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Functional and Nutritional Properties of Stirred Yoghurt Supplemented with Silymarin and its Impact on Chronic Hepatic Damage

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Abstract: The present work was conducted to study the impact of supplementation of stirred yoghurt with different levels of silymarin (0.0, 0.4, 0.6 and 0.8%) on the functional and nutritional properties of the resultant yoghurt. The attained results revealed that the phytochemistry analysis showed that the total phenolic compound in silymarin was 40.92% and radical scavenging activity (RSA) was 86.78%. The higher was the quantity of silymarin added causing the lower in the values of protein, fat and pH, also the higher in the values of ash, total solids (TS) and acidity of the resultant stirred yoghurt. Advancing storage period increased acidity and decreased pH. The lactic acid bacteria counts in stirred yoghurts increased in presence of silymarin. The sensory evaluation revealed that the fresh yoghurt was not affected organoleptically by the applied treatments, while the stored samples were affected at different manner. Moreover, the colour of stirred yoghurt samples had the best value of total colour (88.19%) in stirred yoghurt supplemented with 0.6% silymarin. The results of the biological evaluation of the stirred yoghurt samples supplemented with silymarin (0.4, 0.6 and 0.8%) and CCl_4 illustrated that the experimental rats fed on standard diet plus stirred yoghurt (7ml) by epi gastric tube had no observed differences in the serum levels of biochemical parameters and less abnormal signs throughout rats group along the tested period (28 days) compared with rats groups treated with CCl_4. The histopathological investigation of liver, kidney and spleen in rats injected with CCl_4 and fed on standard diet had been improved in groups treated with 0.8% silymarin than occurred in the rats groups treated with 0.4% and 0.6% silymarin, respectively.

Key words: Albino rats · CCl_4 · Histopathology · Kidney functions · Liver disease · Liver enzymes Silymarin · Stirred yoghurt

INTRODUCTION

Functional foods are diverse groups, including conventional foods, such as yoghurt, or they can be specifically enhanced, such as supplemented yoghurt with probiotics and fruit [1]. Yoghurt is known for its therapeutic, nutritional and sensory properties for a long time and it is obtained by the lactic acid fermentation of milk by addition of homo-fermentative yoghurt starter culture. It is well-known and consumed as fermented dairy product around the world. It plays an important role in human nutrition, health maintaining, therapeutic and dietetic functions [2]. Yoghurt and yoghurt-like products have been marketing and modifying successfully to meet consumers' demands [1]. The increasing yoghurt consumption trends in many countries have been attributed to increase the variety of flavoured yoghurt in markets. To create these flavors, a number of ingredients are incorporated in the yoghurt recipe. This includes whole foods, juices, jams, herbs, spices and sweeteners. The addition of different fruits into milk may enhance the taste and the therapeutical values of milk products as well. Therefore, the consumption of foods containing these compounds as a part of diet may also be healthful for people. In recent years, there has been increasing interest in the use of natural food additives into the diet [3]. The fermentation of milk by lactic acid bacteria releases a large number of peptides and amino acids with varying

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biological actions such as angiotensin-converting enzyme (ACE) inhibitory [4], immune modulatory [5] and antioxidant activities [6]. Yoghurt is a good matrix for enrichment with omega-3 fatty acids [7]. In contrast to omega-3 enriched milk, yoghurt enriched with omega-3 fatty acids had a very good oxidative stability [8]. *Silybum marianum,* commonly known Shoak el-gamal in Egypt, milk thistle, Mary thistle or deve dikeni (Turkish), is one of the oldest and thoroughly researched plant in the treatment of liver diseases. The active constituent of milk thistle is silymarin, a mixture of flavonolignans comprised of 4 isomers: silibinin, isosilibinin, silichristin and silidianin. Most supplements are standardized according to their silibinin (often called silybin) content, the main component of the silymarin [9]. Silymarin/silybin has being currently used as the supportive treatment for a liver-protective action without notable adverse effects, liver cirrhosis, chronic hepatitis [10], liver diseases associated with alcohol consumption and environmental toxin exposure [11]. Furthermore, Silymarin can be used as antifibrotic activity [12] in hepatic fibrosis in animal model induced by CCl_4 [13-16] and it was diminish the oxidative stress that results from a state of redox imbalance which is caused by the elevated generation of reactive oxygen species and a decreased antioxidant capacity [17]. Interestingly, not only silymarin can exert beneficial effects on the balance of cell survival and apoptosis via anti-inflammatory and anti-oxidative effects to prevent and/or treat liver diseases [18,19] but also, silymarin has an inhibitory effect on multiple cancer cell lines, including prostate, lung, colon, skin and bladder cancers cell line [20].

The objective of the present research was to manufacture stirred yoghurt supplemented with silymarin powder in order to increase its function properties i.e. serum function of liver and kidney and to stimulate antifibrotic activity in liver, kidney and spleen tissues in the experimental rats.

MATERIALS AND METHODS

Materials

Ingredient: Fresh cow's milk was obtained from the herd of the dairy cattle, at Faculty of Agriculture, Cairo University. Skim milk powder made in Poland was obtained from the local market (96% TS, 1.5% milk fat).

Bacterial Starter Cultures: Commercial yoghurt starter culture containing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* was obtained from Chr. Hansen Lab., Denmark. Silymarin powder was obtained from Bio-Egypt for biological products (New Cairo, Cairo, Egypt).

