BIOMEDICINE, 2006 VOL. 26 (3&4) 18-26

ISSN : 0907-2067

EFFICACY OF SILYMARIN AND CURCUMIN ON DIMETHYL NITROSAMINE INDUCED LIVER FIBROSIS IN RATS

Joseph George
Department of Medicine
Columbia University
New York 10032
USA

Lonchin Suguna
Email: slonchin@yahoo.co.uk

Ramachandran Jayalakshmi
Email: rjai7@yahoo.com

Corresponding author

Dr.Gowri Chandrakasan
Emeritus Scientist, Department of Biochemistry
Central Leather Research Institute
Adyar, Chennai-600 020, India
Phone: 91-44-24911386, Fax: 91-44-24911589
Email: gowrichandrakasan@hotmail.com

ABSTRACT

The efficacy of Silymarin and Curcumin treatment in preventing biochemical and histological alterations in Dimethylnitrosamine (DMN) induced liver fibrosis in rats was studied. Six groups chosen for the study were control, Silymarin, Curcumin, DMN treated, DMN and Silymarin treated, DMN and Curcumin treated. All animals were sacrificed 72hrs after the end of treatments. The activities of Aspartate Transaminase (AST), Alanine Transaminase (ALT), Bilirubin, Glucose 6 phosphatase (G6Pase) and Triglycerides (TG) content were determined. $Na^{+}K^{+}$ATPase and Ca^{++}ATPase activities were measured in isolated plasma membranes. Lipid peroxide and triglyceride content were measured in liver homogenates. Liver fibrosis was evidenced by significant increase in liver collagen and lipid peroxidation, increased activities of Aspartate Transaminase (AST), Alanine Transaminase (ALT), glucose 6 phosphatase (G6Pase), Bilirubin levels and liver triglycerides. ATPase activities were significantly reduced in plasma membranes. Immunohistochemical staining of α-smooth muscle actin a marker for activated stellate cells were vivid during DMN induction and treatment with Silymarin and Curcumin decreased the α-smooth muscle actin, which showed absence of activated stellate cells. Both Silymarin and Curcumin treatment completely reversed all the changes observed in DMN induced fibrotic rats. The protective effects of Silymarin and Curcumin can be attributed to their antioxidants and membrane stabilizing actions. The results clearly exhibit potential antiproliferative and antifibrogenic effects of Silyarin and Curcumin.

Key words: Hepatic Fibrosis; Silymarin; Curcumin; Hepatic stellate cells; DMN

INTRODUCTION

Liver fibrosis results from the excessive secretion of matrix proteins by hepatic stellate cells, which proliferate during fibrotic liver injury. The development of hepatic fibrosis is associated with many biochemical alterations leading to structural and metabolic abnormalities in liver. Hepatic stellate cells are the key mediators of hepatic fibrosis.

The flavonoid Silymarin (5,7,4-trihydroxy-3-methoxy-3-01 (Legalon), which occurs in the thistle Silybum marianum Gaertn, was introduced as a hepatoprotective agent, two decades ago (1-4). Research in experimental

©*Publication of Indian Association of Biomedical Scientists*

Material may be protected by copyright law (Title 17, U.S. Code)

animals has shown that this flavonoid has a protective action on the liver, which is particularly effective in poisoning by several hepatotoxic substances such as carbon tetrachloride, thioacetamide, D-galactosamine or phalloidine (1, 4). The protective action of the flavonoid was explicable in terms of its capacity for trapping free radicals (5). In addition the effect has been ascribed to stabilizing effect on the cytoplasmic membranes (6).

Curcumin, 1,6-heptadiene-3, 5-dione-1, 7-bis (4-hydroxy-3-methoxyphenyl) (diferuloyl methane), isolated from the rhizomes of Curcuma longa is proved to have anti-inflammatory, antioxidant, membrane stabilizing actions (7,8). Preventive actions of this compound on *in vitro* lipid peroxide formation have also been reported (9,10).

