

Reprinted from

International Journal
of
Health Research

Peer-reviewed Online Journal

<http://www.ijhr.org>

Abstracting/Indexing

Embase, Index Corpenicus, Chemical Abstracts, Socolar, EBSCO, African Journal Online,
African Index Medicus, Open-J-Gate, Directory of Open Access Journals (DOAJ)

PORACOM
Academic Publishers

International Journal of Health Research

The *International Journal of Health Research* is an online international journal allowing free unlimited access to abstract and full-text of published articles. The journal is devoted to the promotion of health sciences and related disciplines (including medicine, pharmacy, nursing, biotechnology, cell and molecular biology, and related engineering fields). It seeks particularly (but not exclusively) to encourage multidisciplinary research and collaboration among scientists, the industry and the healthcare professionals. It will also provide an international forum for the communication and evaluation of data, methods and findings in health sciences and related disciplines. The journal welcomes original research papers, reviews and case reports on current topics of special interest and relevance. All manuscripts will be subject to rapid peer review. Those of high quality (not previously published and not under consideration for publication) will be published without delay. The maximum length of manuscripts should normally be 10,000 words (20 single-spaced typewritten pages) for review, 6,000 words for research articles, 3,000 for technical notes, case reports, commentaries and short communications.

Submission of Manuscript: The *International Journal of Health Research* uses a journal management software to allow authors track the changes to their submission. All manuscripts must be in MS Word and in English and should be submitted online at <http://www.ijhr.org>. Authors who do not want to submit online or cannot submit online should send their manuscript by e-mail attachment (in single file) to the editorial office below. Submission of a manuscript is an indication that the content has not been published or under consideration for publication elsewhere. Authors may submit the names of expert reviewers or those they do not want to review their papers.

Enquiries:

The Editorial Office
International Journal of Health Research
Dean's Office, College of Medicine
Madonna University, Elele Campus, Rivers State
E-mail: editor_ijhr@yahoo.com or editor@ijhr.org

PORACOM
Academic Publishers

Original Research Article

Open Access
Online Journal

Hepatoprotective and Antioxidant Activities of Fruit pulp of *Limonia acidissima* Linn

Received: 25-Apr-09

Revised: 04-Nov-09

Accepted: 29 Dec 2009

Abstract

Purpose: To investigate the possible hepatoprotective and antioxidant activities of methanolic extract of fruit pulp of *Limonia acidissima* Linn (MELA) against carbon tetra chloride (CCl_4) induced liver damage in rats.

Methods: 200 and 400 mg/kg p.o doses of MELA was administered to group of animals for 10 days. Silymarin (100mg/kg) served as a standard and carbon tetrachloride at a dose of 0.5 ml/kg i.p was used to induce liver damage. The various hepatic biochemical parameters like aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total protein (TP), total bilirubin (TB), gamma glutamyl transferase (GGT) and in vivo antioxidant biochemical parameters like super oxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) levels were evaluated to determine the hepatoprotective and antioxidant activity.

Results: Significant dose dependant hepatoprotective effect of both doses of MELA was observed ($p < 0.001$). The fruit extract significantly restored the antioxidant enzyme level in the liver cells.

Conclusion: MELA exhibited significant dose dependant protective effect against CCl_4 induced liver damage which can be mainly attributed to the antioxidant property of the extract. This study rationalized the ethno-medicinal use of the plant for curing hepatic injuries.

Keywords: *Limonia acidissima*, hepatoprotective, carbon tetrachloride, antioxidant enzymes, Silymarin.

Ilango K^{1*}

Chitra V²

¹Department of Pharmaceutical Chemistry, SRM College of Pharmacy, SRM University, Kattankulathur, Kanchipuram District – 603203, Tamilnadu, India.

²Department of Pharmacology, SRM College of Pharmacy, SRM University, Kattankulathur, Kanchipuram District-603203. Tamilnadu, India.

***For Correspondence:**

Tel: +91-44-27453160

Fax: 91-44 - 27455717

E-mail: ilangok67@gmail.com

Introduction

Liver is the most important organ, which plays a pivotal role in regulating various physiological processes in the body. It is involved in several vital functions, such as metabolism, secretion and storage. It has great capacity to detoxicate toxic substances and synthesize useful principles. Therefore, damage to the liver inflicted by hepatotoxic agents is of grave consequences¹. Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorders. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages². In addition, serum levels of many biochemical markers like aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and bilirubin were also elevated.

