The Protective Effect Of *Morus Alba* And *Calendula Officinalis* Plant Extracts On Carbon Tetrachloride- Induced Hepatotoxicity In Isolated Rat Hepatocytes

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ABSTRACT: Liver is prone to xenobiotic-induced injury because of its central role in xenobiotics metabolism, its portal location within the circulation, and its anatomic and physiologic structure **(Jones, 1996).** Herbal medicine is the most widely used form of medicine in the world today where the medicinal plants contain curative bioactive ingredients such as alkaloids, coumarins, saponins and flavonoids **(Halberstein, 2005).** The present work was planned to evaluate the potential hepatoprotective effects of *morus alba* and *calendula officinalis* extracts against cytotoxicity and oxidative stress induced by carbon tetrachloride (CCl_4) in isolated primary rat hepatocytes. Hepatocytes were isolated by collagenase perfusion two steps technique. Cytotoxicity was determined by assessing cell viability and leakage of cytosolic enzymes, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). Oxidative stress was assessed by determining reduced glutathione (GSH) level and lipid peroxidation as indicated by thiobarbituric acid reactive substances (TBARS) production. Exposure of isolated rat hepatocytes to CCl_4 caused cytotoxicity and oxidative injury, manifested by loss of cell viability and significant increase in ALT, AST and LDH leakages. As well as, CCl_4 caused progressive depletion of intracellular GSH content and significant enhancement of TBARS accumulation. Pre- incubation of hepatocytes with either *morus alba* and *calendula officinalis* extracts ameliorated the hepatotoxicity and oxidative stress induced by CCl_4, as indicated by significant improvement in cell viability and enzymes leakages (ALT, AST and LDH). Also, significant improvement of GSH content and significant decrease in TBARS formation as compared to CCl_4 treated cells. The present study indicate the *morus alba* and *calendula officinalis* extracts possess a highly promising hepatoprotective effects against CCl_4 - induced hepatotoxicity. [Journal of American Science 2010;6(10):762-773]. (ISSN: 1545-1003).

Keywords: Egyptian medicinal plants, heptotoxicity, isolated hepatocytes.

INTRODUCTION

Liver plays a vital role in maintaining health and in the same time is highly susceptible to disease and injury. The liver diseases are a major cause of illness and death worldwide; Hepatitis and cirrhosis are particularly common liver disorders **(Cubero and Nieto, 2006 and Ajith et al., 2007).**

Large number of xenobiotics is reported to be potentially hepatotoxic. Free radicals generated from the xenobiotic metabolism can induce lesions of the liver and react with the basic cellular constituents such as proteins, lipids, RNA and DNA **(Ajith et al., 2007).**

CCl_4 is a potent environmental hepatotoxin , has been served as a model compound for study of hepatotoxicity and the cellular mechanisms behind oxidative damage and further was used to evaluate the therapeutic potential of drugs and dietary antioxidants **(Basu , 2003 and Prasenjit *et al.*, 2006) .**

Nowadays, many investigators have been focused for searching for the best approach in treatment of liver diseases using the effective herbal preparations.

Natural products are gaining a revitalized attention in medical community and their therapeutic uses are gradually increasing. As many synthetic drugs have revealed serious side effects. Therefore, a better strategy is to look for natural substances with strong pharmacological action and less cytotoxicity. In the last few years much attention was directed to the potential health promoting properties of phenolic phytochemicals **(Block, 1992; Block and Langseth, 1994 and Kartal, 2007)**. Plants containing phenolic compounds have been proved to possess many pharmacological effects such as hepatoprotective, antioxidant, anti-inflammatory, cardio protective and anticancer properties **(Croft, 1999).**

Mulberry trees especially *Morus Alba* is a widely found plant in Egypt. It is a wild plant available all over the year and found in a large amount in Beni-Suef governorate mainly in Beni-Suef villages. Morus Alba leaves used in flavored mulberry tea and also used as a feed for ruminants and other animals due to its high contents of crude protein (15-25%) **(Sanchez, 2000).**

Calendula Officinalis is a widely used plant in Egypt. It is a cultivated plant and found in large quantities in Beni-Suef governorate mainly in Beni-Suef gardens. Its leaves are very rich in vitamins and minerals. The fresh petals are chopped and added to salads, however the dried petals have a more concentrated flavor and used as a seasoning in soups, cakes. In addition the petals offers an edible yellow dye used as a saffron substitute to color and flavor rice, soups etc **(Allardice, 1993)**.

Therefore; the aim of the present study is to investigate and shed some light on the hepatoprotective effects of ethanolic extracts of *morus alba* and *calendula officinalis* as a most popular and available plants in Beni-suef governorate against cytotoxicity and oxidative stress induced by carbon tetrachloride in primary isolated rat hepatocytes in comparable with silymarin as standard hepatoprotective agent.

MATERIALS AND METHODS

Animals and Chemicals

Thirty Male Sprague–Dawley rats of locally bred strains (200–250 g) were used in this study. They were supplied from Faculty of Veterinary Medicine, Cairo University, Egypt. They were kept under good ventilation and standard hygienic conditions and allowed free access to balanced standard diet pellets and tap water ad libitum. Bovine serum albumin, carbon tetrachloride, collagenase (type IV), dimethyl sulfoxide (DMSO), GSH, thiobarbituric acid (TBA), triton X-100 and Silymarin were purchased from Sigma Chemical Co. (St. Louis, MO., USA. All chemicals were of the highest analytical grade.

A- Preparation and Extraction of the plant materials:

Four kilograms from *Morus Alba* plant (leaves) were collected from mulberry trees which cultivated in Faculty of Veterinary Medicine, Beni-Suef University in Beni-Suef governorate. One kilogram from *Calendula officinalis* plant (flowers) was collected from Beni-Suef gardens.

The collected plant samples were identified by the department of botany, Faculty of Science, Beni-Suef University. The selected fresh parts of the plants were dried at a temperature not exceeding 40 oC and powdered (2mm mesh size). The investigated dried powdered plant materials were separately extracted with 70% ethanol. The ethanolic plant extracts were filtered and the combined filtrates evaporated to dryness in vacuo at a temperature not exceeding 50 oC. The dried plant extracts were kept in dark bottle for investigation.