It has been demonstrated that N-nitrosodimethylamine (DMN) induced liver injury in rats is a good and reproducible animal model for studying biochemical and pathophysiological alterations associated with the development of hepatic fibrosis and cirrhosis (11-14).

It has been shown that lipid peroxidation is associated with hepatic fibrosis and stellate cell activation. In the present study we report the hepatoprotective efficacy of Silymarin and Curcumin in preventing DMN induced liver fibrosis in rats.

MATERIAL METHODS

Materials

Chemicals are of analytical grade and obtained from Sigma-Aldrich, India. a-smooth muscle actin antibodies, peroxidase labeled IgG mouse immunoglobulin and Avidin-Biotin peroxidase complex were obtained from Sigma, India. All other chemicals were of analytical grade.

Animal Experiments

Male albino rats of wistar strain weighing around 150gms were used for the experiments. The animals were maintained with commercial rat feed and water available ad libitum.

The animals were divided into six groups and six animals per group were used.Control animals received injections of 0.15M NaCl without DMN.

SILYMARIN: 50mg/Kg of Silymarin was given as suspension in 0.05 % carboxy methylcellulose through an intra gastric tube.

CURCUMIN : 200mg/Kg was suspended in 1 % gum acacia, and given orally.

DMN: Hepatic fibrosis was induced by intraperitoneal injections of Dimethylnitrosamine in doses of 1mg (prepared in 0.15M NaCl)/100g body weight for 7 consecutive days.

DMN+SILYMARIN: Same dosage as above

DMN+CURCUMIN: Same dosage as above

All the animals were sacrificed 72h after the last administration of DMN, Silymarin and Curcumin. Treated and control animals were anesthetized with diethylether, and the blood was collected by cutting the right jugular vein on the neck. Serum and liver were collected for the determination of activities of enzymes such as Aspartate Transaminase and Alanine Transaminase according to the method of Reitman and Frenkel, (15), glucose 6 phosphatase by the method of Nordlie, (16) and Bilirubin content according to the method of Malloy and Evelyn (17). Na^+K^+ATPase and Ca^{++}ATPase activities were measured in isolated plasma membranes according to the procedure of Bonting (18) and Hjerten and Pan (19) respectively. Lipid peroxides and triglyceride content were also measured in liver homogenates according to the method of Ohkawa et al (20) and Foster and Dunn, (21) respectively. Liver collagen content was estimated by measuring hydroxyproline by the method of Woesnner (22).

Isolation and Purification of Ito Cells from Rat Liver

The procedure for isolation of rat hepatic cells (Ito Cells) was followed according to the procedure of Knook et al (23) by portal vein perfusion method. Briefly, hepatic stellate cells were isolated by pronase-collagenase digestion and were activated by culture on uncoated plastic. The resulting cell suspension was filtered through sterile gauze and centrifuged at

Material may be protected by copyright law (Title 17, U.S. Code)

50 x g for 3 min to remove hepatocytes and debris. The suspension after removal of sediments containing non-parenchymal cells were layered over 25% preformed (20,000 x g for 10 min) percol gradient and centrifuged at 800 x g for 30 min. The gradient centrifugation produced a top layer of yellowish white oily debris with a band of cells immediately below. The band mainly contained stellate cells. These cells were identified by autofluorescence at 325nm that rapidly faded under fluorescent microscope and exhibited round morphology. Staining for desmin was used as the confirmatory marker. The cells also displayed > 95% viability following trypan blue exclusion protocol.

Cell culture

The freshly isolated hepatic stellate cells were washed thoroughly in Dulbecco's Minimal Essential Medium and plated in DMEM supplemented with 10% FBS at a density of 1.0 x 106 cells per 100 mm tissue culture dish. The cells were maintained at 37°C under humidified 5% CO_2 - 95% air. The medium was changed every 24 hrs. As the cells reached confluence, they were passaged at a 1:4 split ratio after being removed from the dish with a brief exposure to 0.25% trypsin - 0.02% EDTA in phosphate buffered saline (PBS).

