Sequential Liver *Gss* and *sod1* Genes Expression Levels in Silymarin Treated Male Rats

[a]Jabbar A.A.Al-Sa'aidi, [b]Hussein J. Shoabith,
[a]BVMS, MS, PhD; Professor, Physiology, Dept. Physiol. & Pharmacol., College Vet. Med., Al-Qadisiya Univ, Iraq.
[b]MS student, Iraq

Abstract

To investigate wheather silymarin has its own antioxidant activity and/or it acts through induction of the endogenous antioxidants, the present study was conducted on intact adult male rats. Seventy males were assigned to control and silymarin treated groups (35 each), and were drenched with drinking water and silymarin suspension (200 mg/ kg b.w) daily for 40 days. Each group was allocated to 5 equal subgroups; sacrificed before treatment (0 day), after 10, 20, 30, and 40 days of treatment. At the end of each period, evaluation of liver mRNA expression levels of *Gss* and *sod1* genes has been performed using qRT-PCR technique based on Syber Green dye. Silymarin treated liver samples recorded significant higher total RNA concentrations at 10, 20, 30, and 40 day periods compared with control. Significant elevation of both liver *Gss* and *sod1* genes expression levels (fold changes) shown in silymarin treated males compared with control, started after 10 days of treatment. In comparison between period, both genes showed no significant difference in control, whereas silymarin treated males recorded significant gradual elevation of both genes as the treatment period progress. In conclusion, silymarin was an inducer for endogenous superoxide dismutase and glutathione even in intact male rats.
KEYWORDS: silymarin, Gss gene, sod1 gene, antioxidants.

Introduction:

Seeds of *Silybum marianum* (Milk Thistle) were used traditionally for treatment of liver and gallbladder disorders, such as hepatitis, icterus, cirrhosis and recently for protection of liver against chemical and environmental poisoning, included insect stings, snake bites and mushroom poisoning *(Amanita phalloides)* (Radko and Cybulski, 2007). Silymarin is the main flavonoid (70%) found in the herb *Silybum marianum*, whereas the remaining 20-30 % are chemically undefined fraction, mostly composed of oxidized polyphenolic and polymeric compounds (Vladimir and Daniela, 2005). In silymarin complex, the main components are isosilybin (5%), silydianin (10%) and silychristin (20%) (Burgess, 2003). Silymarin and its major isomer silybin are used for along time to manufacture the therapeutic products against liver diseases, gallstones and jaundice (Vogel, 1968).

It has been reported that silymarin is considered as a potent antioxidant in protection of liver cells (as well as other body cells particularly those of brain) from different toxins (Saller *et al.*,,2001). Sonnenbichler and Zetl, (1988) mentioned that silymarin may able to reduce the incidence of certain cancer forms. In other study, Burgess (2003) demonstrated that silymarin protective activity is due to its activity for scavenging of produced free radical. A hepatoprotective role of silymarin has been examined in paracetamol and thioacetamide treated male rats (Singh and Handa, 1995). They proposed that silymarin can act in the initiation stage of peroxidation by interfering with the metabolism of oxidative agent either by free radicals scavenging mechanism or by impairing the microsomal enzymatic system necessory for this

metabolism. In a recent study, Alozy (2013) concluded that silymarin has a positive beneficial effect, when given at the dose (200 mg/ kg, bw), as hepatoprotective, hypolipidemic, and antioxidant agent in streptozotocin-induced diabetic mature male rats. Therefore, further investigations are needed to be performed in order to determine: is silymarin act as exogenous antioxidant or it can induce the endogenous enzymatic and/or non-enzymatic antioxidants? The present study aimed to evaluate the sequential antioxidant potency of silymarin as well as its activity in the induction of endogenous enzymatic and non-enzymatic antioxidants in intact adult male rats.