Isolation of Hepatocytes:

Hepatocytes were isolated using a collagenase two-step perfusion technique as described by **Berry and Friend (1969)** with slight modifications as published by **El-Tawil and Abdel-Rahman (1997).**

Briefly, a rat was anaesthetized by subcutaneous injection with 100 mg ketamine/kg (Ketalar, Park-Davis, Morris Plains, NJ, USA), restrained, and an incision was made in the abdominal cavity to expose the portal vein. A polyethylene canola was inserted into the portal vein and the liver was perfused in *situ* for 8 min with calcium-free Hank`s bicarbonate buffer maintained at 37^oC. The liver was then mechanically dislocated from the abdomen with the cannula in place and recirculated for 10 min in collagenase (0.67 mg/ ml) containing 5 mM calcium chloride. The isolated liver cells were filtered through four layer of cotton gauze and centrifuged for two minutes at 50 g. The cells were washed twice and suspended in HEPES-bicarbonate buffer (pH 7.4) containing 0.5% bovine albumin. The isolated hepatocytes were counted in a hemocytometer, while the viability of the cells was assessed by 0.4% trypan blue exclusion technique **(Baur *et al.,* 1985).** Freshly prepared cell suspension had 90% or greater viability prior to each experiment.

Incubation and treatment of hepatocytes

Freshly isolated hepatocytes ($2 X 10^6$ cells/ ml) were suspended in a HEPES-bicarbonate buffer (pH 7.4) and incubated at 37^oC in a shaking water bath at 30 oscillations per minute.The hepatocytes were incubated in plastic vials equipped with covers and used for determination of CCl_4 cytotoxicity and the possible protection with *Mentha longifolia* and *Chichorium endivia* and compared with silymarin as a known hepatoprotective agent at different incubation time intervals (30, 60, 120 min).

CCl_4 and silymarin, were dissolved in 0.5% dimethyl sulphoxide (DMSO) and their concentrations in the incubation medium were adjusted to reach a final concentration of 5mM CCl_4 **(Dvorak *et al.*, 2003)** and 0.5mM silymarin **(Farghali *et al.*, 2000).** The concentrations of *Mentha longifolia* and *Chichorium endivia* plant extracts were selected according to the dose response experiment using 4 different concentrations from each plant extract (1, 10, 100 and 1000µg/ml) dissolved in DMSO. Five replicates were used for each chemical and plant extracts. Cytotoxicity and Cytoprotection were determined by assessing of cell viability using trypan blue exclusion method, cytosolic enzymes leakage percent [lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], GSH content and thiobarbituric acid reactive substances (TBARS) accumulation. Control replicates were carried out simultaneously under the same conditions and at the same time intervals, using DMSO at a final concentration of 0.5% (**Dvorak *et al.*, 2003**).

Sample preparation for enzyme leakage

Enzyme activities (LDH, ALT and AST) were monitored using Sigma kits (Sigma Chemical Co., St. Louis, MO., USA) in an aliquot of cell-free medium and compared to the total activity achieved after lysis of the cells (**Moldeus *et al.*, 1978**). The cell-free medium was obtained by centrifugation of the aliquots at 1300 g for 15 min to obtain the supernatant. Lysate was obtained by addition of 1% triton X-100 and shaking for 15 min followed by centrifugation at 1300 g. The leakage was expressed as percentage of total lysate activity at each time point.

Assay for cellular GSH

Because GSH accounts for the majority of soluble- reduced sulphydryls in cells (**Kosower and Kosower, 1978**), Reduced GSH levels in hepatocytes were determined by measuring total soluble-reduced sulphydryl content using a ready made kit according to the method described by **Beutler *et al.* (1963**).

Lipid peroxidation assay

Lipid peroxidation was assessed by determining thiobarbituric acid reactive substances (TBARS) in hepatocyte culture media by the method of **Uchiyama and Mihara (1978)**. TBARS content was always expressed as nanomoles per milligram protein. Protein was determined by the method of **Lowry *et al.* (1951)** with bovine serum albumin as the standard.

Data Analysis

The GRAPHPAD (ISI Software, Philadelphia, PA, USA) computer program was used to conduct regression analysis and to plot collected data. Data were expressed as means ± standard error of means (SEM). Assessment of the results was performed using one-way analysis of variance (ANOVA) procedure followed by Tukey-Kramer multiple comparison post-tests. Statistical analyses were performed using Software GRAPHPAD INSTAT (Version 2). The 0.05 level of probability was used as the criterion for significance.

RESULTS

Table (1) demonstrates the dose response effect of different concentrations of *Morus Alba* and *Calendula Officinalis* extracts (1, 10, 100 and 1000 µg/ml) on CCl4 induced decrease in the viability% of isolated rat hepatocytes. The results revealed that CCl4 (5 mM) induced significant decrease in the viability% of isolated rat hepatocytes after 30 min of incubation period. This decrease in the viability was a time dependant compared to a control group.

However, preincubation of hepatocytes with *Morus alba* or *Calendula officinalis* plant extracts at concentrations (1 and 10µg/ml) for 30 min before CCl4 addition showed protective effect against CCl4 effect, but the data of *Morus alba* and *Calendula officinalis* plant extracts at concentrations (1 and 10µg/ml) are not closely related to control values.

While preincubation of hepatocytes with *Morus Alba* or *Calendula Officinalis* plant extract at concentrations (100 and 1000 µg/ml) for 30 min before CCl4 addition exhibited a significant protective effect against CCl4 effect. A marked protection was detected as early as 30min after CCl4 exposure. There was a simple significant difference between the effects of (100 and 1000 µg/ml) as compared to CCl4 group.

Table (2,3) shows the dose response effect of different concentrations of *Morus Alba* or *Calendula Officinalis* extracts (1, 10, 100 and 1000 µg/ml) on CCl_4 induced lactate dehydrogenase leakage % and lipid peroxidation as idicated by TBARS formation in isolated hepatocytes respectively. The results revealed that CCl_4treated group showed a significant increase in LDH leakage % and increase in a the rate of lipid peroxidation formation which indicated by elevation in TBARS level as early as 30 min after addition of CCl4 as compared to control group. These effects of CCl4 continued throughout the whole experimental period. While pre-incubation of hepatocytes with *Morus Alba* or *Calendula Officinalis* extracts at concentrations (100 and 1000 µg /ml) for 30 min before CCl_4addition exhibited a significant protection against CCl_4-induced LDH enzyme leakage % or increase in the rate of lipid peroxidation formation at 60 and 120 min incubation time.

Cell survival was assessed by trypan blue exclusion method. CCl_4 induced significant progressive time dependent decrease in cell viability as early as 30 min after exposure compared to control cells. On the other hand, concomitant incubation of the cells with 100 µg /ml of *Morus Alba* or *Calendula Officinalis* extracts inhibited the decrease in the cell viability caused by CCl_4. Co-incubation with silymarin showed similar protective effect **(Figure 1**).