In spite of tremendous advances in modern medicine, there are not many effective drugs available that stimulate liver function, offer protection to the liver from damage or help to regenerate hepatic cells³. In the absence of reliable liver-protective drugs in modern medicine, there are a number of medicinal preparations in ayurveda recommended for the treatment of liver disorders⁴ that have been in use for centuries which are quite often claimed to offer significant relief.

Limonia acidissima Linn syn *Feronia limonia* (Rutaceae) is a moderate sized deciduous tree grown throughout India. Its fruits are woody, rough and used as a substitute for bael in diarrhoea and dysentery while the bark and leaves are used for vitiated conditions of *vata* and *pitta*. The fruits are used for tumors, asthma, wounds, cardiac debility and hepatitis⁵. It has been reported that this part of the plant contains flavanoids, glycosides, saponins, tannins⁶, coumarins⁷ and tyramine derivatives⁸. Fruit shells of *L. acidissima* have been reported to have antifungal compounds namely psoralene, xanthotoxin, 2,6-dimethoxybenzoquinone and osthenol⁹ while the leaves have hepatoprotective activity¹⁰. The stem bark of

the plant yielded (-) - (2S) - 5, 3' - dihydroxy-4'-methoxy-6",6"-dimethylchromeno-(7, 8, 2",3")-flavanone along with several known compounds including an alkaloid, five coumarins, a flavanone, a lignan, three sterols and a triterpene which were found to possess antimicrobial activity¹¹.

In spite of the medicinal claim for the fruit as a hepatoprotective, there are no reports in the literature regarding this pharmacological effect use as a hepatoprotective agent. Thus the present study was performed to evaluate the hepatoprotective activity of the methanolic extract of *L. acidissima* fruit pulp against CCl₄-induced hepatic injury in rats.

Materials and Methods

Plant Materials

Fruits of *L. acidissima* were purchased from local market in Chennai in August 2007, separated into pulp and shell, and authenticated by Prof P. Jayaraman, a botanist at Plant Anatomy Research Centre (PARC), Tambaram, Chennai. A voucher specimen (SRMCP/08/07) was deposited in the Department of Pharmacognosy, SRM College of Pharmacy for future reference. The fruit pulp was shade dried, coarsely powdered using a cutter mill and stored in an air-tight, light resistant container for further use.

Preparation of extract

The coarsely powdered fruit pulp was defatted with hexane using soxhlet apparatus. The defatted material was further extracted with methanol using soxhlet apparatus and the extract obtained was concentrated using rotary evaporator and the extractive value, found to be 23% w/w, was then stored in a vacuum desiccator.

Animals

Inbred adult male Wistar albino rats (150-200 g) and albino mice were obtained from the animal house of SRM College of Pharmacy.

The animals were maintained in a well-ventilated room at a temperature of 25 ± 1 °C with 12:12 hr light/dark cycle in polypropylene cages. Standard pellet feed (Hindustan lever, Bangalore) and tap water were provided *ad libitum* throughout the experimentation period. Animals were acclimatized to laboratory conditions for 10 days prior to initiation of experiments. Prior to the study, approval was obtained from the Institutional Animal Ethical Committee (IAEC) with approval number IAEC/32/2007.

Acute toxicity studies

Acute oral toxicity of MELA was determined using nulliparous, non-pregnant female mice. Albino mice were fasted for 3 hr prior to the experiment and were administered single dose of MELA dissolved in water and observed for mortality up to 48 hr. Based on the short term toxicity, the dose of the test animals were determined as per OECD guidelines 425. All the animals were observed for lethal or toxic signs up to 2000 mg/kg.

Study design

The animals were randomly divided into five groups consisting of six animals each: group I served as control and received double distilled water orally for 10 days; group II received CCl_4 0.5 ml/kg i.p. for 3 days; group III received Silymarin¹³ 100 mg/kg p.o for 10 days while groups IV and V received MELA at the dose of 200 and 400 mg/kg p.o for 10 days, respectively.