Experimental Procedures

Production of Stirred Yoghurt: Fresh cream was mechanically separated from fresh cow's milk and mixed with different levels of silymarin powder to be nil (the control), 0.4, 0.6, 0.8% in the final milk mixtures which were homogenized using homogenizer (Jank & GmbH & Co KG, Germany (27000 rpm/min) and then mixed with skim cow's milk to standardize it to 3 % fat. All treatments were enriched with 2% skimmed milk powder. Stirred Yoghurts of different treatments were manufactured according to the procedure of Erdogan and Zekai [21], with some modifications as follows: Cow's milk (fat 3%, protein 3.72%, total solids 12.3% and acidity 0.17%) was used for yoghurt production after supplementation with 2% skim milk powder to increase total solids. The mixes were heated to 85°C for 10 min. and then rapidly cooled to 45°C. The working yoghurt culture was added at the rate of 2% (w/v). The yoghurt milks were incubated at 43°C. Incubation was terminated till pH 4.6. At this point, the yoghurt was put overnight in a refrigerator then; the samples of yoghurt were stirred and filled into 100g plastic cup. Three replicates were done for each treatment. The resultant stirred yoghurt samples were stored in refrigerator at 5±1°C for 14 days. The samples were analyzed at 0, 3, 7, 10 and 14 days intervals during cold storage.

Analytical Methods

Total Phenolics Contents of Silymarin Powder: The total phenolics contents of silymarin powder were determined using Folin-Ciocalteu reagent according to Yu *et al.* [22]. The reaction mixture contained 100µl of extracts and 500µl of the Folin-Ciocalteu reagent and 1.5ml of 20% sodium carbonate. The final volume was made up to 10ml with water. After 2h of reaction, the absorbance at 765 nm was determined and used to estimate the phenolic contents using a standard curve prepared using gallic acid.

Assay of DPPH Radical Scavenging Activity: DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacities of silymarin powder were determined by the reduction of the reaction colour between DPPH solution and sample extracts as previously described by Huang *et al.* [23]. A final concentration of DPPH solution

used was 0.15 mM for phenolic extracts instead of 0.075 mM for silymarin powder. DPPH solution (3.9ml) was mixed with sample solution (0.1ml). The mixture was kept in the dark at ambient temperature. The absorbance of the mixtures was recorded at 515 nm for exactly 30 min. Blank was made from 3.9 ml of DPPH and 0.1ml methanol and measured absorbance at t = 0. The scavenging of DPPH was calculated according to the following equation [24].

% DPPH scavenging ={(Abs (t=0)- Abs(t=30))/Abs (t=0)} x 100

where: Abs (t=0 min) = absorbance of DPPH radical + methanol at t = 0 min; Abs (t=30 min) = absorbance of DPPH radical + phenolic extracts at t = 30 min.

Physiochemical Analysis of Stirred Yoghurt: Protein, ash and total solids contents of stirred yoghurt samples and fat of silymarin were determined according to AOAC [25]. Titratable acidity as lactic acid (TA %) and fat of stirred yoghurt were determined according to Ling [26]. The pH value of stirred yoghurt samples was measured electrometrically using Lab. pH meter with glass electrode, Hanna model 8417 digital pH meter. Colour parameters of stirred yoghurt samples were measured according to Hunter (Lab.) Colour scale using a spectrophotometer (MOM, Hungary) and as out lined by Calvo *et al.* [27]. The CIE-coordinates X, Y, Z were converted to L, a and b-values using conversion equations provided by the manufacture.

Microbiological Examination of Stirred Yoghurt: Lactic acid bacteria (LAB) were counted using MRS agar medium according to De Man *et al.* [28] The plates were incubated at 37°C for 48 h. Coliform was enumerated using Violet Red Bile Agar medium as reported by American Public Health Association (APHA) [29]. The plates were incubated at 37°C for 48 h. Yeasts and moulds were counted on Malt-Extract Agar medium as suggested by Harrigan and McCance [30]. The plates were incubated at 25-27°C for 4 days.

Sensory Evaluation: Sensory evaluation of stirred yoghurt was done during cold storage period by ten staff members of Food Science Department, Faculty of Agriculture, Ain Shams University. To score stirred yoghurt samples, a maximum number of points were assigned to each sample characteristic identified according to the scheme of Keating and Rondwhit [31].

Biological Analysis

Animal, Housing and Diet: Sixty male albino rats of one month old weighting about 150±5g were obtained from the Research Institute of Ophthalmology, Giza, Egypt. The animals groups were placed in a cage at in an atmosphere of filtered, pathogen-free air and water and maintained at a temperature between 20-25°C with a 12 h light/dark cycle and light cycle (8-20 h) and relative humidity of 50%. The animals acclimatized for one week as an adaptation period. The animals were randomly divided into two main groups each groups divided to 5 subgroups of six rats each. The first group of rats (control) was fed on standard diet, while the remaining groups were fed a standard diet plus stirred yoghurt supplemented with different ratios of silymarin by epi-gastric tube. The second group was subcutaneous injection with CCl_4 in paraffin oil (50% v/v 2ml/kg) twice per week for 4 weeks to induce liver tissue damage [32] (Table 1). The rats were weighed weekly till the end of the experimental period, all animals were fasted for 12 hrs, Blood samples were withdrawn from orbital plexus venous by using fine capillary glass tubes [33], blood samples were collected into plain tubes without anticoagulant and allowed to clot, blood samples were centrifuged at 3000 rpm for 10 min at 4°C, to obtain clear serum. Serum was frozen at -80°C until analyzed. The animals were anesthetized with ether and sacrificed. They were quickly dissected to excise the liver, kidney and spleen. These organs were weighed and then kept in 10% formaldehyde until histological investigations.