Materials and methods:

Experimental animals: Mature male Wistar rats have been used in the experiment. Male rats were allowed one week to acclimate to the animal house environment before beginning of experiment. Animals were housed in polypropylene cages inside a well-ventilated room. Each cage consist of not more than five rats. Animals were fed on the standard chow and drinking water *ad libitum* throughout the experiment. Room temperature was maintained at 23±2 ºC., the light-dark cycle was on a 12:12 hr with light on at 06:00 a.m. and off at 06:00 p.m. throughout the experimental period.

Preparations of Silymarin suspension: Milk thistle seeds (silymarin) were purchased from Natures manufacturers for herbal extract, USA. The seeds were grinded by coffee mill into powder. To prepare the dose of 200 mg/ kg bw (Capasso *et al*., 2009), 40g. of seeds were suspended in one litter of drinking water (20 mg/ 0.5 ml). Each 100 g of body weight need to be drenched 0.5 ml of silymarin suspension (20 mg/ 0.5 ml/ 100 g bw). According to the body weight, male rats were drenched the suitable dose of silymarin suspension. For example, for male rat weighted 150 g, it need to drenched 0.75 ml of silymarin suspension which contain 30 mg of silymarin seeds.

Experimental design: 70 mature male rats were assigned equally (35 each) to control (C) and treated (S) groups. Male rats were drenched with drinking water and silymarin suspension (200 mg/ kg b.w) daily for 40 days. Each group has been allocated to five subgroups (periods), where sacrificed before treatment (0 day as subgroup 1), after 10, 20, 30, and 40 days of treatment (as subgroups 2-5), respectively.Male rats have been monitored throughout the experimental periods. At the end of each treated and control subgroup period, male rats were anaesthesized (by injection of 0.3ml ketamine + 0.1 ml of xylazine/ kg b.w. *ip*), dissected and liver samples from each male have been obtained and kept directly at –70 ºC for evaluation of mRNA expression levels of GAPDH as housekeeping gene and *Gss* and *sod1* genes using qRT-PCR technique based on Syber Green dye.

Quantitative real time RT-PCR

RNA isolation from rat tissues: RNA was isolated from rat liver tissues according to the protocol described by the TRIzol® reagent manufacturer with some modification.

Assessing RNA yield and qualit: There are three quality controls were performed on isolated RNA. The first one is to determine the quantity of RNA (ng/µL) that has been isolated using Nanodrop UV/VIS spectrophotometer (OPTIZEN POP. MECASYS Korea), the second is the purity of RNA by reading the absorbance in spectrophotometer at 260 and 280 nm in the same Nanodrop machine, and the third is the integrity of the RNA by prepared gel electrophoresis.

DNase inactivation (DNase I) Treatment: The extracted total RNA samples were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme) and done according to method described by promega company, USA.

cDNA synthesis: DNase-I treatment total RNA samples were used in cDNA synthesis step using AccuPower® RocktScript RT PreMix kit that provided from Bioneer company, Korea and done according to company instructions.

qRT-PCR based SYBER Green I Dye Detection: qRTPCR was performed using AccuPower® Greenstar™ qPCR PreMix reagent kit (Bioneer, Korea) and Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer, Korea).The Syber Green based qRT-PCR PreMix reagent kit is designed for PCR amplification of cDNA for target genes (*Gss* and *sod1*genes) by using it's primers and (*GapdH*) Housekeeping gene, as well as for quantification of PCR amplification copy numbers comparatively to copy numbers of Genomic DNA qRT-PCR standard curve. The Syber Green dye that was used in this kit is DNA binding dye which reacted with new copies of amplification specific segment in target and housekeeping gene, then the fluorescent signals were recorded in RT-PCR thermocycler. A genomic DNA standard curve was generated from *GapdH* gene of *Rattus norvegicus* (27.9Mbp) were taken from NCBI-Gene Bank information was approximately (~1×107) copies, and serial dilution representing (1×107, 1×106 and 1×105) gDNA copies were used as genomic DNA standard curve.