Plasma membrane damages assessed by monitoring LDH, ALT and AST enzyme leakages from hepatocytes**. Figure (2)** demonstrates the time course of LDH leakage in the perfusion medium of hepatocytes treated with CCl_4 alone and those pretreated with *Morus Alba* or *Calendula Officinalis* extracts or silymarin. Exposure of hepatocytes to CCl_4 resulted in a significant increase in the leakage of LDH enzyme into the culture medium as early as 30 min of incubation. Pretreatment of hepatocytes with both plant extracts or silymarin ameliorated the effects of CCl_4 on LDH enzyme leakage.

Figure (3) demonstrates the effect of CCl_4, *Morus Alba* or *Calendula Officinalis* extracts and silymarin on ALT leakage % of isolated hepatocytes. CCl_4 caused time- dependent significant increase in the leakage of ALT in comparison to control. On the other hand, Pre-incubation of isolated hepatocytes with *Morus Alba* or *Calendula Officinalis* extracts or silymarin decreased the ALT leakage compared to CCl_4 treated cells.

The effect of CCl4 and *Morus Alba* or *Calendula Officinalis* extracts on AST leakage from isolated hepatocytes is demonstrated in **Figure (4).** CCl_4 caused significant time-dependent increase in AST leakage in comparison to control. Both *Morus Alba* or *Calendula Officinalis* extracts significantly decreased the AST leakage in hepatocyte medium induced by CCl_4. Silymarin showed a similar protective effect on AST leakage at the time points studied.

Assessment of oxidative stress-induced by CCl_4 in isolated hepatocytes was done by measuring of lipid peroxidation and cellular GSH level .

The effects of CCl_4, *Morus Alba* , *Calendula Officinalis* extracts and silymarin on lipid peroxidation, as indicated by TBARS formation, was estimated. **Figure (5)** shows a significant increase of TBARS production in hepatocytes exposed to CCl_4 as early as 30 min of incubation. Both plant extracts and silymarin significantly decreased the TBARS formation induced by CCl_4.

Figure (6) depicts the time-course effects of CCl_4 on hepatocytes glutathione content and its possible protection by either *Morus Alba* or *Calendula Officinalis* extracts or silymarin. CCl_4 caused significant depletion of glutathione content from isolated rat hepatocytes compared to control cells during the 2-h incubation period. Concomitant incubation of cells with *Mentha longifolia* or *Chichorium endivia* or silymarin prevented the depletion of glutathione induced by CCl_4 exposure.

Table (1): Effects of Carbon Tetrachloride (CCl4) and Different Concentrations of *Morus Alba* and *Calendula officinalis* Plant Extracts on Viability % of Isolated Rat Hepatocytes

Groups \ Incubation times	30 Min	60 Min	120 Min
Control	91.07 ± 0.82	88.69 ± 1.16	86.12 ± 0.71
CCl_4	57.94 ± 1.18[a]	47.61 ± 2[a]	41.47± 2.28[a]
CCl_4+Silymarin	87.63 ± 0.91 [b]	83.01±1.14 [b]	80.85± 1.02[b]
CCl_4+*Morus*(1µg)	78.63±1.93 [ab]	71.13 ± 2.9[ab]	67.02±2.53[ab]
CCl_4+*Morus*(10µg)	81.22 ± 1.6 [ab]	75.11±1.59[ab]	72.5 ± 1.79[ab]
CCl_4+*Morus*(100µg)	89.19 ± 1.37 [b]	82.2 ± 1.59 [b]	79.99 ±2.31[b]
CCl_4+*Morus*(1000µg)	90.59 ± 1.63 [b]	84.57±1.56 [b]	81.56±1.12 [b]
CCl4+C.officinalis (1µg)	79.28±1.66[ab]	75.88±1.67[ab]	72.09±2.25[ab]
CCl4+C.officinalis (10µg)	81.82±1.45[ab]	79.45±1.08[ab]	73.01±2.66[ab]
CCl4+C.officinalis (100µg)	90.95±1.32 [b]	84.04 ± 1.1 [b]	81.73± 1.32[b]

CCl4+C.officinalis (1000µg)	91.64 ± 2.1 [b]	87.35±1.42 [b]	83.06±1.63 [b]

-Data expressed as mean ± S.E. (n= 5replicates).

-([a]) Significantly different from control group by One-way **ANOVA** at $P \leq 0.05$.

([b]) Significantly different from CCl4 treated group by One-way **ANOVA** at $P \leq 0.05$.

- CCl4 incubated with hepatocytes at concentration (5 mM).

- Different concentration of *Morus Alba and Calendula officinalis* plant extracts (1, 10, 100, 1000 µg/ml) and silymarin (0.5 mM) were preincubated with hepatocytes 30 min prior to CCl4.

Table (2): Effects Of Carbon Tetrachloride (CCl4) And Different Concentrations of *Morus Alba* and *Calendula officinalis* Plant Extracts on LDH Leakage % of Isolated Rat Hepatocytes

Groups \ Incubation times	30 Min	60 Min	120 Min
CONTROL	27.13 ± 1.43	29.70 ± 1.26	31.93 ± 1.25
CCl_4	68.04±1.26[a]	71.53 ±2.18[a]	74.65 ±2.97[a]
CCl_4+Silymarin	30.71± 1.66[b]	32.65± 1.34[b]	34.3 ± 1.47[b]
CCl_4+*Morus*(1µg)	59.55±3.53[a]	63.76± 3.1[a]	66.57±3.1[a]
CCl_4+*Morus*(10µg)	58.35±3.8[a]	62.69±5.27[a]	65.12± 3.43[a]
CCl_4+*Morus*(100µg)	36.22 ± 1.8[ab]	38.09± 1.96[b]	40.01± 1.88[b]
CCl_4+*Morus*(1000µg)	34.77± 1.74[b]	36 ± 1.79[b]	38.02± 1.58[b]
CCl4+C.officinalis (1µg)	54.69±2.17[ab]	57.28±3.19[ab]	60.77±2.91[ab]
CCl4+C.officinalis (10µg)	52.03±2.68[ab]	55.89±2.26[ab]	58.35±2.26[ab]
CCl4+C.officinalis (100µg)	29.15± 1.12[b]	31.54± 1.54[b]	33.85±1.84[b]
CCl4+C.officinalis (1000µg)	28.56±1.59[b]	30.93± 1.69[b]	32.85± 1.97[b]

- Data expressed as mean ± S.E. (n= 5 replicates).

- ([a]) Significantly different from control group by One-way **ANOVA** at $P \leq 0.05$.

- ([b]) Significantly different from CCl4 treated group by One-way **ANOVA** at $P \leq 0.05$.

