dependent manner. The best protection in architecture of liver was found at 600

mg/kg bw, which was found to be parallel to control and silymarin treated groups. Hence, the histopathological examinations of AEAM treated group of mice showing hepatoprotective effects and this is supported by biochemical studies.

Natural antioxidants are present in all parts of plant. Phytochemical constituents are responsible for medicinal activity of plant species. Phytochemical screening of the *A. marmelos* leaves extract revealed the presence of alkaloids, tannins, saponin, flavonoids and phenols, which are able to scavenge free radicals such as superoxide or lipid peroxides. These results suggest that the phytochemical constituents possess potential anti-inflammatory, antimicrobial and antioxidant for curing various ailments

The antioxidant effect of *A. marmelos* may be due to the presence of cineol, chromane, eugenol and myristic acid (Maity *et al.*, 2009). Results pertaining to GC-MS analysis led to the identification of number of compounds from the GC fractions of the extract of *A. marmelos*. Our GC-MS results have shown that it contains high amount of it. Cineol, chromane, eugenol and myristic acid belong to an extensive class of polyphenolic flavonoid compounds almost ubiquitous in plants and plant food sources. These compounds are considered to be a strong antioxidant due to its ability to scavenge free radicals and bind transition metal ions. These properties of compounds allow these to inhibit lipid peroxidation and have anti-inflammatory properties (Sharma *et al.*, 2011). In addition to these compounds, *A. marmelos* extract also contains citronellol, limonene, menthol, limonene oxide, piperitone, linolenic acid, surfynol, phthalic acid, ocimenol, silane, oleic acid, campholonic acid, sinapic acid, ledol, carvenone, verbenol etc. and citronellol is known to be potential antioxidant due to its ability to scavenge free radicals and active oxygen species such as singlet oxygen, superoxide anion radical and hydroxyl radicals (Sharma *et al.*, 2011). The presence of these compounds could explain the antioxidant activity found in the crude extract.

In the last decade, epidemiological studies suggested the importance of plant polyphenols against degenerative diseases (Wanasundara *et al.*, 2008). The over abundance of phenolic compounds from several plant extracts have been reported to possess strong antioxidant activities (Anish *et al.*, 2013). The phenolic groups can accept an electron to form relatively stable phenoxyl radicals, thereby disrupting chain oxidation reactions in cellular components. There are increasing evidences that as antioxidants, phenols may protect cell constituents against oxidative damage and therefore, limit the risk of drug induced with oxidative stress (Maniana *et al.*, 2008). The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites.

Flavonoids are the most common groups of polyphenols in the human diet, which are found in plants and reported to be efficient as antioxidants (Samira and Ulrike, 2012). Major dietary sources of flavonoids include wine, plants extracts, vegetables, cereals and fruit juices. Therefore, the flavonoids were found to have higher radical scavenging activity and reducing power of free radicals (Ebrahimzadeh *et al.*, 2010).

Tannins bind to proline rich protein and interfere with protein synthesis. The plant extract revealed to contain saponins, which are known to produce inhibitory effect on inflammation. Saponins have the property of precipitating and coagulating red blood cells. Some of the characteristics of saponins include formation of foams in aqueous solutions, hemolytic activity, cholesterol binding properties and bitterness (Okwu, 2004). Alkaloids are associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity. Several workers have reported the analgesic, antispasmodic and antibacterial properties of alkaloids (Okwu, 2004). Glycosides are known to lower the blood pressure. The results obtained in this study thus suggest that identified phytochemical compounds may be bioactive constituents and protect the drug induced hepatotoxicity in a dose dependent manner. The dose 600 mg/kg bw was found to be the best for protection against CPA-induced toxicity in mice in the present study. Therefore, this study provides an experimental evidence for the protective and beneficial role of AEAM against CPA intoxication.

A. marmelos has an important place in traditions of Indian folk medicines. Our investigations have shown that AEAM may be used as hepatoprotectant. Different active components present in AEAM, may be responsible for the hepatoprotectivity. The present study suggested that AEAM has a preventive and curative role in CPA-induced hepatotoxicity in mice. However, further investigations and analysis are required in order to establish the mechanism of these active compounds which are responsible for the hepatoprotection against CPA-induced toxicity. Thus, it may be hypothesized that AEAM protects the liver tissues by scavenging the toxic metabolites, which is evidenced by the normalization of the clinical chemistry.