Biological Determination: Biological evaluation of the different tested diets was carried by determination of initial body weight (IBW), final body weight (FBW) and body weight gain% (BWG %) and organs weight/body weight % according to Chapman *et al.* [34]. The serum uric acid was determined at 510 nm according to method reported by Barham and Trinder [35]. Serum urea nitrogen was determined at 550 nm according to the method described by Fawcett and Soctt [36]. Serum creatinine was determined at 510 nm as given by Larsen [37]. Alanine amino transferase (ALT) and aspartate amino transferase (AST) activities were determined colorimetrically at 505 nm according to the method of Reitman and Frankel [38], for all parameters under studying which had been estimated in serum samples were made by using spectrophotometer (model DU 4700) and were analyzed by using biodiagnostic kits.

Table 1: Experimental diet of rats used in the biological study

Groups	Experimental diets
First group	
I	Standard diet (control negative)
II	Standard diet + 7ml stirred yoghurt by epi gastric tube.
III	Standard diet + (7ml stirred yoghurt supplemented with 0.4% silymarin by epi gastric tube).
IV	Standard diet + (7ml stirred yoghurt supplemented with 0.6% silymarin by epi gastric tube).
V	Standard diet + (7ml stirred yoghurt supplemented with 0.8% silymarin by epi gastric tube).
Second group	
VI	*CCl_4 (control positive) + Standard diet
VII	*CCl_4 + Standard diet + 7ml stirred yoghurt by epi gastric tube
VIII	*CCl_4 + Standard diet + (7ml stirred yoghurt supplemented with 0.4% silymarin by epi gastric tube).
IX	*CCl_4 + Standard diet + (7ml stirred yoghurt supplemented with 0.6% silymarin by epi gastric tube).
X	*CCl_4 + Standard diet + (7ml stirred yoghurt supplemented with 0.8% silymarin by epi gastric tube).

*Subcutaneous injection

Histopathology Technique: The tissues of liver, kidney and spleen were fixed immediately after dissection in 10% neutral formalin for 24h, then dehydrated in ascending concentration of alcohol, cleaned in xyline and embedded in paraffin wax. Tissues were sectioned at a thickness of 3 micron and stained with hematoxylin and fosin stains [39]. All tissues were examined by the light microscope for detection of any histopathological alteration.

Statistical Analysis: The obtained data were exposed to analysis of variance. Duncan's multiple range tests at 5% level of significance was used to compare between means. The analysis was carried out using the PROC ANOVA procedure of Statistical Analysis System (SAS) [40].

RESULTS AND DISCUSSION

Chemical Properties of Silymarin: The obtained results of silymarin powder analysis indicated that, total phenols content was 40.92mg gallic acid/g dry matter. While, radical scavenging activity (RSA) was 86.78% and fat content was only 1.81%.

Physicochemical Properties of Stirred Yoghurts: Data presented in Table 2 indicated that control sample and stirred yoghurt supplemented with 0.4% silymarin had the highest protein content being 3.95 and 3.90 % respectively. This was followed by stirred yoghurt supplemented with silymarin at levels 0.6 and 0.8% since concentrations of protein were 3.85 and 3.79 %, respectively. These differences were significance ($P \leq 0.05$). The fat content showed the highest level in the control stirred yoghurt sample ($P \leq 0.0.5$) being 3.06% and the other treated stirred yoghurt samples had nearly the same level. So silymarin levels didn't affect the level of the fat of treated stirred yoghurts. With respect to the ash content, exception of stirred yoghurt with silymarin (0.8%) that the other samples recorded the values of 0.72 0.75 and 0.78% for control and stirred yoghurt supplemented with 0.4 and 0.6% silymarin, respectively. Such differences were significant. Ali *et al.* [41] noticed that, ash is a measure of the total amount of minerals present within stirred yoghurt. It is important to the mineral contents of stirred yoghurt during processing because they play roles in physiochemical properties of stirred yoghurt. In general, the addition of different ratios of silymarin in stirred yoghurt manufacture also increased the total solids (14.30-14.97%) and ash contents and decreased contents of fat and protein in the final product (Table 2). These results are in agreement with those obtained by Erdogan and Zekai [21] and Farahat and El-Batawy [42].

Acidity in Stirred Yoghurt: The changes of titratable acidity in stirred yoghurt samples during storage period as affected by the applied treatments are shown in Table 3. The acidity in silymarin added stirred yoghurts were significantly higher than those of control. This variation was probably due to the high acidity of silymarin added to the stirred yoghurts and also to the activity of yoghurt starter cultures. The titratable acidity of control and silymarin-added stirred yoghurts increased significantly during the storage period ($P \leq 0.05$). The lowest titratable acidity value was obtained from the fresh and 3 days. Yoghurt control samples being 0.70%, but the highest value (0.98%) were found in sample from T3 after14 day and these differences were statistically ($P \leq 0.05$) significant. Samples from T3 of 14 days storage had the highest acidity value of 0.98%.This was followed by values of 0.95, 0.89 and 0.85% for T2, T1 and control of the same age, respectively. Our results are in accordance with some researchers reported that the titratable acidity of fruit-flavoured yoghurts increased during storage [43, 44].

Table 2: Major chemical composition of fresh stirred yoghurt supplemented with different ratios of silymarin powder.

	Major chemical constituent (%)			
Treatments	Protein	Fat	Ash	Total solids
Control	3.95±0.01[a]	3.06±0.01[a]	0.72±0.01[d]	14.30±0.01[d]
T1	3.90±0.01[b]	2.95±0.01[b]	0.75±0.01[c]	14.50±0.01[c]
T2	3.85±0.01[c]	2.90±0.01[b]	0.78±0.01[b]	14.75±0.01[b]
T3	3.79±0.01[d]	2.84±0.01[c]	0.82±0.01[a]	14.97±0.01[a]

Control: stirred yoghurt without any silymarin,T1: stirred yoghurt supplemented with 0.4% silymarin, T2: stirred yoghurt supplemented with 0.6% silymarin; T3: stirred yoghurt supplemented with 0.8%.silymarin

a, b, c, d: Means with different letters among treatments in the same column are significantly different ($P \leq 0.05$).