- CCl4 incubated with hepatocytes at concentration (5 mM).

- Different concentrations of *Morus Alba* and *Calendula officinalis* plant extracts (1, 10, 100, 1000 µg/ml) and silymarin (0.5 mM) were preincubated with hepatocytes 30 min prior to CCl4.

Table (3): Effects of Carbon Tetrachloride (CCl4) and Different Concentrations of *Morus Alba* and *Calendula officinalis* Plant Extracts on Lipid Peroxidation of Isolated Rat Hepatocytes

Groups \ Incubation times	30 Min	60 Min	120 Min
CONTROL	25.3 ± 1.14	27.62 ± 0.93	29.64 ±0.52

CCl4	49.1± 1.68 [a]	54.22± 1.61[a]	60.2 ± 1.44[a]
CCl4+Silymarin	28 ± 0.71[b]	31.12± 0.99[b]	33.23±1.31[b]
CCl4+*Morus*(1µg)	46.36± 1.27[a]	50.99± 0.88[a]	55.82±1.36[a]
CCl4+*Morus*(10µg)	45.05± 1.08[a]	49.2 ± 1.21[a]	53.79±1.27[a]
CCl4+*Morus*(100µg)	28.17± 0.51[b]	32.26± 0.86[b]	35.06±1.23[b]
CCl4+*Morus*(1000µg)	28.05 ±0.47[b]	30.23± 0.33[b]	33.31±1.47[b]
CCl4+C.officinalis (1µg)	44.06± 2.16[a]	51.41± 1.13[a]	57.31±1.29[a]
CCl4+C.officinalis (10µg)	43.39± 1.51[a]	50.27± 0.87[a]	56.09±1.49[a]
CCl4+C.officinalis (100µg)	25.99± 0.59[b]	29.61± 1.66[b]	32.9 ± 1.02[b]
CCl4+C.officinalis (1000µg)	25.23± 0.51[b]	28.9 ± 1.53[b]	31.94±1.13[b]

- Data expressed as mean ± S.E. (n=5 replicates).

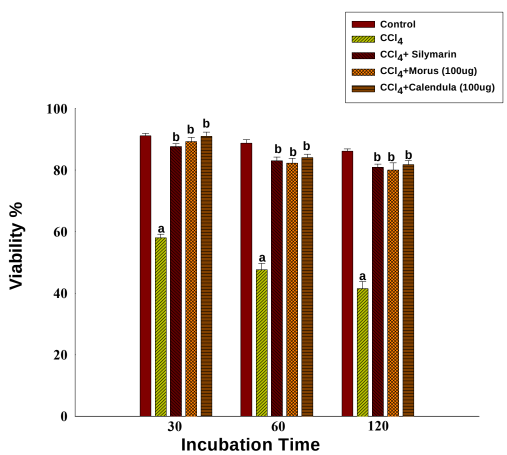
- ([a]) Significantly different from control group by One-way **ANOVA** at P≤0.05.

- ([b]) Significantly different from CCl4 treated group by One-way **ANOVA** at P≤0.05.

- CCl4 incubated with hepatocytes at concentration (5 mM).

- Different concentration of *Morus Alba* and *Calendula officinalis* plant extracts (1, 10, 100, 1000 µg/ml) and silymarin (0.5 mM) were preincubated with hepatocytes 30 min prior to CCl4.

Figure(1): Effects of Carbon tetrachloride (CCl_4) and Plant Extracts Of *Morus Alba* (100ug) and *Calendula Officinalis* (100ug) On Viability % of Isolated Rat Hepatocytes



Data expressed as mean ± S.E. (n= 5 replicates).

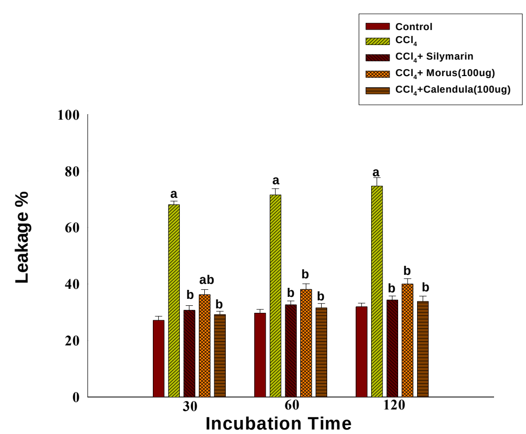
-([a])Significantly different from control group by One-way **ANOVA** tP≤0.05.

-([b]) Significantly different from CCl4 treated group by One-way **ANOVA** at P≤0.05.

- CCl4 incubated with hepatocytes at concentration (5 mM)

- The *Morus Alba* and *Calendula officinalis* plant extracts (100µg/ml) and silymarin (0.5 mM) were preincubated with hepatocytes 30 min prior to CCl4.

Figure (2): **Effects of Carbon tetrachloride(CCl_4) and Plant Extracts of *Morus Alba*(100ug) and *Calendula Officinalis* (100ug) on LDH Leakage% of Isolated Rat Hepatocytes**



Data expressed as mean ± S.E. (n= 5 replicates).

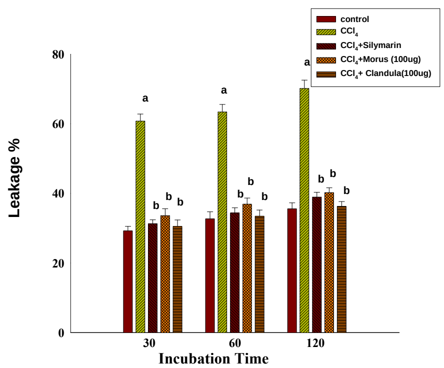
- ([a])Significantly different from control group by One-way **ANOVA** at p≤0.05.

- ([b]) Significantly different from CCl4 treated group by One-way **ANOVA** at p≤0.05.

- CCl4 incubated with hepatocytes at concentration (5 mM).

- The *Morus Alba* and *Calendula officinalis* plant extracts (100µg/ml) and silymarin (0.5 mM) were preincubated with hepatocytes 30 min prior to CCl4.

Figure(3): Effects of Carbon Tetrachloride (CCl_4) and Plant Extracts of *Morus Alba* (100ug) and *Calendula Officinalis* (100ug) On ALT Leakage% of Isolated Rat Hepatocytes



- Data expressed as mean ± S.E. (n= 5 replicates).