Hepatoprotective activity

The rats were kept overnight fasting after 10 days and blood samples were collected by retro orbital puncture under ether anesthesia and the serum was used for the estimation of biochemical markers like AST, ALT¹⁴, ALP¹⁵ and bilirubin¹⁶ using standard kits¹⁷. The enzyme levels were assayed using standard kits from Erba Diagnostics, Germany and the results were expressed as U/l. The animal

livers were dissected out and washed immediately with ice cold saline to remove blood and the wet weight noted. Liver homogenates were prepared in cold 50 mM potassium phosphate buffer pH 7.4 using Remi homogenizer. The unbroken cells and debris were removed by centrifugation at 2000 rpm for 10 min using a Remi cooling centrifuge and the supernatant was used for the estimation of GSH, SOD¹⁸, CAT¹⁹, TP²⁰, GGT levels²¹.

Histopathological studies

The liver tissues were dissected out, washed with normal saline and fixed in 10% formalin. Paraffin sections were prepared and stained with haematoxylin and eosin and examined using light microscopy (x100 magnification).

Statistical analysis

The data are expressed as mean \pm SEM (n=6). Statistical significance was determined by one way ANOVA followed by Dunnet's test. At 95% confidence interval, p values less than 0.05 were considered significant.

Results

Acute toxicity

The methanolic extract of the fruit pulp was found to be non-toxic up to the dose of 2000 mg/kg. There was a significant ($p < 0.05$) increase in the serum hepatic enzyme levels in the group after CCl_4 treatment which was prevented by MELA and indicative of a decrease in serum marker enzyme levels (Table 1) when compared with that of the control group. Pretreatment with Silymarin and MELA significantly prevented the biochemical changes induced by CCl_4 . The hepatoprotective effect offered by MELA 400 mg/kg was found to be greater than that of 200 mg/kg treatment.

Histopathological evaluation of control group as shown in (Figure 1a) and CCl_4 treated rats

Table 1: Effect of Methanolic extract of the fruit pulp of *Limonia acidissima* (MELA) on hepatic marker enzymes in CCl₄ induced hepatotoxicity in rats

Treatment	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Total protein (g/dl)	Bilirubin		GGT (IU/L)
					Total (mg/dl)	Direct (mg/dl)	
Vehicle control	71.5±3.2	74.3± 3.4	194.6±14.3	9.4±0.02	0.4± 0.03	0.4±0.05	60.4±0.2
CCl ₄ induced	232.8±3.3	162.7±2.6	324.5±15.6	5.9±0.06	2.9±0.06	2.5±0.04	80.2±0.2
Silymarin (100mg/kg)	79.9± 5.1**	74.4±2.6**	196.4±16.3***	8.7 ±0.2**	0.5±0.02**	0.5±0.04**	56.2±0.3**
MELA (200mg/kg)	92.4±3.6**	93.2±3.5**	206.6±13.7**	7.4±0.03*	0.6±0.05*	0.6±0.03*	62.4±0.3
MELA (400mg/Kg)	74.6±4.0***	75.5±3.2***	192.6±15.4***	8.8 ±0.1***	0.5 ±0.04***	0.5±0.06**	55.1±0.3**

Values are mean ± SEM expressed as (n=6)

P * < 0.05; ** < 0.01; *** < 0.001 as compared with CCl₄ induced group

Table 2: Effect of methanolic extract of the fruit pulp of *Limonia acidissima* (MELA) on antioxidant enzymes in CCl₄ induced hepatotoxicity in rats

Treatment	GSH	LPO	GPX	CAT	SOD
Normal	15.13±1.26	119.9±21.5	16.89±0.13	35.83±5.43	2.04±0.05
CCl ₄ induced	11.47±0.21	185.2±11.69	14.91±0.09	15.73±0.69	1.79±0.06
Silymarin 100mg/kg	13.1±0.99*	144.31±6.91**	15.03±0.11*	20.69±0.65**	1.75±.05
MELA 200mg/kg	14.6±1.07**	108.5±10.93***	16.01±0.14***	30.23±0.77***	1.99±0.13*
MELA 400mg/kg	15.09±1.11***	120.2±10.02***	16.87±0.15***	34.31±0.79***	2.02±0.08**

Values are mean ± SEM for 6 animals in each observation

P * < 0.05; ** < 0.01; *** < 0.001; as compared with CCl₄ induced group

Values are expressed as GSH, glutathione (µg/mg protein); LP, lipid peroxidation [nmoles of malonaldehyde (MDA) formed/mg protein/h]; GPX, glutathione peroxidase (µg of glutathione consumed/min/mg protein); CAT, catalase (unit/min/mg protein); SOD, superoxide dismutase (unit/min/mg protein)

revealed fatty degeneration, necrosis and fibrosis (Figure 1b). Concurrent administration of MELA preserved the histological structure of the liver though there was a mild congestion and regeneration of liver tissues (Figure 1c and 1d).

