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RESEARCH ARTICLE

The prophylactic effect of silymarin on hepatic damage and IL-1β, IL-6 and TNF-α expression in rats with hepatic fibrosis.

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Abstract

The present study aimed to evaluate the protective effect of silymarin (Sy) on liver fibrosis induced by carbon tetrachloride (CCl_4). Rats were distributed as follows: 1st group is the normal control group administered only with olive oil. 2nd group is the positive control group administered with Sy. 3rd group of rats intoxicated with CCl_4 to induce hepatic fibrosis. 4th group of rats treated with Sy+CCl_4. CCl_4 intoxication were followed the Sy oral protection treatments by 2h. Hepatic fibrosis, augmentation of aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin (ALB), total protein (TP), IL-1β, IL-6, TNF-α expression and decreased levels in reduced glutathione (GSH) and oxidized glutathione disulfide (GSSH) contents; occurred as a result of CCl_4 intoxication. Pre-administration of Sy reversed liver injury by reversing AST, ALT, TB, ALB, improving antioxidant status, and pro-inflammatory cytokine expression.

Introduction:-

Hepatic fibrosis represents the final common pathological outcome for the majority of chronic liver insults. Its final stage is cirrhosis. Liver cirrhosis, the irreversible terminal stage of chronic liver disease, characterized by widespread fibrous scaring, and it is considered as a major cause of morbidity and mortality worldwide, with no effective therapy (Huang et al., 2006). The liver regulates many important metabolic functions, so the hepatic injury is associated with distortion of these functions (Wolf, 1999). Liver damage ranges from acute hepatitis to hepatocellular carcinoma, through apoptosis, necrosis, inflammation, immune response, fibrosis, ischemia, altered gene expression and regeneration. Loguercio and Federico (2003) stated that all processes that involve hepatocyte, Kupffer, stellate and endothelial cells which induce liver disease are related to the crucial role of reactive oxygen and nitrogen species. The main sources of free radicals are represented by hepatocyte mitochondria and cytochrome P450 enzymes, by endotoxin-activated macrophages (Kupffer cells) and by neutrophils.

The extracts of the flowers and leaves of *Silybum marianum* (St. Mary's thistle, milk thistle, or silymarin) has been used for centuries to treat liver, spleen and gallbladder disorders (Rainone, 2005). One of the important issues about this plant that it has accepted as a safe herbal product, since no health hazards or side effects are known in conjunction with the proper administration of designing therapeutic dosages (Medical Economic Company, 2000).

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The most constituents of silymarin are silibinin, isosilibinin, silicristin and silidianin (Sonnenbichler et al. 1999). Recently oxidized derivatives of silybin (the major component forming 70–80% of silymarin) and their antiradical, and antioxidant activity was studied by Gazak et al. (2004). The antioxidant, antiinflammatory and anticarcinogenic properties were demonstrated in the studies conducted with silymarin against oxidative stress, inflammatory responses and tumor promotion in mice and rats (Katiyar 2005; Fahmy & Soliman, 2007; Shaker et al., 2010). Other studies have focused on mechanistic studies regarding possible molecular targets of silymarin for cancer prevention (Ramasamy and Agarwal, 2008). Silymarin (Sy) modulates imbalance between cell survival and apoptosis through interference with the expressions of cell cycle regulators and proteins involved in apoptosis.
Cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL-6) have been related to inflammation. Several reports have shown an increase in serum levels of TNF- α and its receptors, in addition to IL-6 levels in HCV-infected patients (Knobler & Schattner, 2005; Hung et al., 2009).

This work was conducted to investigate the possible protective role of silymarin against the exposure to CCl_4 on histopathological, immunohistochemical distribution of IL-1β, IL-6, and TNF-α in rat hepatic tissue.