Microscopic observation

Cultured cells were viewed with a WILOVERT inverted microscope and photographed using KODAK Ektachrome film. Immunostaining of α-smooth muscle actin (α- SMA) was done according to the procedure of Buchwalow et al (24).

Statistical analysis

Arithmetic and standard deviations were calculated for the data. The results were statistically evaluated using one-way analysis of variance (ANOVA). The control mean values were compared with the treated mean values by the least significant difference method. The value of $P<0.05$ was considered as statistically significant.

RESULTS

DMN induced liver fibrosis in rat is a well-established, reproducible model and has several similarities with human liver cirrhosis (13). In the present study preventive role of Silymarin and Curcumin was studied in DMN induced hepatic fibrosis in rats.

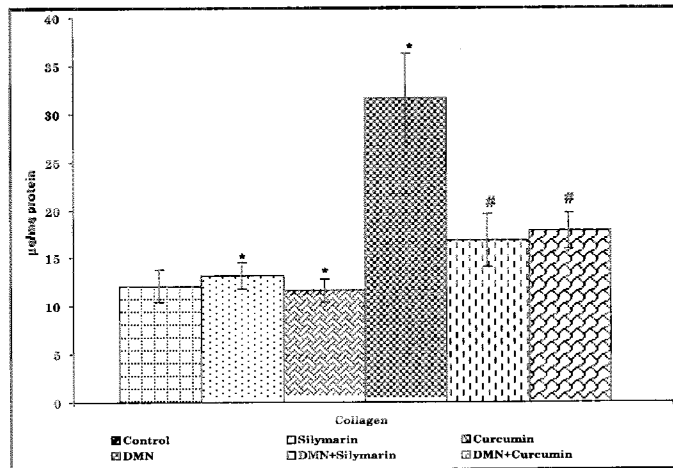
Table 1 depicts the activities of serum AST and ALT in control and experimental groups. Higher enzymactivity levels of AST and ALT were observed in DMN induced groups while treatment with Silymarin and Curcumin restored the increased enzyme activity levels to near normal, which shows the therapeutic efficacy of Curcumin as well as Silymarin.

Table 2 shows the levels of liver lipid peroxides in control and experimental groups. Increased malondialdehyde (MDA) levels during DMN induction were observed. Treatment with Silymarin and Curcumin groups showed lesser MDA levels, which could be attributed to preventive effect of Silymarin and Curcumin in hepatic fibrosis formation. Figure 1 shows the liver collagen content of control and experimental groups. A significant increase in liver collagen content of 31.6 ± 4.7 mg / mg of protein was observed in DMN treated rats when compared to the untreated controls. A notable reduction in collagen level in both DMN+Silymarin and DMN+Curcumin proves the antifibrotic effect of these drugs. Silymarin and Curcumin treated groups did not show much change from control, which shows the non-toxic nature of the drugs. Table 3 shows the levels of bilirubin, G6Pase and triglycerides in control and experimental groups. The levels of bilirubin, G6Pase and TG seem to be higher in DMN induced rats when compared to control rats. Treatment with Silymarin and Curcumin brought back the elevated levels to near normal values. When compared to control groups, all differences were found to be significant.

Figure 2 shows the $Na^{+}K^{+}$ATPase and Ca^{++}ATPase activities in control and experimental groups. DMN treated group exhibits increased activity of $Na^{+}K^{+}$ATPase and Ca^{++}ATPase when compared to control group. Silymarin and curcumin treatment prevented the increase of ATPases.

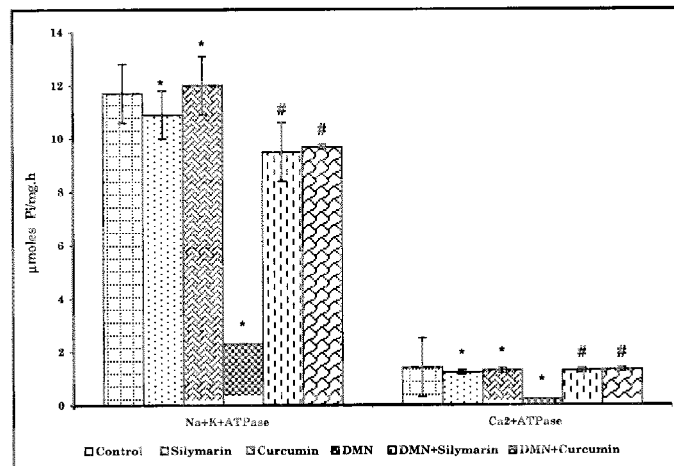
Material may be protected by copyright law (Title 17, U.S. Code)