***In vivo* lipid peroxidation**

The effect of MELA on localization of radical formation resulting in lipid peroxidation is shown in Table 2. LPO content in the liver homogenate was significantly increased in CCl₄ treated group when compared to normal

group (p < 0.001). LPO level of MELA 200 and 400 mg/kg and Silymarin treated groups were significantly inhibited by 40%, 90% and 96%, respectively, when compared to the control group.

Effect of MELA on anti-oxidant enzyme levels

Effect of MELA on GSH, SOD and CAT of liver homogenate is shown in the Table 2. The antioxidant enzyme levels in normal control group were observed to be significantly higher than CCl₄ control group.

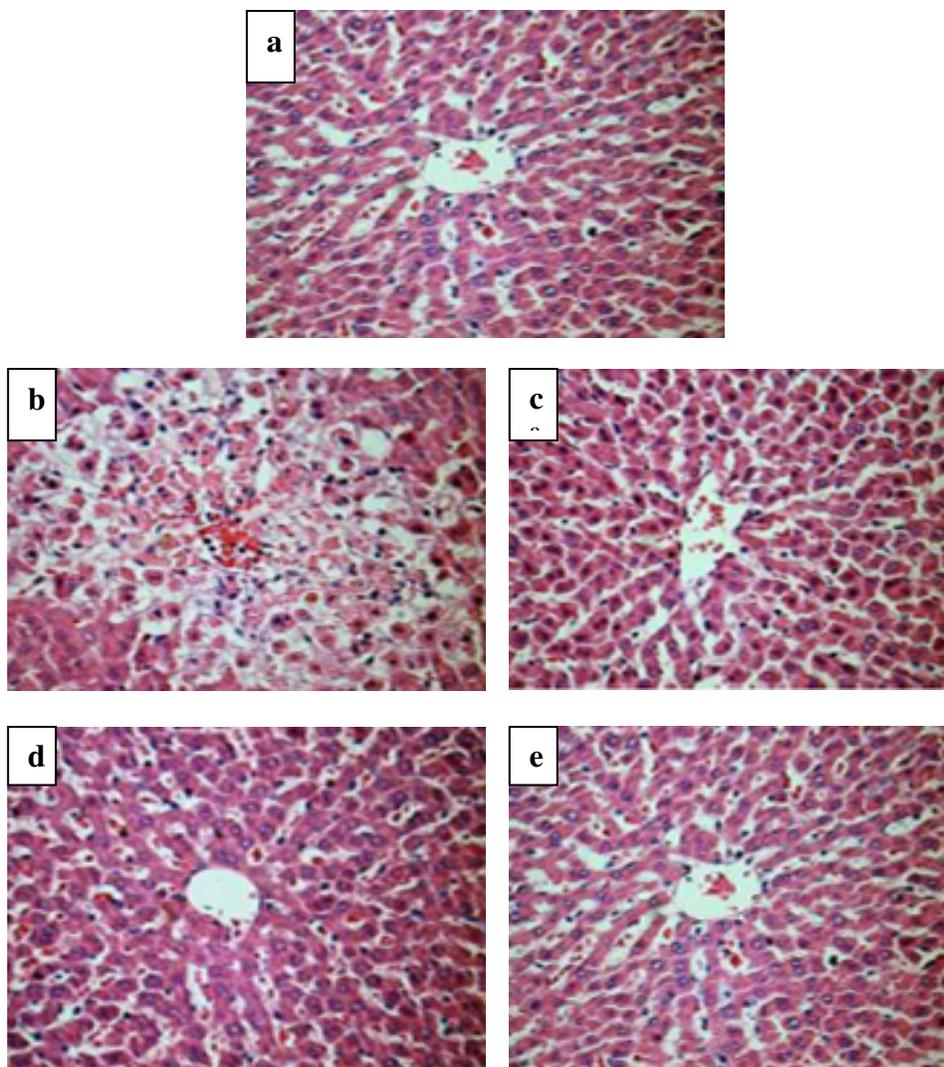


Figure 1: Histopathology of liver sections of animals (x100). (a) control group (received distilled water for 10 days) showing normal architecture of hepatic cells; (b) CCl_4 (0.5ml/kg i.p for 3 days) treated group showing centrilobular degeneration, necrosis of hepatic cells; (c) CCl_4 (0.5ml/kg i.p for 3 days) + methanolic extract of *L. acidissima* (MELA) 200 mg/kg for 10 days) showing mild degeneration and reverting to regeneration; (d) CCl_4 (0.5ml/kg i.p for the last 3 days) + MELA 400 mg/kg (for 10 days) showing complete regeneration and almost normal architecture of hepatocytes; (e) CCl_4 (0.5ml/kg i.p for 3 days) + Silymarin 100 mg/kg (for 10 days) showing complete regeneration and normal architecture of hepatocytes.

Antioxidant enzyme levels of MELA 200 and 400 mg/kg group were significantly increased by 45 and 90%, respectively, when compared to CCl_4 treated group and Silymarin treated group almost restored the enzyme levels.

Discussion

Carbon tetrachloride is the most commonly used hepatotoxin in the experimental study of liver diseases²². The hepatotoxic effects of CCl_4 are largely due to its active metabolite,

trichloromethyl radical. These activated radicals bind covalently to the macromolecules and induce peroxidative degradation of membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acid leading to the formation of lipid peroxides. The lipid peroxidative degeneration of biomembrane is one of the principle causes of hepatotoxicity of CCl_4 ²³. In the assessment of liver damage by CCl_4 , hepatotoxin (the determination of enzyme levels such as AST and ALT) is largely used. This is evidenced by an elevation in the serum marker enzymes namely AST, ALP and ALT. At the two dose levels (200 and 400 mg/kg), the significant reduction of the enzyme levels in a dose dependant manner and increase in the level of TP by MELA indicate hepatoprotective activity as stimulation of protein synthesis accelerates the regeneration process and production of liver cells.