Table 3: Titratable acidity (% as lactic acid) of stirred yoghurt supplemented with different ratios of silymarin powder during storage period

	Treatments*				
Storage period (days)	Control	T1	T2	T3	Means of storage ***
Fresh	0.70±0.01	0.70±0.005	0.73±0.005	0.76±0.005	0.72[E]
3	0.71±0.005	0.72±0.005	0.78±0.005	0.79±0.005	0.74[D]
7	0.74±0.005	0.85±0.005	0.89±0.005	0.90±0.005	0.84[C]
10	0.80±0.005	0.87±0.01	0.90±0.005	0.92±0.005	0.87[B]
14	0.85±0.005	0.89±0.005	0.95±0.005	0.98±0.01	0.90[A]
Means of treatment**	0.76[d]	0.80[c]	0.84[b]	0.86[a]	--

* Data are presented as means ± SDM ($n=6$). **Data in a row with different superscript small letters are statistically different; ($P \leq 0.05$) and ***Data in a column with different superscript capital letters are statistically different ($P \leq 0.05$); Control: stirred yoghurt without any silymarin,T1: stirred yoghurt supplemented with 0.4% silymarin, T2: stirred yoghurt supplemented with 0.6% silymarin; T3: stirred yoghurt supplemented with 0.8%.silymarin

Table 4: The pH value of stirred yoghurt supplemented with different ratios of silymarin powder during storage period.

	Treatments*				
Storage period (days)	Control	T1	T2	T3	Means of storage ***
Fresh	4.68±0.01	4.65±0.01	4.62±0.01	4.61±0.01	4.64[A]
03	4.66±0.01	4.62±0.01	4.61±0.01	4.60±0.01	4.62[B]
07	4.62±0.01	4.61±0.01	4.60±0.01	4.57±0.01	4.60[C]
10	4.60±0.01	4.59±0.01	4.57±0.01	4.54±0.01	4.57[D]
14	4.59±0.01	4.55±0.01	4.53±0.01	4.51±0.01	4.54[E]
Means of treatment**	4.62[a]	4.60[b]	4.58[c]	4.56[d]	

*Data are presented as means ± SDM ($n=6$). **Data in a row with different superscript small letters are statistically different ($P \leq 0.05$) and ***Data in a column with different superscript capital letters are statistically different ($P \leq 0.05$)

Control: stirred yoghurt without any silymarin, T1: stirred yoghurt supplemented with 0.4% silymarin, T2: stirred yoghurt supplemented with 0.6% silymarin; T3: stirred yoghurt supplemented with 0.8%.silymarin

The pH Value: As seen from the data in Table 4 that the highest pH at any given storage time was recorded for the control. This was followed by decreased values in the silymarin treated samples. The higher was the amount of silymarin added, the lower were the values of pH, this was true at any given storage time. In general, the maximum pH value was recorded for the fresh control, where samples from T3 (with the highest amount of silymarin) had the lowest pH (4.51) at the end of storage period. These results agree with statistical analysis the highest significant was 4.64 at fresh time in the mean of storage, also control samples record the same trend in mean of treatment 4.62 at $P \leq 0.05$. Zekai and Erdogan [45] showed that, the decrease in pH was accompanied by an increase in the alcoholic aroma and acidic taste of stirred yoghurt samples resulting in decreased flavour scores. These results are in general with the trends given in the literature [46].

Colour Characteristics of Stirred Yoghurt Samples: Table 5 shows the colour parameters of stirred yoghurt samples measured according to the three stimulus colour coordinates. The lightness (L-values) of the treatments of stirred yoghurt supplemented with different

Table 5: Colour of stirred yoghurt supplemented with different ratios of silymarin powder at the end of storage at 5±1°C for 14 days

Treatments	Lightness (L- value)	Redness (a-value)	Yellowness (b-value)	Chroma*	HUE Arctan b/a	Total Color Index**
Control	90.29	-15.83	7.80	17.65	26.23	92.00
T1	84.88	-13.02	19.98	23.85	56.90	88.17
T2	84.60	-11.92	21.85	24.89	61.38	88.19
T3	82.89	-11.22	23.70	26.22	64.66	86.94

* $va^2 + b^2$; ** $va^2 + b^2 + L^2$

Control: stirred yoghurt without any silymarin, T1: stirred yoghurt supplemented with 0.4% silymarin, T2: stirred yoghurt supplemented with 0.6% silymarin; T3: stirred yoghurt supplemented with 0.8%.silymarin

ratio of silymarin were lower than those of control sample, while the redness (a-values) of the treatments were higher than those of control samples. The a-readings were negative indicating a greenish colour shade in control and treated stirred yoghurt samples. One the other side, the yellowness (b-values) of the treatments was remarkably higher in treated samples than that of control with less difference in b-values among the treatments by changing the different concentration of the added ratio of silymarin. Furthermore, the Hue-angle (colour type) of the treated samples was much higher than that of control indicating the tendencies of treated samples to become yellower than the control. Also, the addition of different ratio of silymarin (0.4, 0.6 and 0.8%) caused the treated samples were more saturated in colour concentration than control. Total colour index should the highest value for the control sample (92) than the other treatments (88.17, 88.19 and 86.94) in T1, T2 and T3, respectively. According to Jovanka *et al*. [47] the increase in b-value is associated with increase in acidity of the dairy products resulting from the reaction between aldehyde groups of lactine sugar and amino acids producing millard reaction with brown - coloured judgments. Also the a-values have a negative sign indicating the presence of components of a green (-) colour which may be due to the presence of tryptophan, tyrosine and riboflavin. Also, Bosch *et al*. [48] reported a decrease in L- value and increase in both a- and b- values by increasing amounts of hydrolyzed cereals flour added to milk.