Figure 1: Liver collagen content of control and experimental groups



Values are expressed as mean ± SD
P <0.05, * Compared with Control group; # Compared with Control and DMN treated group

Figure 2: $Na^{+}K^{+}$ATPase and Ca^{++}ATPase activity in control and experimental groups.

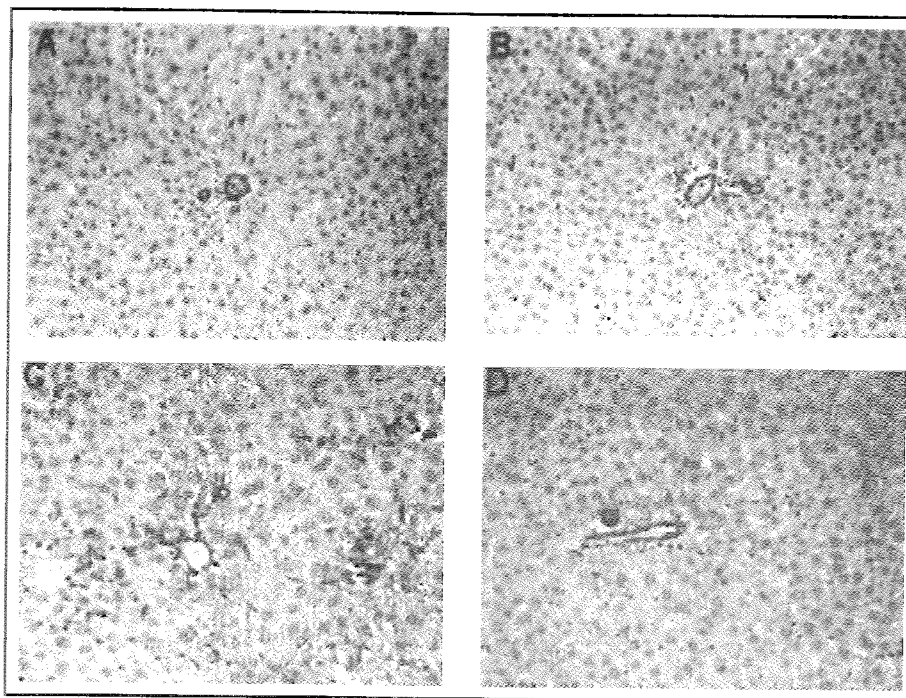


Values are expressed as mean ± SD
P <0.05, * Compared with Control group; # Compared with Control and DMN treated group

Figure-3 shows the immunohisto-chemical studies on SMA demonstrating activated stellate cells during DMN induced experimental fibrosis in rats and the effect of concurrent administration of curcumin on activated stellate cells

Material may be protected by copyright law (Title 17, U.S. Code)

Figure 3: Immunohistochemical a - smooth muscle actin demonstrating activated stellate cells during DMN induced experimental fibrosis in rats and the effect of concurrent administration of curcumin on activated stellate cells



A - Control Liver shows absence of a - smooth muscle actin staining
B - Liver after Curcumin treatment shows absence of a - smooth muscle actin Staining
C - DMN induced liver showing activation of hepatic stellate cells and Necrosis
D - DMN + Curcumin shows absence of activated stellate cells

Table 1: Activities of serum ALT & AST in control and experimental groups

Parameters	ALT (IU/L)	AST (IU/L)
Control	192.8 ± 25.4	108.1 ± 12.1
Silymarin	201.7 ± 23.2*	105.3 ± 9.4*
Curcumin	190.6 ± 24.1*	104.0 ± 14.1*
DMN	369.2 ± 59.2*	266.2 ± 72.3*
DMN+Silymarin	210.1 ± 27.03	186.4 ± 18.1
DMN+Curcumin	206.5 ± 23.7	215.6 ± 31.7

Values are expressed as mean ± SD
P <0.05, * Compared with Control group; # Compared with Control and DMN treated group

Material may be protected by copyright law (Title 17, U.S. Code)

Table 2: Levels of liver lipid peroxides in control and experimental groups.