In the present study, elevation in the levels of antioxidant enzymes in CCl_4 treated animal was observed when compared to control group. Increase in GSH, CAT, SOD, LPO levels in the liver homogenates of CCl_4 treated group suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms. Treatment with MELA significantly prevented these changes and this may be due to its antioxidant effects. Since MELA treated groups has significantly elevated the GSH, SOD, CAT contents of the liver, it may also be helpful in treating the hepatotoxicity induced by other agents.

Estimation of γ -GTP levels is a valuable parameter with a high negative predictive value for liver diseases²⁴. The γ -GTP levels increase proportionately with the increase in microsomal enzymes²⁵. There is usually a severe damage to tissue membrane at CCl_4 toxic doses because γ -GTP is a membrane bound enzyme. But the γ -GTP levels have significantly decreased in MELA treated groups as shown in the Table 1 which is indicative of the membrane stabilizing activity of MELA in improvement of liver function.

Histopathological studies has shown that the pretreatment with MELA exhibited protection against CCl_4 induced fatty degeneration and necrosis of the liver tissue, confirming the results of biochemical studies and indicating the hepatoprotective properties of MELA..

Conclusion

Our results indicate that MELA exhibits significant dose dependant protective effect against CCl_4 -induced liver damage which may be due to its anti-lipid peroxidative and free radical scavenging properties. Further studies are in progress to isolate and identify the active principle involved in the hepatoprotective and antioxidant activities of this plant extract.

Acknowledgements

Authors are grateful to Dr R Shivakumar (Pro-Vice Chancellor) and Dr KS Lakshmi (Dean, College of Pharmacy), SRM University, Kattankulathur, for providing necessary facilities to carry out this work.

Contribution of Authors

We declare that this work was done by us named as authors of this article and all liabilities pertaining to claims relating to the content of this article will be borne by us. We both conceived and designed the study, collected and analysed the data and prepared the manuscript which was approved by both of us.

References

1. Shahani S. Evaluation of hepatoprotective efficacy of APCL-A polyherbal formulation in vivo in rats. *Ind Drug* 1999;36:628–631.
2. Dianzani MU, Muzia G, Biocca ME, Canuto RA. Lipid peroxidation in fatty liver induced by caffeine in rats. *Int J Tissue React* 1991;13:79–85.
3. Chattopadhyay RR. Possible mechanism of hepatoprotective activity of *Azadirachta indica* leaf extract: part II. *J Ethnopharmacol* 2003; 89:217–219.