Microbiological Analyses of Stirred Yoghurts Supplemented with Different Ratios of Silymarin:

Lactic Acid Bacteria Counts: The presence of silymarin increased lactic acid bacteria (LAB) counts in all stirred yoghurts samples compared to the control stirred yoghurt as seen in Table 6. Viable LAB counts reduced from day 3 to day 14 of storage for all stirred yoghurts samples. Viable LAB counts on day 14 of storage for stirred yoghurt supplemented with 0.8% silymarin was higher (6.28×10^6cfu/ml) than corresponding control stirred yoghurt (6.11×10^6cfu/ml). The same trend was noticed in other treated samples of T1 and T2 ($6.23, 6.28 \times 10^6$cfu/ml, respectively). The decrease in the viable cell counts for both stirred yoghurt bacteria can be attributed to the organic acids accumulation as a result of growth of LAB and fermentation. The gradual decrease in lactic acid bacterial counts may be also due to the sensitivity of these bacteria to acid developed during the storage period. However, impact of the amount of silymarin should be taken in consideration in this respect. These results are in harmony with those obtained by Oliveira *et al*. [49] and Paseephol and Sherkat [50].

Coliform, Yeasts and Moulds Counts: Coliform bacteria were not detected (ND) in all fresh or stored yoghurt samples. It can be clearly seen from Table 6 that yeasts and moulds were not detected in fresh and after 3 days except the control sample. The highest values were recorded after 14 days of storage in all samples. This may attributed to contamination from air or the post contamination from silymarin homogenates and during filling the products. The highest values of Yeast and moulds are in harmony with those given by Con *et al.* [51] and Zekai and Erdogan [45], who reported that, all the stored stirred yoghurt samples showed significant increases in yeast and mould count. Farahat and El Batawy [42] found that, Yeasts and moulds count was higher in fruit stirred yoghurt compared with the control and increased significantly during storage at 5±1°C.

Sensory Evaluation of Stirred Yoghurts Supplemented with Different Ratios of Silymarin: As shown in Table 7 sensory evaluation results showed that increasing levels of silymarin negatively influenced the sensory scores of some properties of stirred yoghurts. The maximum attainable score was given for appearance of all fresh samples, whereas after 14 days of storage the samples supplemented with the highest amount of silymarin ranked the lowest score for appearance (7.3). Body and texture of all fresh samples ranked 40 out of 40 points, while adding 0.4 and 0.8% silymarin decreased the score given in samples of 7, 10 and 14 days to the minimum levels. This was also true for the flavour, since the higher was the amount of silymarin added (0.8%). The lower was

Table 6: Counts of Lactic acid bacteria and yeasts & moulds (log CFU/ml) of fresh and stored stirred yoghurt supplemented with different ratios of silymarin

	Storage period (days)				
Treatment	Fresh	03	07	10	14
	Lactic acid bacteria counts				
Control	7.04	7.08	7.00	6.56	6.11
T1	7.30	7.28	7.26	6.74	6.23
T2	7.32	7.32	7.23	6.76	6.28
T3	7.40	7.41	7.32	6.79	6.28
	Yeasts and Moulds				
Control	ND	1.18	2.48	3.04	3.60
T1	ND	ND	2.30	2.90	3.51
T2	ND	ND	2.18	2.86	3.55
T3	ND	ND	2.18	2.83	3.48

Control: stirred yoghurt without any silymarin, T1: stirred yoghurt supplemented with 0.4% silymarin, T2: stirred yoghurt supplemented with 0.6% silymarin; T3: stirred yoghurt supplemented with 0.8%.silymarin. ND: not detected-

Table 7: Sensory evaluation of stirred yoghurts supplemented with different ratio of silymarin during storage at 5±1°C for 14 days

		Storage period (days)					
Criteria	Treatments	Fresh	3	7	10	14	Means of treatments**
Flavour (50)	Control	49.5±0.57	47.6±0.5	46.3±0.5	42.0±0	38.00± 0	44.80 ^{a}
	T1	46.6±0	44.3±2.3	40.3±1.5	37.6±0.57	34.00±1.73	39.80^{b}
	T2	46.0±0	43.0±1.7	39.6±2.8	35.3±0.57	33.00±0	38.53^{c}
	T3	46.3±0.57	43.3±2.3	39.3±2.3	35.3±0.57	33.00±0	38.60^{c}
Means of storage ***	--	47.17^{A}	43.67^{B}	40.08^{C}	36.83^{D}	34.42^{E}	--
Body &Texture (40)	Control	40.0±0	40.0±0	38.3±1.15	34.0±1.73	30.66±1.52	36.60^{a}
	T1	40.0±0	38.0±1.7	32.3±0.5	28.3±1.15	25.0±1.73	32.73^{b}
	T2	40.0±0	38.3±1.15	35.3±0.5	32.3±1.15	27.6±1.15	34.73^{c}
	T3	40.0±0	35.6±0.5	33.6±1.15	30.3±1.15	26.0±1.73	33.13^{c}
Means of storage ***	--	40.00^{A}	38.00^{B}	34.92^{C}	31.25^{D}	27.33^{E}	--
Appearance (10)	Control	10.0±0	10.0±0	9.0±0	8.6±0.57	8.3±0.57	9.20^{a}
	T1	10.0±0	9.6±0.5	8.6±0.5	8.3±0.57	7.6±0.57	8.87^{b}
	T2	10.0±0	9.3±0.5	9.0±0	7.6±0.57	7.3±0.57	8.67^{b}
	T3	10.0±0	9.6±0.5	8.0±0	7.6±0.57	7.3±0.57	8.53^{b}
Means of storage ***	--	10.00 ^{A}	9.67^{A}	8.67^{B}	8.08^{C}	7.67^{D}	--