Parameters	Lipid peroxides (nmoles of malondialdehyde/mg protein)
Control	0.08 ± 0.001
Silymarin	0.10 ± 0.009
Curcumin	0.12 ± 0.007
DMN	1.1 ± 0.071
DMN+Silymarin	0.13 ± 0.006
DMN+Curcumin	0.14 ± 0.005

Values are expressed as mean ± SD
P <0.05, * Compared with Control group; # Compared with Control and DMN treated group

Table 3: Levels of Serum Bilirubin, serum G6Pase and liver Triglycerides in control and experimental groups.

Parameters	Bilirubin (mol/I)	G6Pase (units/mg protein)	TG (g/mg Prot.)
Control	0.42 ± 0.1	2.5 ± 0.4	6.0 ± 0.9
Silymarin	0.51 ± 0.2*	2.2 ± 0.23*	6.3 ± 0.8*
Curcumin	0.39 ± 0.1*	2.6 ± 0.2*	5.9 ± 1.0*
DMN	4.6 ± 0.5*	5.5 ± 0.5*	12.3 ± 1.5*
DMN+Silymarin	1.8 ± 0.3#	3.2 ± 0.8#	7.3 ± 0.7#
DMN+Curcumin	1.3 ± 0.2#	2.8 ± 0.4#	6.4 ± 0.5#

Values are expressed as mean ± SD
P <0.05, * Compared with Control group; # Compared with Control and DMN treated group

DISCUSSION

Hepatic fibrosis, a consequence of most forms of liver diseases, is a dynamic process that forms a part of tissue response to hepatocyte injury. It involves interactions between several cell types, resulting in the enhanced synthesis of matrix components specifically collagen, with a concomitant decrease in the expression of matrix degrading proteases, the net result of which is the accumulation of several ECM proteins. In this present study, the efficacy of two hepatoprotective drugs, Silymarin and Curcumin on DMN induced hepatic fibrosis was investigated. The onset of liver fibrosis was characterized by the analyses of different biochemical parameters such as liver collagen, lipidperoxidation, serum activities of Aspartate

Material may be protected by copyright law (Title 17, U.S. Code)

Transaminase (AST), Alanine Transaminase (ALT), glucose 6 phosphatase, bilirubin content and liver triglycerides and immunohistochemical staining of a-SMA. Findings from our investigation reveal that DMN induced fibrotic rats exhibit a characterized pattern of alterations which indicate chronic liver damage.

The immunohistochemical findings reveal the absence of a SMA staining in control group while induction with DMN showed staining of this protein implicating activation of hepatic stellate cells and necrosis during liver fibrosis. The collagen levels were significantly higher in DMN induced rats. Treatment with both silymarin and curcumin brought back the collagen levels of liver to near normal.

Measurement of lipid peroxidation is a convenient method to monitor oxidative damage (25). Lipid peroxidation was measured in all groups as MDA formation per milligram protein. As shown in Table, 2 hepatic MDA formation was increased in DMN treated groups when compared to the control group. The difference was statistically significant. In DMN + Silymarin, MDA formation was similar to control groups. The study reveals that treatment with Curcumin, Silymarin helps to inhibit the hepatocellular damage. Recently, it has been reported that tetrahydrocurcumin and curcumin exert significant protection against Chloroquine induced toxicity by their ability to ameliorate the lipid peroxidation through the free radical scavenging activity, which enhanced the levels of antioxidant defense enzymes (26).