Materials and methods:-

Animals:-

Twenty-eight male rats (180±10g) of the *Rattus rattus* strain were purchased from Agricultural Research Center, Giza, Egypt. Animals were given 2 week acclimation period, during which they were fed a standard rat pellet diet and water *ad libitum*. They were housed under standard laboratory conditions with alternated 12-h dark/light cycle. All animal procedures are in accordance with the recommendations of the Canadian Council on Animal Care (CCAC,1993). Animals were randomly assigned up to the mean weight distribution, as follows:
(1) Normal control group: rats belonging to this group did not receive any treatments.
(2) Rats administered Sy 100 mg/kg daily, orally for 8 weeks.
(3) Hepatic fibrosis group: rats were injected IP with 1 ml/kg of sterile CCl4 in a ratio of 1:1 with olive oil 3 days/week, weekly for 8 weeks.
(4) Prophylactic group: rats administered Sy daily for 8 weeks, followed by CCl_4 after 2 h, 3 days/week, weekly for 8 weeks.

Drugs and Chemicals:-

Carbon tetrachloride (BDH Chemicals, England), the dose of CCl_4 was chosen according to the study of (Yao et al., 2004) to induce acute hepatitis model in rats. Silymarin (SEDICO, Cairo) was dissolved in saline to obtain the necessary doses. The dose of silymarin used in the study was chosen based on other studies in which the drugs produced beneficial effects in models of hepatic injury (Shaker et al. 2010).
Biochemical and Antioxidant Analysis
Blood samples were collected by cardiac puncture, allowed to clot and then centrifuged at 3,000 rpm for 15 minutes to separate serum. Serum kept at -20˚C until required. The activities of aspartate aminotransferase (AST), and alanine aminotransferase (ALT), by using commercial kinetic kits (Prolabo, France). Levels of albumin (ALB), and total protein (TP) were measured coloremetrically according to Lowery et al. (1951) and Dumas and Biggs (1972), respectively.

Determination of GSH and GSSG Contents :-
Reduced glutathione (GSH) and oxidized glutathione disulfide (GSSH) contents were determined by a modification of the method of Hissin and Hilf (1976). For GSH; 0.5 ml of the 10,000 g supernatant, 4.5 ml of the phosphate-EDTA buffer, pH 8.0, was added. The final assay mixture (2.0 ml) contained 100 µl of the diluted tissue supernatant, 1.8 ml of phosphate-EDTA buffer, and 100 µl of the OPT solution, containing 100 µg of OPT. After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette. Fluorescence at 420 nm was determined with by the activation at 350 nm.

For GSSH; 0.5 ml portion of the original 10,000 g supernatant was incubated at room temperature with 200 µl of 0.04 M NEM for 30 min to interact with GSH present in the tissue. To this mixture, 4.3 ml of 0.1 N NaOH was added. A portion of this mixture (100 µl) was taken for measurement of GSSG, using the procedure outlined above for GSH assay, except that 0.1 N NaOH was employed as diluent rather than phosphate EDTA buffer.

Cytokines Activity:-

Cytokine activities of IL-1β, IL-6, and TNF-α in serum were measured via a highly sensitive commercial ELISA (Sandwich Immunoassay Technique) specific kit for rats (Immuno-Biological Laboratories Co., Ltd. USA). Briefly, 96-well microplates were coated with IL-1β, IL-6, and TNF-α antibodies and incubated overnight at room temperature. The plates were washed with PBS containing 0.05% Tween 20 and then blocked with PBS with 1% bovine serum albumin and 5% sucrose. After the addition of diluted samples and standard IL-1β, IL-6, and TNF-α dilutions, plates were incubated for 2h at room temperature. Biotinylated goat anti-rat was used as the detection antibody, and streptavidin-HRP was added as the conjugate to each well. Equal proportions of hydrogen peroxide and tetramethylbenzidine were used as the substrate solution, and the reaction was stopped by adding 2N sulfuric acid. All samples and standards were run in duplicate, and the optical density was determined with a microplate reader at a wavelength of 450nm. The values of plasma cytokine concentration were expressed as pm/ml.

Histopathological Examination:-

Autopsy samples were taken from the liver of rats from different groups and fixed at 10% neutral buffered formalin for 24h and subsequently embedded in paraffin. Paraffin tissue blocks were prepared for sectioning at a 4μm thickness by slide microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained with hematoxylin and eosin stain for histopathological examination through the light microscope (Banchroft et al., 1996).