Experimental design of qRT- PCR: For quantification of gene expression in treatment and control samples at duplicate, internal control house keeping gene (GAPDH) was used to normaliz the gene expression levels, therefore, qRT-PCR master mixes were prepared for gDNA standard curve, target genes, and GAPDH housekeeping gene. Thenafter, qPCR PreMix were added into *AccuPower* GreenStar qPCR PreMix tube. Then, real-time PCR tubes sealed by the optical adhesive film and mixed by vigorous vortexing for resuspension of PreMix pellet. The tubes centrifuge at 3,000 rpm, for 2 min, then Exicycler™ 96 Real-Time Quantitative Thermal Block instrument was started and loaded according to the kit instruction. After completion of reaction, data analysis has been performed.

Data analysis of qRT-PCR: The housekeeping gene (*GapdH*) was represented as a normalize gene that can be used for calculation of the relative gene expression or fold change in target genes (*Gss* and *sod1*genes). Therefore, the quantities (Ct) of target genes were normalized with quantities (Ct) of house keeping gene (*GapdH*) by the relative quantification gene expression levels (fold change). Livak method that described by (Livak and Schmittgen, 2001). The relative quantification method, quantities obtained from q RT-PCR experiment must be normalized in such a way that the data become biologically meaningful. In this method, one of the experimental samples is the calibrator such as (Control samples) each of the normalized target values (CT values) is divided by the calibrator normalized target value to generate the relative expression levels. After that, the ΔCT Method with a Reference Gene was used as following equations:

ΔCT (calibrator) = CT (target, calibrator) – CT (ref, calibrator)

Ratio (target / reference) = $2^{CT\ (reference) - CT\ (target)}$

At first, the CT of the reference (ref) gene has been normalized to that of the target gene, for calibrator sample. Then the CT of the reference (ref) gene has been normalized to that of the target gene, for the test sample:

ΔCT (Test) = CT (target, test) – CT (ref, test)

$\Delta\Delta CT$= ΔCT (test) – ΔCT (calibrator)

Fold change = $2^{-\Delta\Delta CT}$

Ratio (reference/target) = $2^{CT\ (reference) - CT\ (target)}$

So, the relative expression was divided by the expression value of a chosen calibrator for each expression ratio of test sample.

Statistical Analysis: All the values are expressed as mean ± SE. Comparisons were performed using two way analysis of variance (ANOVA2) and newman- keuls to test all groups unpaired values. Differences were considered to be significant at the level of $P<0.05$. All statistical analysis were carried out using the GraphPad Prism (SAS Institute, Inc., USA).

Results:

The cocentration and purity of total RNA: Total RNA concentration (ng/µl) and purity were estimated using Nano- drop spectrophotometer in absorbance reading (260/280 nm). All liver tissue samples that used in the present study gave high concentration of total RNA and appeared quantitively enough to proceed in quantitative real time reverse transcriptase PCR. Liver samples obtained from silymarin treated group recorded significant higher RNA concentrations at 10, 20, 30, and 40 day periods compared with control (figure-1). The purity of RNA samples (also assessed using agarose gel electrophoresis) of liver tissues that obtained from experimental male rats recorded different band thickness (figure-2).

Relative quantification of *Gss* and *sod1* genes expression: significant elevation ($p<0.05$) of both liver *Gss* and *sod1*genes expression levels (fold changes) have been shown in silymarin treated males in comparison with control (figure-3), started after 10 days of treatment and continued in its elevation as treatment progressed, whereas pre-treted period (0 day) showed no significant difference ($p>0.05$) between groups (figure-4 and 5, respectively). In comparison between period, both genes showed no significant difference ($p>0.05$) between periods of control group, whereas silymarin treated group recorded significant gradual elevation ($p<0.05$) of both genes as the treatment period progressed.