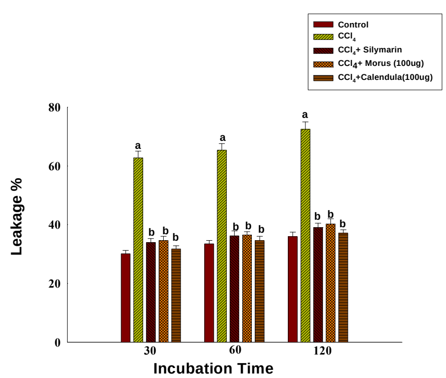
- ([a]) Significantly different from control group by One-way **ANOVA** at P≤ 0.05.

- ([b]) Significantly different from CCl4 treated group by One-way **ANOVA** at P≤ 0.05.

- CCl4 incubated with hepatocytes at concentration (5 mM).

- The *Morus Alba* and *Calendula officinalis* plant extracts at concentration (100µg/ml) and silymarin (0.5 mM) were preincubated with hepatocytes 30 min prior to CCl4.

Figure(4): Effects of Carbon tetrachloride (CCl_4) and Plant Extracts of *Morus Alba* (100ug) and *Calendula Officinalis* (100ug) On AST Leakage% of Isolated Rat Hepatocytes



- Data expressed as mean ± S.E. (n= 5 replicates).

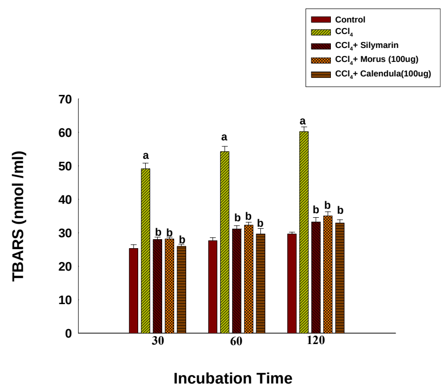
-([a])Significantly different from control group by One-way **ANOVA** at p≤0.05.

- ([b]) Significantly different from CCl4 treated group by One-way **ANOVA** at p≤0.05.

- CCl4 incubated with hepatocytes at concentration (5 mM).

- The *Morus Alba* and *Calendula officinalis* plant extracts at concentration (100µg/ml) and silymarin (0.5 mM) were preincubated with hepatocytes 30 min prior to CCl4.

Figure (5): Effects of Carbon tetrachloride (CCl_4) and Plant Extracts of *Morus Alba* (100ug) and *Calendula Officinalis* (100ug) on Lipid Peroxidation of Isolated Rat Hepatocytes



- Data expressed as mean ± S.E. (n=5 replicates).

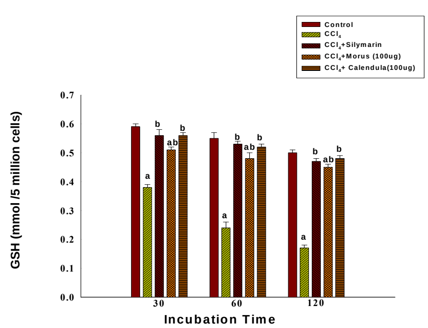
- ([a]) Significantly different from control group by One-way **ANOVA** at P≤0.05.

- ([b]) Significantly different from CCl4 treated group by One-way **ANOVA** at P≤0.05.

- CCl4 incubated with hepatocytes at concentration (5 mM).

- The *Morus Alba* and *Calendula officinalis* plant extracts (100µg/ml) and silymarin (0.5 mM) were preincubated with hepatocytes 30 min prior to CCl4.

Figure(6): Effects of Carbon tetrachloride (CCl_4) and Plant Extracts of *Morus Alba* (100ug) and *Calendula Officinalis* (100ug) on Reduced Glutathion Content of Isolated Rat Hepatocytes



- Data expressed as mean ± S.E. (n= 5 replicates).

- ([a]) Significantly different from control group by One-way **ANOVA** at P≤0.05.

- ([b]) Significantly different from CCl4 treated group by One-way **ANOVA** at P≤0.05.

- CCl4 incubated with hepatocytes at concentration (5 mM).

- The *Morus Alba* and *Calendula officinalis* plant extracts at concentration (100µg/ml) and silymarin (0.5 mM) were preincubated with hepatocytes 30 min prior to CCl4.

DISCUSSION

The liver diseases and its treatment clearly remain an important problem and in the spotlight of society where maintenance of healthy liver is important to over all health **(Smets *et al*., 2008).**

CCl_4 as a potent hepatotoxin cause a wide spectrum of hepatocellular dysfunctions including surface bleeding, decreased lipid secretion, fatty liver **(Masuda, 2006).** Also CCl_4 induced characteristics liver damage which may be ended by liver cirrhosis and in some instance liver cancer **(Park *et al*., 2008)**, CCl_4 is used for cytotoxicity and genotoxicity screening, evaluation of potential hepatoprotective capacity of different compounds, study of toxic injury and characterization of hepatotoxicity mechanisms. So it selected as a model of chemically induced liver injury in this study.

The isolated perfused liver is a one of the most important systems for study of toxicity and metabolic activity of many compounds *in vitro*, where ethical, economic, legislative, research and other reasons do not allow testing all of newly-synthesized compounds *in vivo* conditions. Hence new methods and approaches for hepatotoxicity testing *in vitro* have been developing **(Kucera *et al*., 2006).**

Isolated hepatocytes have been extensively used to characterize the metabolism of xenobiotics. They offer the possibility of analyzing the pathways of metabolism in a model system under different conditions where largely maintaining the cell integrity and the intracellular inter- relation ship between enzyme systems and cofactors **(Schlemper *et al*., 1993).**

Today, Complementary and Alternative Medicine (CAM), including herbal medicine, are popular and gaining attention in the general population worldwide. The reasons for such shift toward the use of herbals include the expensive cost of conventional drugs, adverse drug reactions, and their inefficacy **(Ozcakir *et al*., 2007).**

Silymarin as a mixture of flavonolignans extracted from the seeds of Silybum marianum, is a most widely used as a remedy for liver diseases and is a one of the most important therapeutics strategies in acute alcohol hepatitis treatment **(Naveau, 2001 and Kvasnicka *et al*., 2003).** It is used in our study as the standard hepatoprotective drug.