Statistical Analysis:-

Mean data were calculated and SD was measured for each mean number. The obtained data were statistically analyzed by using the program analysis of variance (ANOVA) followed by Duncan's multiple range test according to Duncan (1955) and Snedecor & Cochran (1982) using a computer program (Costate). Values of $P<0.05$ were considered statistically significant.

Results:-

Biochemical and Antioxidant Analysis:-

Biochemical data for all the studied parameters showed non-significant differences between the control group and Sy treated group. The liver-specific marker AST and ALT increased significantly (173.47 U/l) and (75.27 U/l) after the induction of fibrosis by CCl_4, whereas the TP and ALB values of this group were reduced, thus indicating that liver cell damage was significantly induced; causing liver dysfunction. On the other hand, pre-administration of Sy diminishes hepatic damage by preventing the augmentation of AST, ALT, and by preventing the inhibition of TP, ALB values as appeared in table (1). The antioxidant status of liver tissue reduced significantly after establishment of hepatic fibrosis GSH (3.15 μM/mg) and GSSG (0.18 μM/mg). Sy administration restored these levels near to normal levels as reported in table 1.

Table (1): Biochemical and antioxidant status of control and experimental groups.

Groups	C	Sy	CCl_4	Sy + CCl_4
AST (U/L)	111.10 ± 1.42	109.60 ± 0.75$^{\#}$	173.47 ± 1.86*	139.46 ± 1.35$^{*/\#}$
ALT (U/L)	23.14 ± 0.66	21.44 ± 0.79$^{\#}$	75.27 ± 0.87*	40.38 ± 1.07$^{*/\#}$
TP (g/dL)	5.99 ± 0.11	5.89 ± 0.12$^{\#}$	4.83 ± 0.10*	5.53 ± 0.21$^{*/\#}$
ALB (g/dL)	4.05 ± 0.11	3.95 ± 0.10$^{\#}$	3.13 ± 0.11*	3.62 ± 0.13$^{*/\#}$
GSH (μM/mg)	3.70 ± 0.10	3.63 ± 0.09$^{\#}$	3.15 ± 0.12*	3.41 ± 0.12
GSSG (μM/mg)	0.33 ± 0.06	0.28 ± 0.06$^{\#}$	0.18 ± 0.006*	0.25 ± 0.004

Values are mean±SE significant difference at ($P<0.05$).

* Significant to C # Significant to CCl_4 group

Detection of the cytokines IL-1β, IL-6 and TNF-α by ELISA, revealed that CCl_4 intoxication elevated their concentrations relative to control group, reached 283.44, 30.80 and 19.25 pg/ml respectively as recorded in table 2. This elevation is an obvious evident for hepatic inflammatory. Sy pre-treatment counteracted CCl_4 effect on pro-inflammatory cytokine production where these levels recorded 167.39, 20.95 and 11.37 pg/ml for IL-1β, IL-6, and TNF-α respectively. These results suggested the protective effect of Sy in preventing hepatic injuries.

Table (2): IL-1β, IL-6 and TNF-α cytokines levels in Control and Experimental Groups (pg/ml)

Groups	C	Sy	CCl_4	Sy + CCl_4
IL-1β	72.81 ± 1.25	74.83 ± 0.98$^{\#}$	283.44 ± 1.76*	167.39 ± 1.63$^{*/\#}$
IL-6	9.79 ± 0.34	9.98 ± 0.27$^{\#}$	30.80 ± 0.65*	20.95 ± 0.57$^{*/\#}$
TNF-α	5.27± 0.11	5.16 ± 0.09$^{\#}$	19.25 ± 0.38*	**11.37 ± 0.43**$^{*/\#}$

Values are mean±SE significant difference at (P<0.05).
* Significant to C # Significant to CCl_4 group

Histological Examination:-

Liver sections from control rats illustrated preserved architecture with hexagonal hepatic lobules, each is formed of cords of hepatocytes radiating from the central vein to the periphery of the lobule (Fig.1). The hepatic cords were separated by narrow blood sinusoids lined by endothelial cells and kupffer cells. The acidophilic cytoplasm around a pale stained nucleus could be seen. Sections from liver tissue of rats treated with Sy showed no histopathological changes when compared with control animals (Fig.2).