Discussion:

In the present study, we tried to find out how silymarin could improves the pharmacological intervention in healthy adult male rats. The present clinical findings revealed that silymarin administration had benift improvement of antioxidant activity even in the intact animal model, not only in stressed animals, as mentioned by researchers (Maritim *et al.*, 2003). So, its interesting that silymarin had improved the antioxidant status in intact animals, since in various animal models researchers examine the role of silymarin in liver disorders, from which they pointed out to the observed antioxidant activities of silymarin (Saller *et al.*, 2008) as it could effectively attributed to the hepatoprotection, therefore they suggested that silymarin has direct effects on cell functions which is responsible for the prevention of liver disorders in these animal models (Polyak *et al*., 2007).

In general, the modulatory effects of silymarin, that reveated in the present study, could attributed to its role that derives from its ability to counterarrest the action of free superoxide radicals, which are formed due to cell membrane lipid peroxidation (the damage the cell membranes), competitive inhibition through the hepatocytes external cell membrane modification; stimulation of hepatic cell metabolism, in addition to activating of RNA biosythesis of the ribosomes, and stimulating of protein biosynthesis (Sandoval *et al*., 2008). On the other hand, silymarin may diminishes the activity of Kupffer cells and increase the production of glutathione, and also inhibits its oxidation. Participation has also been shown in the increase of protein biosynthesis in the hepatocyte by stimulating polymerase I RNA activity.

One of the most important potency of silymarin, as proved in the present study, was by increasing the cellular content of GSH. Where high expression level of *Gss* gene in liver cells has been shown in the present experiment. It has been mentioned that *Gss* gene encoded mitochondrial and cytoplasmic glutathion synthetase biosynthesis in

most body cells particularly in hepatocytes, which is responsible for glutathione production (Janaky *et al*., 1999). And therefore silymarin administration increased the antioxidant activity.

The present study reported significant increase of total RNA concentration in liver tisue samples obtained from silymarin treated male rats compared with that from control, which may attributed to the role of silymarin by which, as it has been reported, that silymarin can enter into the nucleus and stimulates RNA polymerase I enzymes and thus the transcription of rRNA, resulting in elevation of ribosomal formation. This may causes acceleration of protein and DNA biosynthesis, and therefore enhances the biosynthetic rate of both structural and functional proteins. This stimulation may enhancing cells to provide more transporters and enzymes. This action has important in the regeneration of hepatocytes and increasing the normal functions of liver (Sonnenbichler and Zetl, 1986).

On the other hand, molecular observations reported increased hepatocytes expression levels of *Gss* and *sod1* genes in liver tissues that obtained from silymarin treated male rats in comparison with control. These findings proves that silymarin treatment causes induction of endogenous enzymatic (SOD) and non-enzymatic (GSH) anioxidant, as well as its role as exogenous antioxidant that confirmed by other researchers (Al-Sa'aidi and Alozy, 2013). Thus, silymarin had induced liver tissue to over express the production of SOD and GSH, and may be others which could need more traits to imrove that.

From these findigs, it can be postulated that silymarin treatment is of great pharmacological value not only as an antioxidant but also as an inducer of endogenous enzymatic and non-enzymatic antioxidants.

References:

Al- Sa'aidi, J.A.A.; and Alozy, M.A.A. (2013). Hypolipidemic and antioxidant activity of silymarin in streptozotocin-induced diabetic male rats.

Alozy, M.A.A. (2013). Effect of Silymarin on lipid profile and oxidant-antioxidant status in Streptozotocin-induced diabetic adult male rats. M. Sc. Thesis, college of Veterinary Medicine, Al-Qadisiya University, Iraq.

Burgess, C.A. (2003). Silybum marianum (Milk Thistle). *J. Pharm. Soc*., 34: 38-40.

Capasso, R.; Avillo, G.; Capasso, F.; Savino, F.; Izzo, A.A.; Lembo, F.; and Borrelli, F. (2009). Silymarin BIO-C, an extract from Silybum marianum fruits, induces hyperprolactinemia in intact female rats. *Phytomedicine*, 16(9): 839-844.