4. Chatterjee TK. Medicinal Plants with Hepatoprotective Properties, in Herbal Opinions, 3rd ed. Books and Allied (P) Ltd, 2000, p.35.
5. Kirtikar KR, Basu BD. Indian Medicinal Plants. Dehradun, India, International book distributors, 1933, vol II, pp. 1039-1040
6. Saima Y, Das AK, Sarkar KK, Sen AK, Sur P. An antitumor pectic polysaccharide from *Feronia limonia*, Int J Biol Macromol 2000; 27:333-335.
7. Ghosh P, Sil P, Majumdar SG, Thakur S. A Coumarin from *Limonia acidissima*. Phytochem 1982; 21: 240-241.
8. Parthasarathi G, Prabal S, Srabani D, Swapnadip T, Kokke WCMC, Akihisa T, Shimizu N, Tamura T, Matsumoto T. Tyramine Derivatives from the Fruit of *Limonia acidissima*. J Nat Prod 1991; 54: 1389–1393.
9. Badikaram NK, Yamuna A, Lesliegunatilaka A, Ratnayuke Bandara BM, Kithsiri EM, Wijeratne. Antifungal activity, acid and sugar content in wood apple (*Limonia acidissima*) and their relation to fungal development. Plant Pathol 2007; 38:258–265.
10. Kamat CD, Khandelwal KR, Bodhankar SL, Ambawade SD, Mhetre NA. Hepatoprotective activity of leaves of *Feronia elephantum* Correa (Rutaceae) against carbon tetrachloride- induced liver damage in rats, J Natural Remed 2003; 3: 148-154
11. Rahman MM, Gray AI. Antimicrobial constituents from stem bark of *Feronia limonia*. Phytochem 2001; 59:73–77.
12. Parthasarathy R, Nivethetha M, Brindha P. Hepatoprotective activity of *Caesalpinia bonducella* seeds on paracetamol induced hepatotoxicity in male albino rats. Indian Drugs 2007; 44:401- 404.
13. Reitman S and Frankel A. Colorimetric method for determination of serum glutamate oxaloacetate and glutamic pyruvate transaminase. Am J Clin Pathol 1957; 28:56–58.
14. Kind PR and King EJ. Estimation of plasma phosphates by determination of hydrolyzed phenol with antipyrin, J Clin Pathol 1954; 7:322–326.
15. Mallay HT and Evelyn KA. Estimation of serum bilirubin level with the photoelectric colorimeter. J Biol Chem 1937; 119:481–484.
16. Rajesh KG, Achyut NK, Geeta W, Murthy PS, Ramesh C, Vibha T. Nutritional and hypoglycemic effect of fruit pulp of *Annona squamosa* in normal healthy and alloxan-induced diabetic rabbits. Ann Nutr Metab 2005; 49:407–413
17. Marklund S and Marklund G. Involvement of superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974; 47:469–474.
18. Sinha AK. Colorimetric assay of catalase, Anal Biochem. 1972; 47:389–394.
19. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with Folin–Phenol reagent. J Biol Chem 1951; 193: 265–275.
20. Szaszi G. A kinetic photometric method for serum gammaglutamyl transpeptidase. Clin Chem 1969; 15: 124–126.
21. Johnson DE, Kroening C. Mechanism of early Carbon tetrachloride toxicity in cultured rat hepatocytes, Pharmacol Toxicol 1998; 83:231-239.
22. Kaplowitz N, Aw TY, Simon FR, Stolz A. Drug induced hepatotoxicity. Ann Intern Med 1986; 104: 826–839.
23. Nemesanszky E. Enzyme test in hepatobiliary disease. In: Donald W Moss and Sidney B Rosarki (Eds). Enzyme test in diagnosis, New York, USA: Oxford University Press, p 25-29.
24. Kim NK, Vasminah WG, Frejar EF, Goldman AL, Theologides A. Value of alkaline phosphatase, 5'-nucleotidase gamma glutamyl transferase and glutamate dehydrogenase activity measurements (Single and combine) in serum in diagnosis of metastases to the liver. Clin Chem 1977; 23: 2034-38.