Increased activities of serum AST and ALT may be explained by the leakage of the enzymes into the blood stream from the damaged liver tissue, which is rich in transaminases (27). In alcoholic liver diseases, a decreased concentration of pyridoxal 5' phosphate may be responsible for the diminished hepatic activities of transaminases, particularly ALT (25). The increased levels of AST when compared to ALT may be due to the release of mitochondrial AST into the blood stream, a consequence of severe hepatocyte damage. Increased enzyme levels by DMN induction was significantly restored by both Silymarin and Curcumin, suggesting that they provide protection by preserving the membrane integrity of hepatocytes against DMN.

Increased levels of bilirubin, glucose 6 phosphatase and triglyceride were found during DMN induction while treatment with both Silymarin and Curcumin significantly decreased these levels. Likewise, induction with DMN showed a significant decrease in the levels of ATPases, which were efficiently restored by treatment with Silymarin and Curcumin.

We thus concluds that DMN induction in rats appears to be a good and reproducible model for hepatic fibrosis. Enzyme activities like AST, ALT and G6Pase were found to be useful markers of hepatocellular necrosis. Lipid, MDA, bilirubin and hydroxyproline observed to be altered by DMN induced hepatic fibrosis.

Curcumin shows inhibitory effect against the activation of liver stellate cells during DMN induced liver fibrosis in rats. The rate of activation of liver stellate cells was monitered by immunohistochemical staining of α–SMA as a marker for activated stellate cells. Curcumin adminstration significantly reduced activated liver stellate cells. It is reported that curcumin inhibits metal induced oxidative damage in cultured hepatocytes and hepatic lysosomal fractions (28).

Thus the present study reveals that treatment with Silymarin and Curcumin, partially reverses the hepatocellular damage. It is suggestive that anti-inflammatory, antioxidant features and membrane stabilizing action of these agents might be responsible for the antifibrotic action.

Acknowledgement

We acknowledge financial assistance from CSIR, New Delhi for carrying out this research project. We also acknowledge the support and help provided by the Director, CLRI during the study.

Material may be protected by copyright law (Title 17, U.S. Code)

REFERENCES

1. Churungoo, V. J., Singh, K., Singh J. (1997)Silymarin mediated differential modulation of toxicity induced by carbon tetrachloride, paracematol and D-galactosamine in freshly isolated rat hepatocytes. Ind J Exp Biol. 35: 611-617

2. Ramadan, L. A., Roushdy, H. M., Abu Senna, G. M., Amin, N. E., El-Deshw, O. A.. (2002) Radioprotective effect of Silymarin against radiation induced hepatotoxicity. Pharmacol Res. 45: 447-454

3. Lieber, C. S., Leo, M. A., Cao, Q, Ren, C, De Carli, L. M., (2003) Silymarin retards the progression of alcohol induced hepatic fibrosis in baboons. J Clin Gastroenterology. 37: 336-339

4. Tasdug, S. A., Peerzada, K., Koul, S., Bhat, R., Johri, R. K., (2005) Biochemical manifestations of antituberculosis drugs induced hepatotoxicity and the effect of Silymarin. Hepatol Res. 31:132-135

5. Najmi, A. k., Pillai, K. K., Pal, S. N., Aqil, M., (2005) Free radical scavenging and hepatoprotective activity of jigrine against galactosamine induced hepatopathy in rats. J Ethnopharmacol. 97: 521-25

6. Valenzuela, A., Garrido, A., (1994) Biochemical bases of the pharmacological action of the flavanoid Silymarin and of its structural isomer silibinin. Biol Res. 27: 105-112

7. Ammon, H. P., Wahl, M. A., (1991) Pharmacology of Curcuma longa. Planta Medica. 57: 1-7

8. Ruby, A. J., Kuttan, G., Babu, K. D., Rajasekaran, K. N., Kuttan, R., (1995) Antitumor and antioxidant activity of natural curcuminoids. Cancer Lett. 94: 79-8.