* Data are presented as means ± SDM ($n=6$). **Data in a row with different superscript capital letters are statistically different ($P \leq 0.05$) and ***Data in a column with different superscript small letters are statistically different ($P \leq 0.05$)

Control: stirred yoghurt without any silymarin, T1: stirred yoghurt supplemented with 0.4% silymarin, T2: stirred yoghurt supplemented with 0.6% silymarin; T3: stirred yoghurt supplemented with 0.8%.silymarin

the score given for the flavour of 7, 10 and 14 days in stirred yoghurt samples. These are in agreement with those obtained by Joseph *et al.* [52], who revealed that, flavour had significant influence ($p<0.05$) on overall acceptability of stirred yoghurt. In recent years, there has been increasing interest in the use of natural food additives into the diet [53]. Also, using different fruit additives gives more stirred yoghurt choices to the consumer. The sensory scores of all the samples in the present study decreased during storage period. This is may be due to the acidity development or the production of microbial metabolism which slightly affected the rheological and sensory properties of the product.

Biological Evaluation of Stirred Yoghurts Supplemented with Different Ratios of Silymarin

Body and Organs Weights: The final body weights (FBW) of rats for different healthy groups are given in Table 8. There were significant differences ($P \leq 0.05$) in the final body weights of rats in the control (-) group (165.8±22.0) and the remaining treated groups (healthy group with yoghurt supplemented with silymarin 0.4, 0.6 and 0.8%) since the FBW values were (186.1±4.1, 186.3±5.3 and 197.2±6.2) respectively. The weights of rats fed on standard diet plus stirred yoghurts (7ml) by epi gastric tube were 165.6±6.4, compared with the control (-) group (fed on standard diet only) record the same rang.

Table 8: Mean body weight gain (g) of rats (healthy and injected groups with CCl_4) fed on stirred yoghurts supplemented with different ratio of silymarin.

		SD + Yoghurt + silymarin (Treatments %)			
Periods (days)	Standard diet (SD)	0.0	0.4	0.6	0.8
	Control (-)	Healthy groups			
IBW	122.0±2.4 [a]	125.0±3.1 [a]	123.3±5.1 [a]	123.3±4.0 [a]	124.1±4.9 [a]
7	150.2±16.5 [b]	177.5±7.4 [a]	169.6±8.2 [a]	165.6±6.4 [a]	175.8±6.8 [a]
14	155.0±18.1 [c]	185.1±8.3 [a]	171.6±4.5 [b]	170.6±6.0 [b]	181.4±5.5 [ab]
21	160.4±20.2 [d]	191.1±8.3 [a]	171.6±5.2 [cd]	175.3±5.7 [c]	190.4±5.0 [ab]
FBW	165.8±22.0[c]	165.6±6.4 [c]	186.1±4.1 [ab]	186.3±5.3[a b]	197.2±6.2 [a]
BWG (%)	10.95±4.9 [d]	10.15±0.81[d]	12.08±0.03[c]	14.5±0.33[b]	18.28±0.33[a]
	Control (+)	Injected groups with CCl_4			
IBW	159.0±10.0 [c]	194.1±15.2 [ab]	183.6±16.8 [b]	196.0±6.2 [ab]	200.0±9.7 [a]
7	160.6±10.8 [c]	197.8±14.3 [ab]	188.1±18.2 [b]	201.6±6.1 [ab]	207.3±8.2 [a]
14	163.4±10.7 [c]	206.8±16.1 [ab]	197.3±21.4 [b]	209.0±8.9 [ab]	215.8±8.2 [a]
21	164.8±9.4 [c]	212.3±15.9 [ab]	202.0±19.9 [b]	213.8±7.7 [ab]	221.5±6.8 [a]
FBW	166.6±8.0 [b]	224.0±21.7 [a]	209.3±20.7 [a]	220.0±7.8 [a]	228.1±5.9 [a]
BWG	1.9±0.5[c]	7.48±1.63[a]	6.43±0.98[b]	6.0±0.4[bc]	7.03±0.95[ab]

IBW: initial body weight (g); FBW: final body weight(g); BWG: body weight gain (%).
Data are presented as means ± SDM. **Data in a row with different letters are statistically different ($P \leq 0.05$)

Table 9: Mean organs weight/body weight (%) of rats fed on stirred yoghurts supplemented with different ratio of silymarin

		SD + Yoghurt + silymarin (Treatments %)			
Organ	Standard diet (SD)	0.0	0.4	0.6	0.8
	Control (-)	Healthy groups			
Liver	2.9±0.41 [b]	4.30±0.65 [a]	3.93±0.45 [a]	4.20±0.41 [a]	4.51±0.42 [a]
Kidney	0.68±0.07 [a]	0.65±0.10 [a]	0.63±0.07 [a]	0.71±0.08 [a]	0.72±0.12 [a]
Spleen	0.41±0.07 [b]	0.38±0.07 [b]	0.43±0.12 [ab]	0.50±0.10 [ab]	0.55±0.12 [a]
	Control (+)	Injected groups with CCl_4			
Liver	3.0±0.33 [a]	2.33±0.42 [b]	1.86±0.34 [c]	2.13±0.27 [bc]	2.41±0.24 [b]
Kidney	0.65±0.10 [a]	0.51±0.04 [b]	0.51± 0.05 [b]	0.55±0.04 [b]	0.60±0.06 [ab]
Spleen	0.33±0.10 [a]	0.21±0.04 [b]	0.21±0.10 [b]	0.23±0.08 [b]	0.25±0.04[a b]