Mulberry trees including *Morus Alba* are widely distributed as ornamental or wild plant in marginal areas it was used in numerous industrial and pharmaceutical purposes **(Sanchez, 2000).** It has been reported to have a great pharmacological activities such as hepatoprotective, antioxidant, neuroprotective (**Oh et al., 2002).**

Calendula officinalis is an aromatic and erect herb. The flowers contain calenduline, which is the major constituent. The extract of flower shows antimicrobial effect. Various studies have proved the antiinflammatory, hepatoprotective, antioxidant, antibacterial, immunomodulatory, and antiedematous activities **(Korakhashvili *et al*., 2007).**

Therefore, the primary target of the present study was to shed some light on the potential hepatoprotective effects of the alcoholic plant extracts of *Morus Alba* and *Calendula Officinalis* against CCl_4-induced cytotoxicity *in vitro* using isolated rat hepatocytes

The data presented reflect the utilization of isolated liver cells to investigate the hepatoprotective effects of *Morus Alba* and *Calendula Officinalis* against CCl_4 induced toxicity using different parameters and compared with silymarin as standard hepatoprotective. As membrane damage occurs, hepatocytes release the cytosolic enzymes into incubation media and lose the ability to exclude trypan blue. In this study, trypan blue exclusion was used to assess cell viability. Staining of the cells by trypan blue indicates severe irreversible damage and reflects the end point to evaluate the toxic effect of CCl_4 (**Baur *et al.,* 1985**). Consequently, cell damage exhibits a good correlation with enzyme leakage (**Du *et al.,* 2003**).

In the present study the toxic effect of CCl_4 on cell membrane integrity was indicated by a significant decrease in the viability% of isolated rat hepatocytes and a significant increase in the leakage% of intracellular enzymes (ALT, AST and LDH) into the incubation medium. These results are in agreement with many reports of **Park *et al*., (2008); Xu *et al*., (2007).**

The decrease in hepatocytes viability% and the increase in leakage% of intracellular enzymes after CCl_4 exposure was a time dependant and detected as early as 30 min post exposure.

Viability% of hepatocyte depletion as well as increasing of intracellular enzymes leakage% were observed in different studies after exposure to CCl_4. These alterations may be attributed to the free trichloromethyl radicals (CCl_3) that initiate lipid per oxidation chain reactions which start with abstracting hydrogen ions from polyunsaturated fatty acids (PUFA) of the endoplasmic reticulum membrane phospholipids and these processes consequently leading to functional and structural disruption of cell membrane and intracellular organelle membrane followed by cell damage and an increasing the leakage% of intracellular enzymes **(Basu, 2003)**

Wu *et al*., (2006) and Weber *et al*., (2003) also mentioned that, CCl_3 affects the permeability of mitochondria, endoplasmic reticulum, and plasma membranes, resulting in the loss of cellular calcium sequestration and homeostasis, which can contribute heavily to subsequent cell damage and decrease hepatocytes viability.

CCl_4 produces decrease in adhesive interaction of cellular surface in area of simple connection that lead to increase deformity of hepatocytes surface leading to decrease viability% and increase leakage% of intracellular enzymes **(James *et al*., 2006).**

The present study indicated that CCl_4 has an oxidative stress on isolated rat hepatocytes where our data revealed significant increase in TBARS and decrease in GSH level. These results are in the harmony with those of other investigators who reported the association between CCl_4 toxicity and lipid per oxidation **(Park *et al*., 2008; and Krasteva *et al*., 2007).** The significant increase in lipid per oxidation which manifested as increase the level of TBARS may be attributed to metabolism of CCl_4 lead to production of highly reactive trichloromethyl radical CCl_3 which attack membrane phospholipids stimulating lipid per oxidation and cell lyses **(Akatay *et al*., 2000).**

Also our results are in agreement with **(Boll *et al*., 2001$_b$ and Visen *et al*., 1998)** who stated that CCl_4 as a model of hepatotoxicty is converted, during their intracellular metabolism, to active species which can be radical species or electrophilic intermediates. In most cases the activation is catalyzed by the microsomal mixed function oxidase system, Radical species can bind covalently to cellular macromolecules and can promote lipid peroxidation in cellular membranes.

Thus, Silymarin used in our study as the standard hepatoprotective drug and it revealed marked protective effects against CCl_4-induced cytotoxicity which was indicated by increasing the viability% of isolated hepatocytes, decreasing of intracellular enzymes leakage (AST, ALT, LDH) in the medium, decreasing of lipid peroxidation and prevent the depletion of GSH content compared with CCl_4 treated group. In parallel with our results those results observed by **Pradeep *et al*., (2007); Vengerovskiĭ and Khazanov (2007).**

Farghali *et al*., (2000) concluded that silymarin hepatoprotective effect is due to the inhibition of lipid peroxidation and that the modulation of hepatocyte Ca (2+). Moreover silymarin plays a pivotal role in maintenance the status of glutathione and its conjugating enzymes, an effect that could have been due to the strong antioxidant and free radical scavenging properties of silymarin **(victorrajmohan *et al*., 2005)**

The results of the present investigation showed that prior incubation of hepatocytes with *Morus Alba* and *Calendula officinalis* plant extracts afforded a protection against CCl_4 induced hepatocyte toxicity. This was manifested by an increase in the viability%, decrease in elevated enzymes leakage% of (ALT, AST, and LDH). They also improve intracellular level of GSH and decrease lipid peroxide level this result was in agreement with **Hyun *et al*., (2005).**

The hepatoprotective effect of *Morus Alba* plant extract against oxidative stress induced by CCl_4 mainly attributed to its antioxidant and free radical scavenging properties which have been demonstrated in various studies **(Oh *et al*., 2002).**

Morus Alba plant extract significantly improved the antioxidant status *in vivo and in vitro* which was more pronounced in the reduction of CCl_4-mediaited lipid per oxidation. This effect was returned to mulberroside A and oxyresveratrol obtained from *Morus Alba* plant extract. Also these compounds showed an inhibitory effect against FeSo4/H2O2-induced lipid per oxidation in rat microsomes and a scavenging effect on1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical **(Chungo *et al*., 2003).**

Oh *et al*., (2002) stated that the hepatoprotective effect of the *Morus Alba* plant extract. This effect returned to presence of some compounds isolated from plant extract. 5, 7-dihydroxycoumarin 7-methyle ether and oxyres-veratrol showed superoxide scavenging effect with IC50 values of 19.1 and 3.81 uM, respectively .In addition to inhibitory effect oxyresveratrol on 1, 1-diphenyl-2-picrylhydrazyl (stable radical). Cudraflavone B as well as oxyresveratrol showed hepatoprotective effects with the EC50 values of 10.3 and 32.3 uM respectively, on tarcine-induced cytotoxicity in liver.