9. Phan, T. T., See, P., Lee, S. T., Chan, S. Y., (2001) Protective effects of curcumin against oxidative damage on skin cells in vitro: its implication for wound healing. J Trauma. 51: 927-31

10. Naik, R. S., Mujumdar, A. M., Ghaskadbi, S., (2004) Protection of liver cells from ethanol cytotoxicity by curcumin in liver slice culture in vitro. J Ethnopharmacol. 95: 31-7.

11. Jenkins, S. A., Grandison, A., Baxter, J. N., Day, D. W., Taylor, I., Shields, R. A., (1985) Dimethylnitrosamine-induced model of cirrhosis and portal hypertension in the rat. J Hepatol. 1: 489-99.

12. Jezequel, A. M., Mancini, R., Rinaldesi, M. L., Macarri, G., Venturini, C., Orlandi, F. A., (1987) Morphological study of the early stages of hepatic fibrosis induced by low doses of dimethylnitrosamine in the rat. J Hepatol. 1987; 5: 174-81.

13. George, J., Chandrakasan, G., (1996a) Molecular characteristics of dimethylnitrosamine induced fibrotic liver collagen. Biochim Biophys Acta. 1292: 215-22.

14. George, J., Chandrakasan, G.,(1996b) Glycoprotein metabolism in dimethylnitrosamine induced hepatic fibrosis in rats. Int J Biochem Cell Biol. 28: 353-61 1

5. Reitman, S., Frankel, S. A., (1957) Colorimetric method for the determination of serum oxaloacetic and glutamic pyruvate transaminases. Am J Clin Pathol. 28: 56-63.

16. Nordlie, R, C., Arion, W. J., (1965) Liver glucose-6-phosphatase and pyrophosphateglucose phosphotrans fersae: effects of fasting.Biochem Biophys Res Commun. 20: 606-10.

Material may be protected by copyright law (Title 17, U.S. Code)

17. Malloy, E., Evelyn, K., (1937) The determination of bilirubin with the photoelectric colorimeter. J Biol Chem. 119:481-85.

18. Bonting, S. L., De Pont, J. J., (1980) Use of phospholipid-converting enzymes for the study of membrane-bound enzymes. Biochem Soc Trans. 8: 40-2.

19. Hjerten, S., Pan, H., (1983) Purification and characterization of two forms of a low-affinity Ca2+-ATPase from erythrocyte membranes. Biochim Biophys Acta.728: 281-8.

20. Foster, L. B., Dunn, R. T. (1973) Stable reagents for determination of serum triglycerides by colorimetric Hantzsch condensation method. Clin Chem. 19: 338-40.

21. Ohkawa, H., Ohishi, N., Yagi, K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 95: 351-8.

22. Woessner, J. F Jr. (1961) The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys. 93: 440-7.

23. Knook, D. L., Blansjaar, N., Sleyster, E., Ch. (1977) Isolation and characterization of Kupffer and endothelial cells from the rat liver. Exp Cell Res. 109: 317-29.

24. Buchwalow, I.B, Minin, E.A., Boecker, W. (2005) A multicolor fluorescence immunostaining technique for simultaneous antigen targeting. Acta Histochem. 107: 143-8..

25. Viani, P., Cervato, G., Fiorilli, A., Cestaro, B. (1991) Age related difference in Synaptosomal peroxidative damage and membrane properties. J Neurochem. 56: 253-58

26. Pari, L., Amali, R. (2005) Protective role of tetrahydrocurcumin (THC) an active principle of turmeric on chloroquine induced hepatotoxicity in rats. J Pharm Pharmaceut Sci. 8: 115-123

27. Sallie R, Tredger JM, William R. Drugs and the Liver. Biopharmaceutical Drug Dispos 1991; 12: 251-59

28. Baretto, R., Kawakita, S., Tsuchiya, J., Minelli, E., Pavasuthipaisit, K., Helmy, A., Marotta, F. (2005) Metal induced oxidative damage in cultured hepatocytes and hepatic lysosomal fraction: beneficial effect of a curcumin/absinthium compound. Clin J Dig Diseases. 6: 31-6

Material may be protected by copyright law (Title 17, U.S. Code)