Janaky, R.; Ogita, K.; Pasqualotto, B.A.; Bains, J.S.; Oja, S.S.; Yoneda, Y.; and Shaw, C.A. (1999). Glutathione and signal transduction in the mammalian CNS, A Review. *J. Neurochem*., 73(3): 889-902.

Livak, K.J.; and Schmittgen, T.D. (2001). Analsis of relative gene expression data using real-time quantitative PCR and the 2(- Delta Delta C(T)) Method. *Methods*. 25: 402-410.

Maritim, A.C.; Sanders, R.A.; and Watkins, J.B. (2003). Diabetes, oxidative stress, and antioxidants: A review. *J. Biochem. Mol. Toxicol*., 17: 24–38.

Polyak, S.J.; Morishima, C.; Shuhart, M.C.; Wang, C.C.; Liu, Y.; and Lee, D.Y. (2007). Inhibition of T-cell inflammatory cytokines, hepatocyte NF-kappaB signaling, and HCV infection by standardized silymarin. *Gastroenterology*, 132: 1925–1936.

Radko, L.; and Cybulski, W. (2007). Application of silymarin in human and animal medicine. J. Pre-Clin. and Clin. Res., 1(1): 22-26.

Saller, R.; Brignoli, R.; Melzer, J.; Meier, R. (2008). An updated systematic review with meta-analysis for the clinical evidence of silymarin. *Forsch Komplementmed*, 15: 9–20.

Saller, R.; Meier, R.; and Brignoli, R. (2001). The use of silymarin in the treatment of liver diseases. *Drugs*, 61: 2035-206.
Sandoval, M.; Lazarte, K.; and Arnao, I. (2008). Antioxidant liver protection of *Vitis vinifera L.* (grape) skin and seed. An. Fac. med., 69(4): 250-259.
Singh, A.; and Handa, S.S. (1995): Hepatoprotective activity of *Apium graveolens* & *hygrophila auriculata* against paracetamol. Mol & thioacetamide intoxication in rats. Ethanopharmacol., Dec., 15; 49(3): 119-126.
Sonnenbichler, J.; and Zetl, I. (1986). Biochemical effects of the flavonolignane silibinin on RNA, protein and DNA synthesis in rat livers. *Prog. Clin. Biol. Res.*, 213: 319-31.
Sonnenbichler, J.; Zetl, I. (1988). Specific binding of a flavonolignan derivative to an estradiol receptor, in *Progress in Clinical and Biological Research* (Cody V, Middleton E, Karborne JB and Berck A eds) vol 280, pp 369–374, Alan R. Liss, Inc., New York.
Vladimir, K.; Daniela, W. (2005). Silybin and silymarin – new effects and application. Biomedical Papers, 149(1): 29-41.
Vogel, G. (1968). Arzneimittel-Foeschung. *Drug Res*., 18: 1063.

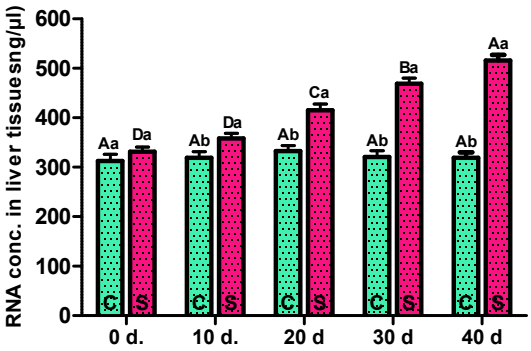

Figure (1): effect of Silymarin on RNA concentration of liver tissue samples at 0, 10, 20, 30, and 40 days of experiment in mature male rats.
The results represented as mean ± SE.
Different small letters denotes the present of significant differences (P<0.05) between groups.
Different capital letters denote the present of significant differences (P<0.05) between periods.
C: male rats drenched with drinking water (0.5 ml).
S: male rats drenched with Silymarin (200 mg/kg suspended in 0.5 ml of drinking water).
0d, 10d, 20d, 30d, and 40d represent the period of treatment.