Consistent with biochemical findings, intoxication of rats with CCl_4 induced moderate fibrosis without formation of septa (Fig. 3). Hepatocytes appeared with focal necrosis and fatty changes (steatosis); beside the increased number of mitotic figures (Fig. 4) and clear vacuolation of the hepatocytes were seen (Fig.). Hepatocyte degeneration and necrosis, lymphocyte infiltration (Fig. 5) and collagen deposition, eosinophilic hepatocytes were detected. Dilated central vein stuffed with RBCs (Fig. 6).

The microscopic examination of liver sections from rats pretreated with Sy followed by CCl_4 showed protective effects. The hepatic tissue revealed the general hepatic architecture with normal arrangement of hepatic cords and narrow hepatic sinusoids and bi-nucleated hepatic cells as a sign of regeneration. The degree of hepatocyte necrosis, degeneration was decreased markedly, and diminution of fibrosis and fatty changes; when compared to the liver sections of rats intoxicated with CCl_4. However, there were a few RBCs infiltrates inside the sinusoids (Fig.7).

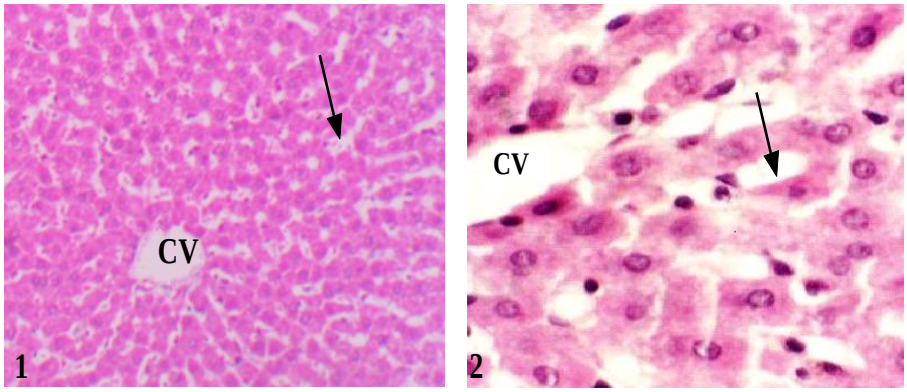

Fig. 1: Photomicrograph of liver section from control rat showing normal lobular architecture with central vein and radiating hepatic cords (H-E, X400).
Fig. 2: Photomicrograph of liver section from Sy administered rat showing the hepatic cords separated by narrow blood sinusoids lined by endothelial cells and kupffer cells (arrows) (H-E, X400).

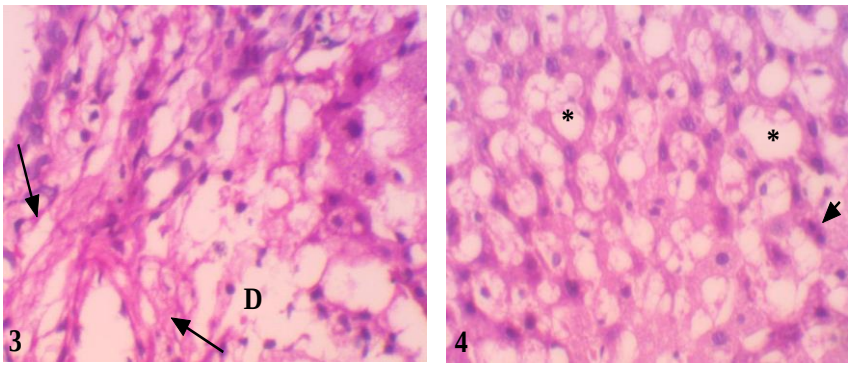

Fig. 3: Photomicrograph of liver section from CCl_4 treated rat showing moderate fibrosis (arrows), disintegrated hepatocytes (D), and lymphocyte infiltration. (H-E, X400).
Fig. 4: Photomicrograph of liver section from CCl_4 treated rat showing vacuolated hepatocytes, steatosis (*), mitotic activity (arrow) (H-E, X400).