Acknowledgement

The financial assistance in the form of SRF to Sangita Singh from the University Grant Commission, New Delhi, is gratefully acknowledged.

Corresponding Author:

Sangita Singh: Department of Zoology, MMV, Banaras Hindu University, Varanasi-

221005, India

Email: bioinfo.sangita@gmail.com

Swarn Lata : Department of Zoology, MMV, Banaras Hindu University, Varanasi-221005, India

E-mail: Swarn Lata: swarn_vns@rediffmail.com / swarnlatabhu12@gmail.com

Tel. +91 9415301826; fax: +91 5422367927

Kavindra Nath Tiwari : Department of Botany, MMV, Banaras Hindu University, Varanasi-221005, India.

Email: K. N. Tiwari: kntiwari@bhu@gmail.com

References

1. Anish N, Maumita B, Anita M. Antioxidant activities and cytotoxicity of *Zingiber zerumbet* (L.) smith rhizome. J Pharmac Phyto 2013; 2: 102-108.
2. Chatterjee T. Medicinal plants with hepatoprotective properties, in Herbal Options. 135 Books and Allied (P) Ltd, Calcutta, India, 3rd edition. 2000.
3. Ebrahimzadeh MA, Seyed MN, Seyed FN, Fatemeh B, Ahmad RB. Antioxidant and free radical scavenging activity of *H. officinalis* L. var. *angustifolius*, *V. odorata*, *B. hyrcana* and *C. speciosum*. Pak J Pharm Sci 2010; 23: 29-34.
4. Evans WC. Trease and Evans Pharmacognosy. Saunders Elsevier, Edinburgh. 2009.
5. Dubey S, Mehta SC. Hepatoprotective activity of *Euphorbia Hirta* Linn. plant against carbon tetrachloride-induced hepatic injury in rats. Int Conf Food, Bio Med Sci Bangkok (Thailand). 2014.
6. Faremi TY, Suru SM, Fafunso MA, Obioha UE. Hepatoprotective potentials of *Phyllanthus amarus* against ethanol-induced oxidative stress in rats. Food Chem Toxicol 2008; 46: 265.
7. Foyerand CH, Shigeoka S. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant Physiol 2010; 155: 93-100.
8. Habibi E, Mohammad S, Aroona C, Farshad N, Razieh K, Amirhossein A. Protective effects of *Origanum vulgare* ethanol extract against cyclophosphamide-induced liver toxicity in mice. Pharma bio 2014; 1-6.
9. Hossain MA, Shah MD, Sakari M. Gas chromatography-mass spectrometry analysis of various organic extracts of *Merremia borneensis* from Sabah. Asian Pac J Trop Med 2011; 4: 637.
10. Khare CP. *Aegle marmelos* In: Indian herbal remedies. Khare C.P. (Ed.). Springer, USA 2004; pp. 27-29.
11. Lata S, Singh S, Tiwari KN, Upadhyay R. Evaluation of the antioxidant and hepatoprotective effect of *Phyllanthus fraternus* against a chemotherapeutic drug