* Data are presented as means ± SDM ($n=6$). Data in a row with different superscripts letters are statistically different ($P \leq 0.05$); a, b, c: Means with different letter among treatments in the same row are significantly different

Healthy group with yoghurt supplemented with 0.8% silymarin was the best result. Body weight gain was recorded the highest (7.48%) for the injected groups with CCl_4 (stirred yoghurt group) (fed on standard diet + 7ml stirred yoghurt by epi gastric tube) while values for the remaining treated groups with silymarin of 0.4, 0.6 and 0.8% ranged between 6.0 and 7.03 g. The lowest rates of body weight gain occurred in control (+) group (1.9), while the best increase in BWG was 7.48 in injected groups fed on stirred yoghurt followed by treated groups fed on stirred yoghurts supplemented with silymarin 0.8% (7.03).

The weights of the various organs/body weight % of the rats are shown in Table 9. The weights of the organs (liver, kidney and spleen) of rats maintained on experimental diets of stirred yoghurt + 0.4 silymarin, were (3.93±0.45, 0.63±0.07 and 0.43±0.12) for 0.6% were, (4.20±0.41, 0.71±0.08 and 0.50±0.10) and for 0.8% were (4.51±0.42, 0.72±0.12 and 0.55±0.12), respectively. In injected groups with CCL_4, there was almost significant difference in the weight of liver, kidney and spleen of rats from control (+) groups. The remaining 3 treatments were either show ratio of weight change or suffered a weight loss ranged from -38 to -19.67% after 28 days. On the other hand, experimental groups with 0.4, 0.6 and 0.8% silymarin of rats showed kidney and spleen weight loss after 28 days, being -21.54, -15.38, -7.69% and -36.36, -30.30, -24.24 respectively.

Biochemical Analysis: Results of biochemical analysis for all tested groups are presented in Table 10. Alterations in the liver enzyme (ALT and AST) were statistically significant ($P \leq 0.05$) in all of tested groups. Kidney function (urea, uric acid and creatinine) was statistically different from control (-) group (fed on standard diet) and four among the other groups (fed on experimental diets).

Table 10: Blood chemical analysis of experimental rats

Parameters	Standard diet (SD)	S.D+ Yoghurt + silymarin (Treatments %) 0.0	0.4	0.6	0.8
	Control (-)	Healthy groups			
Liver function (U/l)					
ALT	34.3±0.6[a]	37.93±7.3[bc]	42.75±0.2[c]	43.9±0.3[d]	36.2±2.2[b]
AST	36.9±5.6[a]	38.6±21.8[cd]	38.6 ±3.5[c]	37.4±1.8[b]	38.5±16.9[d]
Kidney function (mg/dl)					
Urea	33.41±0.12 [a]	37.49±0.09 [d]	36.37±0.08 [cd]	35.24±0.06 [c]	34.73±0.07 [b]
Uric Acid	4.70±3.02[c]	4.83±5.84[d]	4.16±7.35 [a]	4.33±5.16 [b]	4.63±4.91 [cd]
Creatinine	1.16±1.88 [a]	1.38±0.92 [bc]	1.25±1.16 [bc]	1.49±0.85[d]	1.33±3.53 [c]
	Control (+)	Injected groups with CCl_4			
Liver function (U/l)					
ALT	36.1±0.3[a]	46.3±0.4[d]	46.3±0.4[d]	44.0±0.4[c]	43.9±0.4[b]
AST	65.0±9.8[c]	67.8±6.3[d]	57.8±6.3[bc]	49.7±4.3[bc]	38.0±3.1[a]
Kidney function (mg/dl)					
Urea	71.64±0.16 [a]	60.83±2.9 [b]	54.93±5.4 [c]	49.79±3.8 [d]	41.26±2.2 [cd]
Uric Acid	5.40±2.7 [a]	5.63±3.7 [b]	5.33±2.0 [ab]	5.29±5.8 [ab]	5.23±2.0 [c]
Creatinine	3.39±0.7 [a]	2.84±0.3 [b]	2.63±0.5 [bc]	2.21±0.1 [c]	1.90±0.5 [d]

* Data are presented as means ± SDM ($n=6$). Data in a row with different superscripts letters are statistically different (P >0.05)
AST: aspartate amino transferase; ALT: alanine amino transferase

Amounts of uric acid and creatinine were statistically different from control (-). The means values of serum uric acid, urea nitrogen and creatinine increased gradually with increasing the level of protein in the diet. In this respect, Frey [54] mentioned that the serum urea nitrogen is a substance that is formed in the liver when the body breaks down protein.