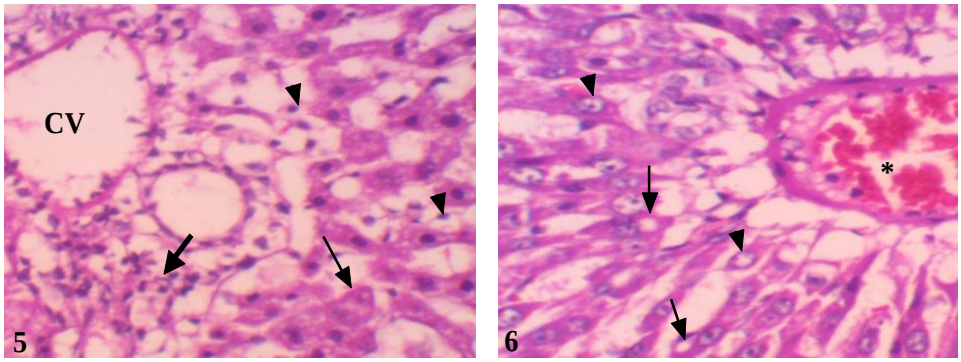

Fig. 5: Photomicrograph of liver section from CCl_4 treated rat showing dilated central vein (CV), degenerated hepatocytes (arrows) with pyknotic nuclei (head arrows), and lymphocyte infiltration (H-E, X400).
Fig. 6: Photomicrograph of liver section from CCl_4 treated rat showing dilated central blood vessel with thickened walls; stuffed with RBCs and lymphocytes (*). Hepatic cells appeared with vacuoles (arrows) and nuclear degeneration (head arrows) (H-E, X400).

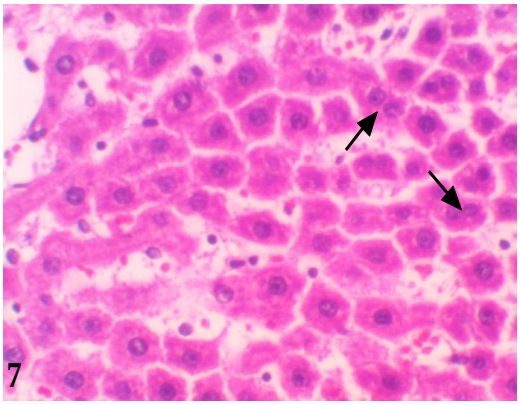

Fig. 7: Photomicrograph of liver section from Sy+CCl_4 treated rat showing near to normal arrangement of hepatic tissue, hepatic cells appeared binucleated (arrows); but sinunsoids filled with few RBCs (H-E, X400).

Discussion:-

Liver is the key organ of metabolism and excretion is continuously exposed to xenobiotics because of its strategic placement in the body. Toxins absorb from the intestinal tract gain access first to the liver resulting in a variety of liver problems (Wolf, 1999). Liver disorders are one of the common recent problems affects on the human health, resulted from the exposure to the environmental polluted sources (Shaker et al., 2010).

Aminotransferase levels are sensitive indicators of hepatocyte injury. Both enzymes are released into the blood in increasing amounts whenever the liver cell membrane is damaged (Abdel-Salam et al., 2007); after CCl_4 intoxication. Rajesh and Latha (2004) stated that elevated activities of these enzymes are indicative of cellular leakage and loss of the functional integrity of liver cell membranes. Liver functions showed significance increase for AST, and ALT; while pre-administration of Sy showing significance decreases in enzyme liver functions. In agreement to the present data, Raja et al. (2007) and Yilmaz-Ozden et al. (2015) found significant rise in levels of AST and ALT. On the other hand, *Cytisus scoparius* extract significantly decreased enzymes levels. The stabilization of these enzymes by *Cytisus scoparius* extract is a clear indication of the improvement of the functional status of the liver and inhibition of hepatic inflammation.