- cyclophosphamide. *App Biochem Biotech* 2014; 173:2163–2173.
12. Lindley C, Geraldine H, Jeannine S, Mccune Stephanie F, Stacy S, Roy S, Hawke Hongbing W, Darryl G, Summer J, Bingfang Y, Edward L. The effect of cyclophosphamide with and without dexamethasone on cytochrome p450 3a4 and 2b6 in human hepatocytes. *Drug Met Disp* 2002; 30: 814–821.
 13. Maity P, Hansda D, Bandyopadhyay U, Mishra DK. Biological activities of crude extracts and chemical constituents of Bael, *Aegle marmelos* (L.). *Corr. In J Exp Bio* 2009; 47: 849–861.
 14. Maniana R, Anusuyab N, Siddhurajub P, Manian S. The antioxidant activity and free radical scavenging potential of two different solvent extracts of *Camellia sinensis* (L.) O. Kuntz, *Ficus bengalensis* L. and *Ficus racemosa* L. *Food Chem* 2008;107: 1000-1007.
 15. Marzine PS, Gilbert R. The effect of an aqueous extract of *A. marmelos* fruits on serum and tissue lipids in experimental diabetes. *J Sci Food Agri* 2005; 85: 569-573.
 16. Oh MS, Chang MS, Park W, Kim DR, Bae H, Huh Y, Park SK. Yukmijihwang-tang protects against cyclophosphamide-induced reproductive toxicity. *Repro Toxicol* 2007; 24: 365-370.
 17. Okwu DE. Phytochemicals and vitamin content of indigenous species of southeastern Nigeria. *J Sus Agri Env* 2004; 6: 30-37.
 18. Palanivel MG, Balasubramanian RR, Senthil K, John WE, Ekambaram PK, Mani RK, Kunchu K, Mohanraj PK, Balasundaram J. Hepatoprotective and antioxidant effect of *Pisonia aculeata* L. against CCl₄-induced hepatic damage in rats. *Sci Pharm* 2008; 76: 203.
 19. Paolo AD, Danesi R, Tacca MD. Pharmacogenetics of neoplastic disease: New trends. *Pharm Res* 2004; 49: 331-342.
 20. Pathak C, Vinayak M. Modulation of lactate dehydrogenase isozymes by modified base queueine. *Mol Bio Rep* 2005; 32: 191–196.
 21. Rceci RJ, Hann IM, Smith OP. *Pediatric hematology* (3rd ed.). Wiley-Blackwell 2006; 763, 3400-2.
 22. Saleem TSM, Madhusudhana SC, Ramkanth SVST, Rajan KKM, Gauthaman K. Hepatoprotective herbs—a review. *Int J Res Pharm Sci* 2010; 1: 1–5.
 23. Samira H, Ulrike M. The role of flavonoids in root rhizosphere signalling: opportunities and challenges for improving plant–microbe interactions. *J Exp Bot* 2012; 430: 1-16.
 24. Shanmugarajan TS, Arunsundar M, Somasundaram I, Sivaraman D, Krishnakumar E, Ravichandran V. Ameliorative effect of *Ficus hispida* Linn. Leaf extract on cyclophosphamide-induced oxidative hepatic injury in rats. *J Pharm Toxicol* 2008; 3: 363-372.
 25. Sharma NG, Dubey SK, Sharma P, Sati N. Medicinal values of bael (*Aegle marmelos*) (L.) *Corr.: A Review. Int J Cur Pharm Rev Res* 2011; 1: 3.
 26. Shokrzadeh M, Chabra A, Ahmadi A, Naghshvar F, Habibi E, Salehi F, Assadpour S. Hepatoprotective effects of *Zataria multiflora* ethanolic extract on liver toxicity induced by cyclophosphamide in mice. *Drug Res* 2014; 10.1055/s-0034-1370932.
 27. Sreelatha S, Dinesh E, Uma C. Antioxidant properties of rajgira (*Amaranthus paniculatus*) leaves and potential synergy in chemoprevention. *Asia Pac J Can Pre* 2012; 13: 2775-2780.
 28. Stankiewicz A, Skrzydlewska E, Makiela M. Effects of amifostine on liver oxidative stress caused by cyclophosphamide administration to rats. *Drug Met Drug Int* 2002; 19: 67-82.
 29. Tobias CF, Philip H. Biomarkers for drug-induced renal damage and nephrotoxicity. *An Over App Toxicol AAPS J* 2012; 13: 615-631.
 30. Vinodhini S, Malairajan S, Hazeena B. The hepatoprotective effect of bael leaves (*Aegle marmelos*) in alcohol induced liver injury in albino rats. *Int J Sci Tech* 2007; 2: 83-92.
 31. Wanasundara PK, Shahidi F. Process induced changes in edible oils. *Adv exp med bio* 1998; 434: 135-60.
 32. Wang CS, Chang Ting-Tsung, Yao, Wei-Jen, Wang, Shan-Tair, Chou, Pesus. Impact of increasing alanine aminotransferase levels within normal range on incident diabetes. *J Form Med Ass* 2012; 111: 201–8.

8/28/2014