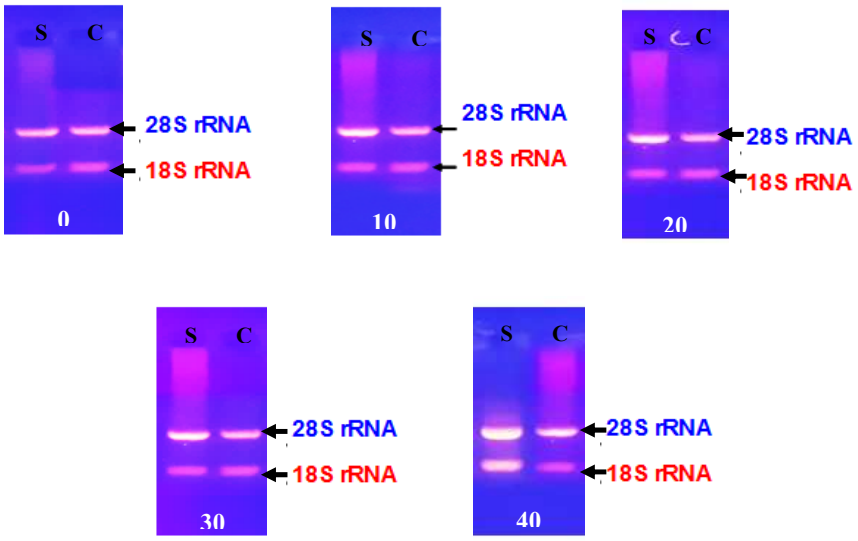

Figure (2): agarose gel electrophoresis for PCR products of liver tissue, where 28s rRNA and 18s rRNA products appear as positive results for S (silymarin treated male rats) and C (control male rats).