The present study revealed a reduction in serum TP and ALB as a result of the damaging effect of CCl_4. This comes in accordance with Castilla-Cortazar et al. (1997). The present investigation revealed inhibition of GSH and GSSG levels as a result of CCl_4 intoxication. This comes in a harmony with (Yilmaz-Ozden et al., 2015, Lin et al., 2016). The production of reactive oxygen species (ROS). The first metabolite, a trichloromethyl free radical ($.CCl_3$) has been formed from the metabolic conversion of CCl_4 and reacts very rapidly with O_2 and forms a second metabolite, a trichloromethyl peroxy free radical (CCl_3OO) or abstract hydrogen atoms to form chloroform (Packer et al., 1978). These free radicals initiate the peroxidation of membrane poly-unsaturated fatty acids and covalently bind to microsomal lipids and proteins (Tom et al., 1984, Srilaxmi et al., 2010). This phenomenon results in the generation of ROS like the superoxide anion O^{2-}, H_2O_2 and the hydroxyl radical, .OH. ROS affect the antioxidant defense mechanisms, decrease the intracellular concentration of reduced glutathione (GSH) and reduces the activity of SOD and CAT; which is considered to be a major factor in oxidative cell injury. CCl_4 is known to decrease GSH of phase II enzyme, and reduces antioxidant enzyme and antioxidant substrates to induce oxidative stress that is an important factor in acute and chronic injuries in various tissues (Preethi & Kuttan, 2009). Reactive oxygen species (ROS) causes oxidative DNA damages, with the formation of DNA adducts, genetic mutation, strand breakage and chromosomal alterations (Jia et al., 2002). Intracellular decrease of the reduced GSH exposes the cell to the destructive effects of oxidative stress (Singh et al., 2008). The antioxidant activity or the inhibition of free radicals generation is important in providing protection against such hepatic damage (Vitaglione et al., 2004).

Glutathione exists in reduced (GSH) and oxidized (GSSG) states. GSH prevent formation of reactive oxygen species (ROS) and their damaging effects. GSH effectively scavenges free radicals and other ROS and oxidized to form GSSG, then glutathione reductase (GR) recycles GSSG to GSH. In addition, GSH reacts with various electrophiles, physiological metabolites and xenobiotics to form mercapturates, which are catalyzed by GST (a family of Phase II detoxification enzymes) (Wu et al., 2004). In healthy cells and tissues, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the disulfide form (GSSG). An increased GSSG-to-GSH ratio is considered as the indicative of oxidative stress (Pompella et al., 2003). The present investigation indicated that Sy could restore the antioxidant status in the rat liver tissues.

The present work showed that CCl4 intoxication caused an increase in TNF-α, IL-1β, and IL-6 production. ROS up-regulates NF-κB, which is required for the induction of pro-inflammatory cytokines, such as IL-1ß, TNF-α and IL-6 (Rocha et al., 2014). TNF-α is a key mediator of the immune and inflammatory responses and controls the expression of the inflammatory gene network. Therefore, the overproduction of TNF-α contributes significantly to the pathological complications observed in many inflammatory diseases. Hepatic injury is associated with the up-regulation of TNF-α gene expression that was observed in the CCl_4 group. Consequently, the over-production of TNF-α contributed to the manifestation of the systemic inflammatory response and ultimately to the development of organ failure (Chehl et al., 2009). Also; Ebaid et al. (2013) found that the up-regulation of TNF-α expression was accompanied by the up-regulation of the Fas genes in CCl4-induced liver injury. The Fas protein is a type I membrane receptor that belongs to the TNF-receptor superfamily. While; Mita et al. (2005) found that the expression of FasL by macrophages plays a role in their pathogenesis. TNF-α is a pro-inflammation cytokine and a major endogenous mediator of hepatotoxicity. TNF-α is expressed in chronic liver injuries by both infiltrating inflammatory cells and hepatocytes and plays an important role in tissue damage (Hernandez-Munoz, et al., 1997).