Mulberry extract contain anthocyanins as natural colorant , have been well characterized to be involved in various bioactive properties and are widely used for their antioxidant properties .The number of peroxyl radicals trapped by these molecules in the trapping reaction, was the fundamental aspect of the antioxidant action **(Rossetto *et al.*, 2007).**

The results of the present investigation showed that prior incubation of hepatocytes with plant extract of *Calendula officinalis* at concentration (100 µg/ml) induced hepatoprotective effect against CCl_4 induced hepatocyte toxicity. This was manifested by an increase in the viability%, decrease in the enzymes leakage% of (ALT, AST, and LDH). It also improve intracellular level of GSH and decrease lipid peroxide level, this results was in parallel with the result obtained by **Rusu *et al.*, (2005)** and **Bele *et al.*, (2004).**

The hepatoprotective effect of *Calendula officinalis* plant extract may be attributed to a significant free radical scavenging and antioxidant activity as mentioned by **Cordova *et al.*, (2002)** who mentioned that *Calendula officinalis* plant extract is rich in a variety of bioactive metabolites including flavonoids and terpenoids. These bioactive ingredients have potent activities for scavenger the Superoxide radicals (O (2) (*-)) and hydroxyl radicals (HO (*)) resulted from CCl_4 metablites.

Herold *et al.*, (2003) measured the antioxidant effect of the hydroalcoholic extract of *Calendula officinalis* using a colorimetric assay. *Calendula officinalis* extract showed strong reactive oxygen species scavenging property so, the plant extract can be used in different anti-inflammatory/allergic diseases and it could be a useful tool for obtaining new antioxidant/anti-inflammatory agents.

In conclusions, our study revealed that *Morus Alba* and *Calendula officinalis* plant extracts significantly improved cell survival and played an essential role to maintain the cellular membranes integrity against CCl_4 that indicated by reduction of (ALT, AST and LDH) elevated enzymes. Moreover, plant extracts of *Morus Alba* and *Calendula officinalis* protect the intracellular antioxidant defense system as shown by preservation of GSH and inhibition of lipid peroxidation.

The previous mentioned effects were probably returned to presence of the predominant biologically active compounds in plant extracts that have potant antioxidant and free radical scavenger properties. Thus, the present study confirmed that the *Morus Alba* and *Calendula officinalis* plant extracts have hepatoprotective effect against CCl_4 induced cytotoxicity and oxidative stress in isolated rat hepatocytes.

REFERENCES

Ajith, T.A.; Hema, U. and Aswathy, M.S. (2007): Zingiber officinale Roscoe prevents acetaminophen-induced acute hepatotoxicity by enhancing hepatic antioxidant status. Food Chem. Toxicol.; 45(11): 2267-72.

Aktay, G.; Deliorman, D.; Ergun, E.; Ergun, F.; Yeilada, E. and Cevik, C. (2000): Hepatoprotective effects of Turkish folk remedies on experimental liver injury. Ethnopharmacology J.Turky., 73(1-2): 121-129.

Allardice, P. (1993): A-Z of Companion Planting. Cassell Publishers Ltd. 1993 ISBN 0-304-34324-2.

Basu, S. (2003): Carbon tetrachloride-induced lipid peroxidation: eicosanoid formation and their regulation by antioxidant nutrients. Toxicology., 189(1-2): 113–127.

Baur, H.; Kasperek, S. and Pfaff, E. (1985): Criteria of viability of isolated liver cells. Hoppe-Seyler's Z Physiol.Chem.J., 356: 827-838.

Bele, C.; Andrei, S.; Pintea, A. and Bara, A. (2004): The evaluation of the hepatoprotective effect of Calendula officinalis L. seed oil. Buletinul Universitatii de Stiinte Agricole si Medicina Veterinara Cluj-Napoca, Seria Medicina Veterinara., 61: 125-130

Berry, M.N. and Friend, D.S. (1969): High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. Cell Biol. J., 43: 506-520.

Berry, M.N.; Grivell, A.R.; Grivell, M.B. and Phillips, J.W. (1997): Isolated hepatocytes - past, present and future. Cell Biology and Toxicology J., 13(4-5): 223-233.

Beutler, E.; Duron, O. and Kelly, M.B. (1963): A colorimetric method for determination of glutathione reduced content in tissue. Lab. Clin. Med. J., 61:882.

Block, G. (1992): The data support a role for antioxidants in reducing cancer risk. Nutr. Rev. J., 50: 207-213.

Block, G. and Langseth, L. (1994): Antioxidants vitamins and disease prevention. Food technology J., 80-84.

Boll, M.; Weber Lutz, W.D.; Becker, E. and Stampfl, A. (2001_b): Mechanism of Carbon Tetrachloride-Induced Hepatotoxicity. Hepatocellular Damage by Reactive Carbon Tetrachloride Metabolites. Z. Naturforsch., 56: 649-659.

Chungo, K.O.; Kim, B.Y.; Lee, M.H. and Kim, Y.R. (2003): In-vitro and in-vivo anti-inflammatory effect of oxyresveratrol from morus alba. J. Pharm. Pharmacol., 55(12): 1695-700

Cordova, C.A.; Siqueira, I.R.; Netto, C,A.; Yunes, R.A.; Volpato, A.M.; Cechinel Filho, V.; Curi-Pedrosa, R. and Creczynski-Pasa, T.B. (2002): Protective properties of butanolic extract of the Calendula officinalis L. (marigold) against lipid peroxidation of rat liver microsomes and action as free radical scavenger. Redox Rep., 7(2): 95-102.

Croft, K.D. (1999): Antioxidant effects of plant phenolic compound. In: Antioxidants in human health and disease, Basu, T. K.; Temple, N. J. and Garg, M. L. (eds.), CABI publishing., London. Pp.109-115.

Cubero, F.J and Nieto, N. (2006): Kupffer cells and alcoholic liver disease. Rev Esp Enferm Dig., 98(6):460-72.

Du, J.; He, Z.D. and Jiang, R.W. (2003): Antiviral flavinoids from the root bark of morus alba L. Photochemistry., 62(8): 1235-8.

Dvorak, Z.; Kosina, P.; Walterova, D.; mSimanek, V.; Bachled, P. and Ulrichova, J. (2003): Primary cultures of human hepatocytes as a tool in cytotoxicity studies: cell protection against model toxins by flaonolignans obtained from Silybum marianum. Toxicology J., 137: 201-212.

El-Tawil, O.S. and Abdel-Rahman, M.S. (1997): Effect of cypermethrin on isolated male and female rat hepatocytes. Toxicology and Enviromental Health J., 52: 461-474.

Farghali, H.; Kameniková, L.; Hynie, S. and Kmonicková, E. (2000): Silymarin effects on intracellular calcuim and cytotoxicity: a study in perfused rat hepatocytes after oxidative stress injury. Pharmacol. Res., 41(2): 231-7.

Herold, A.; Cremer, L.; Calugăru, A.; Tamaş, V.; Ionescu, F.; Manea, S. and Szegli, G. (2003): Antioxidant properties of some hydroalcoholic plant extracts with antiinflammatory activity. Roum. Arch. Microbiol. Immunol., 62(3-4): 217-27.