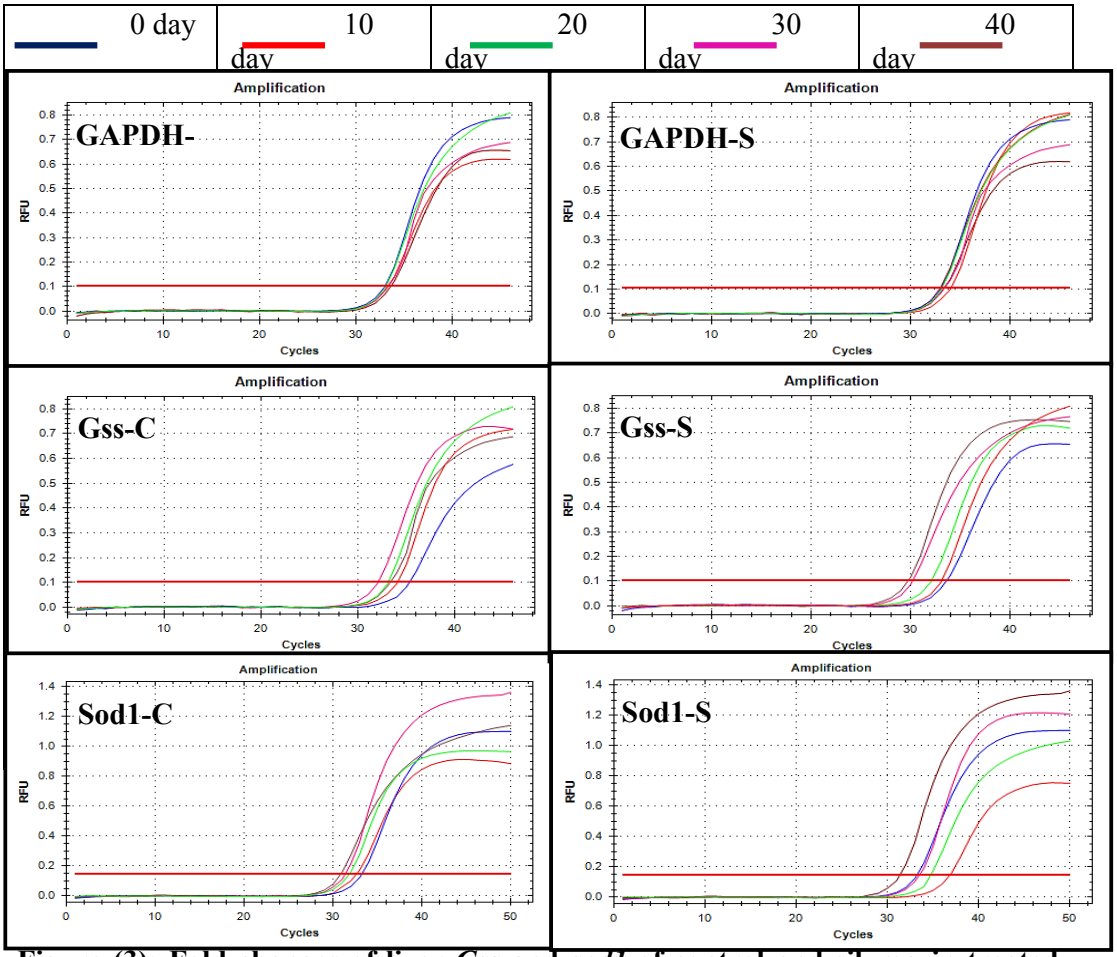

Figure (3): Fold changes of liver *Gss* and *sod1* of control and silymarin treated mature male rats.

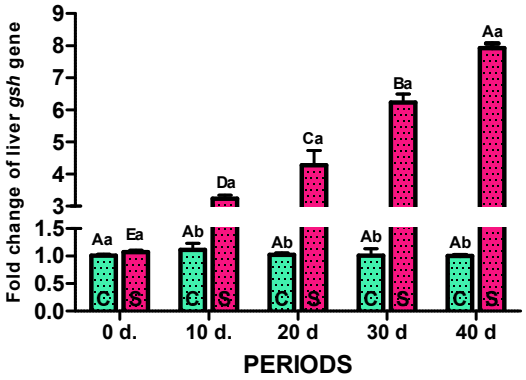

Figure (4): effect of Silymarin on expression level (fold changes) of liver *Gss* gene at 0, 10, 20, 30, and 40 days of treatment in mature male rats.

The results represented as mean ± SE.

Simillar small letters denotes the abscence of significant differences (P>0.05) between groups.

Simillar capital letters denotes the abscence of significant differences (P>0.05) between periods.

C: male rats drenched with drinking water (0.5 ml).

S: male rats drenched with Silymarin (200 mg/kg suspended in 0.5 ml of drinking water).

0d, 10d, 20d, 30d, and 40d represent the period of treatment.

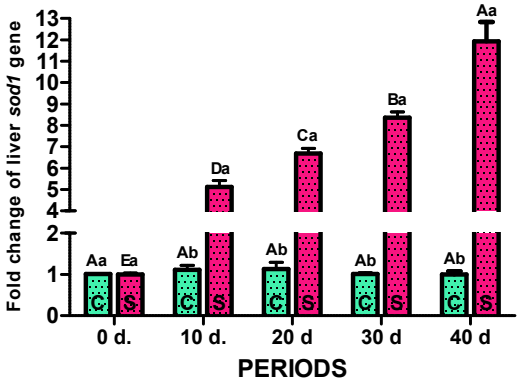

Figure (5): effect of Silymarin on expression level (fold changes) of liver *sod1* gene at 0, 10, 20, 30, and 40 days of treatment in mature male rats.

The results represented as mean ± SE.

Simillar small letters denotes the abscence of significant differences (P>0.05) between groups.

Simillar capital letters denotes the abscence of significant differences (P>0.05) between periods.

C: male rats drenched with drinking water (0.5 ml).

S: male rats drenched with Silymarin (200 mg/kg suspended in 0.5 ml of drinking water).

0d, 10d, 20d, 30d, and 40d represent the period of treatment.