The significant increase in TNF-α, IL-1β, and IL-6 production, inhibited by Sy pre-administration. These results were confirmed in a study of Reyes-Gordillo et al. (2007). Sy markedly suppressed the expression of TNF-α in liver, suggesting it's exerts its inhibitory effect on hepatic fibrosis by blocking the release of inflammatory mediators such as TNF-α and preventing hepatic fibrosis. A similar result has been reported by Nakamuta et al. (2001) and Issa et al. (2004) who studied a model of cirrhosis to determine the mechanisms mediating and limiting spontaneous recovery, and found that micronodular cirrhosis undergoes remodeling to macronodular cirrhosis; and reverse hepatic fibrosis gradually (Lee et al., 2001). Intoxication of CCl_4 induced the translocation of NF-κB to the nucleus; CCl_4induced NF-κB DNA binding activity was blocked by Sy; which prevents acute liver damage by at least two mechanisms: acting as an antioxidant and by inhibiting NF-κB activation and thus production of pro-inflammatory cytokines (Reyes-Gordillo et al., 2007).

In the present study, CCl4 intoxication; induced moderate fibrosis without formation of septa, architectural distortion, which in accordance with other Studies (Gonzalez-Reimers et al., 2003, Lee et al., 2004, Ebaid et al., 2013). Marked inflammatory changes associated with fatty changes were seen in CCl_4 treated rats as reported by Manjrekar, et al. (2008) and Abdel-Wahhab et al. (2011). Bonis et al. (2001) mentioned that fibrosis and necrosis defined as a passive and irreversible chronic damage. Liu et al. (2007) postulated that the liver macro and micro fatty changes (Steatosis) attributed to a defect in the synthesis, as well as secretion, of lipoprotein resulting in interference of the CCl4 with assembly of tubulin in microtubules. Fat metabolism is responsible for fatty disease which may be due to imbalance in energy consumption and combustion resulting in lipid storage or may be a consequence of peripheral resistance to insulin, where by the transport of fatty acids from adipose tissues to the liver is increased (Reddy & Rao, 2006). Increased oxidative stress is a feature of CCl_4-induced liver injury in which Kupffer cells and neutrophils have a significant role (Poli, 2000; Shaker et al., 2010).

Some improvements have been shown in the protective group as dilatation in the hepatic sinusoids associated with inflammatory cell infiltration and diffuse kupffer cell proliferation in between the degenerated hepatocytes.
It is worth to note that Sy possess important anti-inflammatory properties, which are likely to be of relevance to their hepatoprotective and anti-fibrotic effects (Abdel-Salam et al., 2007). Sy inhibited the migration of neutrophils into the inflamed site (DeLa Puerta et al., 1996), an important early event in the inflammatory cascade. In experimental models of hepatic injury e.g., CCl_4 (Muriel & Mourelle, 1990), Sy exerted protective effects and reduced liver fibrosis and reduced serum transaminases (Abdel-Salam et al., 2007). Mechanism of action for Sy conducted mainly to the antiradical and anti-carcinogenic roles. Ethyl acetate (100 mg/kg bw) and ethanol seed extracts for S. marianum (100 mg/kg bw) were tested against the injection by CCl_4 (2 ml/kg bw). Their activity was compared with standard hepatic drug hepaticum (100 mg/kg bw) for 10 days. Ethanolic extract showed the most significantly decrease in the liver enzymes (Medical Economics Company, 2000). Sy has metabolic and cell-regulating effects at concentrations found in clinical conditions, namely carrier-mediated regulation of cell membrane permeability, inhibition of the 5-lipoxygenase pathway, scavenging of ROS and an action on DNA-expression, for example, via suppression of nuclear factor (NF)-kappaB (Saller, et al., 2001); beside its effect on cell proliferation (Tyagi et al., 2004) suggesting that Sy may be a useful additive therapy in patients with chronic liver disease.

Conclusion:-

Increasing requirements for natural plant products could modify the biological harmful molecules by the antioxidant potential. Pre-administration of Sy could mask the harmful effect of CCl_4 on hepatic fibrosis, blocking the free radical formation, preserving the cellular integrity, and thus elicit a reduction in the inflammatory response, restoration of cytokine expression.

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