Hyun, S.H.; Choung, S.Y. and Kwon, H.J. (2005): Traditional chinese medicine improves dysfunction of peroximes proliferators receptorα and microsomal triglyceride transfer protein on abnormalities in lipid metabolism in ethanol-fed rats. Biofactors., 23(3): 163-176.

James, A.; Dolak, Robert L.; Waller, Dr. Eric A.; Glende, J.r. and Recknagel, Richard O. (2006): Liver Cell Calcium Homeostasis in Carbon Tetrachloride Liver Cell Injury: New Data With Fura2[1]. **Journal of Biochemical Toxicology**., 3(4): 329-342.

Kartal, M. (2007): Intellectual property protection in the natural drug discovery. Tradional herbal medicine and herbal medicinal products. Phytother. Res. J., 21(2): 113-9.

Korakhashvili, A.; Kacharava, T. and Kiknavelidze, N. (2007): Biochemical structure of calendula officinalis.

Georgian Med. News., (148-149):70-3.

Krasteva, A.Z.; Mitcheva, M.K.; Kondeva-Burdina, M.S. and Descatoire, V.A. (2007): In vitro study of lovastatin interactions with amiodarone and with carbon tetrachloride in isolated rat hepatocytes. World J. Gastroenterol., 13(15): 2198-204.

Kucera, O.; Lotková, H.; Kriváková, P.; Rousar, T. and Cervinková, Z. (2006): Model systems for study of toxic injury of hepatocytes in vitro. Cesk. Fysiol., 55(3): 103-10.

Kvasnicka, F.; Bíba, B.; Sevcík, R.; Voldrich, M. and Krátká, J. (2003): Analysis of the active components of silymarin. J. Chromatogr A., 990(1-2):239-45.

Masuda, Y. (2006): Learning Toxicology from Carbon Tetrachloride-induced Hepatotoxicity. Journal of the Pharmaceutical Society of Japan., 126(10): 885-899.

Moldeus, P.; Hogberg, J. and Orrenius, S. (1978): Isolation and use of liver cells. In: Methods in enzymology. Fleisher, S. and Packer, L. (eds), Academic Press., New York. PP.60-71.

Naveau, S. (2001): Acute alcoholic hepatitis: treatments Presse. Med., 9;30(20):1024-30.

Oh, H.; Ko, K.E.; Oh, M.H.; Park, S.U. and Jun, J.Y. (2002): Hepatoprotective and free radical scavenging activities of prenylflavonoids ,coumarin, and stilbene from morus alba. Planta Med., 68(10): 932-4.

Ozcakir, A.; Sadikoglu, G.; Bayram, N.; Mazicioglu, M.M.; Bilgel, N. and Beyhan, I. (2007): Turkish general practitioners and complementary/alternative medicine. J. Altern. Complement. Med., 13(9): 1007-10.

Park, S.W.; Lee, C.H.; Kim, Y.S.; Kang, S.S.; Jeon, S.J.; Son, K.H. and Lee, S.M. (2008): Protective Effect of Baicalin Against Carbon Tetrachloride-Induced Acute Hepatic Injury in Mice. J. Pharmacol Sci. 2008 Jan 11.

Pradeep, K.; Mohan, C.V.; Gobianand, K. and Karthikeyan, S. (2007): Silymarin modulates the oxidant-antioxidant imbalance during diethylnitrosamine induced oxidative stress in rats. Eur. J. Pharmacol., 560(2-3): 110-6.

Prasenjit, M.; Mahua, S. and **Parames, C. S. (2006):** Aqueous extract of **Terminalia arjuna** prevents carbon tetrachloride induced hepatic and renal disorders. BMC Complementary and Alternative Medicine., 6:33.

Rossetto, M.; Vanzani, P.; Lunelli, M.; Scarpa, M.; Mattivi, F. and Rigo, A. (2007): Peroxyl radical trapping activity of anthocyanins and generation of free radical intermediates. Free Radic Res., 41(7):854-9.

Rusu, M.A.; Tamas, M.; Puica, C.; Roman, I. and Sabadas, M. (2005): The hepatoprotective action of ten herbal extracts in CCl4 intoxicated liver. Phytother Res., 19(9): 744-9.

Sanchez, M.D. (2000): Mulberry: an expentional forage available almost worldwide. FAO Electronic conference on mulberry for animal production. http:/www.fao.org/livestock.

Schlemper, B.; D.J. Siegers; J.W. Paxton and I.G.C. Robertson (1993): Rat hepatocyte -mediated metabolism of the experimental anti-tumor agent N-[2'-(dimethylamino)ethyl]acridine-4-carboxamide. Xenobiotica, 13: 361–71.

Smets, F.; **Najimi, M**. **and Sokal, E.M. (2008):** Cell transplantation in the treatment of liver diseases. Pediatr. Transplant., 12(1): 6-13.

Vengerovskiĭ, A.I. and Khazanov, V.A. (2007): Effects of silymarin and its combination with succinic acid on brain bioenergetics in rats with experimental inhibition of beta-oxidation of fatty acids. Eksp. Klin. Farmakol., 70(2): 51-5.

Victorrajmohan, C.; Pradeep, K. and Karthikeyan, S. (2005): Influence of silymarin administration on hepatic glutathione-conjugating enzyme system in rats treated with antitubercular drugs. Drugs R. D., 6(6): 395-400.

Visen, P.K.; Saraswat, B. and Dhawan, B.N. (1998): Curative effect of picroliv on primary cultured rat hepatocytes against different hepatotoxins: an in vitro study. J. Pharmacol. Toxicol. Methods., 40(3): 173-9.

Weber, W. D.; Boll, M. and Stampfl, A. (2003): Hepatotoxicity and mechanism of action of haloalkanes: Carbon tetrachloride as a toxicological model. Critical reviews in toxicology., 33(2): 105-136.

Wu, Y.; Wang, F.; Zheng, Q.; Lu, L.; Yao, H.; Zhou, C.; Wu, X. and Zhao, Y. (2006): Hepatoprotective effect of total flavonoids from Laggera alata against carbon tetrachloride-induced injury in primary cultured neonatal rat hepatocytes and in rats with hepatic damage. J. Biomed. Sci., 13(4): 569-78.

Xu, D.; Wu, Y.; Liao, Z.X. and Wang, H. (2007): Protective effect of verapamil on multiple hepatotoxic factors-induced liver fibrosis in rats. Pharmacol. Res., 55(4): 280-6.

8/6/2010