The positive control group in Table 10 recorded a significant increase ($P \leq 0.05$) in the activities of serum ALT, as compared to negative control group or the treated samples (different ratio of silymarin 0.4, 0.6 and 0.8%) respectively. Feeding rats on stirred yoghurt or stirred yoghurt + silymarin 0.4, 0.6 and 0.8% decreased serum AST enzyme. It was noticed also that there was a decrease in the mean values of urea and creatinine in all tested groups, compared to the control positive group (71.64±0.16 and 3.39±0.7 respectively) as observed in Table (10). However, there was a significant decrease in the level of uric acid in the tested groups than the control (+) group (5.40±2.7) and significantly elevated ($P < 0.05$) compared to the untreated control group (negative control). These results are in agreement with those obtained by Ozturk *et al.* [55], who tried to test the efficiency of *Silybum marianum* in different doses to treat carbon tetrachloride (CCl_4) induced liver damage. Alkaladi and Abdelazim [56] noticed the protective role of silymarin in rats- liver tissues damage induced by chemical carcinogenesis. The results demonstrated that ALT, AST, levels were significantly lower in groups treated with silymarin compared with group treated with CCl_4. Increased serum creatinine above normal levels may reflect a destroy of 50% of renal nephrone [57]. These results were performed by Wafay *et al*. [58], who stated that the CCl_4 caused an increase in serum levels of the diagnostic enzymes (ALT, AST and GGT) in rats that received CCl_4 as compared to the control group.

Histopathological Examination:

Liver: Fig. 1 showed that the histopathological examination of the liver sections from control negative (-) (normal rats fed on standard diet only) showed normal appearance of hepatic cells with well preserved cytoplasm, prominent nucleus and nucleolus, central vein and portal area (Fig. 1A). While in the control positive group (normal rats fed on commercial diet + injection with CCl_4) showing fibroplasia in the portal triad control positive (+), kupffer cells activation and portal infiltration with leucocytes and small focal hepatic necrosis associated with leucocytic cells infiltration. As shown in Fig. 1A the rats fed on stirred yoghurt showing hydropic degeneration of hepatocytes. While, the animals affected by CCl_4 plus fed on stirred yoghurt showing kupffer cells activation as seen in Fig. 1B. As seen in Fig.1A-F the animals fed (stirred yoghurt supplemented with silymarin 0.4, 0.6 and 0.8%), showed normal histopathological changes. In contrast, animals affected by CCl_4 and fed (stirred yoghurt supplemented with silymarin 0.4%. Fig. 1C-F showed congestion, mild periportal inflammation and mild steatosis in stirred yoghurt protected rat's liver at the end of the 4th week.

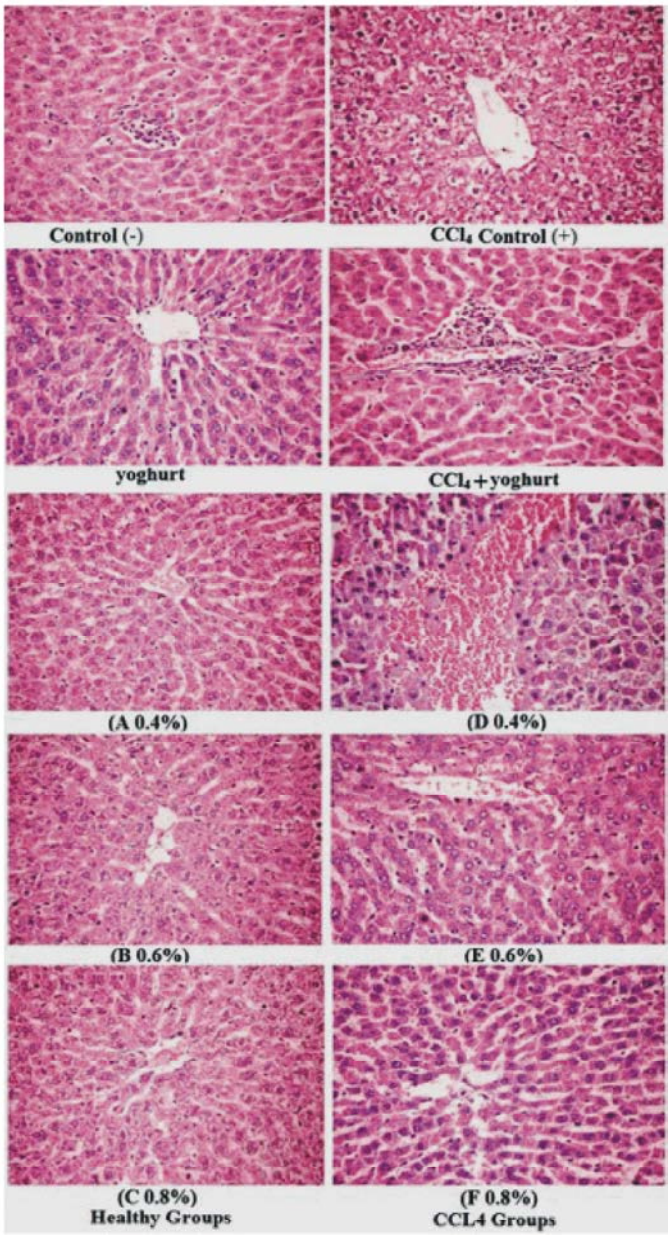

Fig. 1: Microscopical images between histological changes in the liver tissues of the different rats groups fed on stirred yoghurt (H & E x 400). Control: stirred yoghurt without any silymarin, T1: stirred yoghurt supplemented with 0.4% silymarin, T2: stirred yoghurt supplemented with 0.6% silymarin; T3: stirred yoghurt supplemented with 0.8%.silymarin

But, Fig. 1D at the end of the 0.8 % silymarin, those histopathological parameters mild (portal inflammation, necrosis, fibrosis with minimal steatosis). Suja *et al*. [59] found that hepatic injury induced by CCl_4 caused histopathological alterations such as massive fatty changes, gross necrosis and broad infiltration of lymphocytes and Kupffer cells around the central vein and loss of cellular boundaries. Treatment with HZ and silymarin showed similar positive effect on these changes. Ozturk *et al.* [55] reported that, CCl_4 caused hepatocyte degeneration, central ven dilatation, congestion and increased in the number of Kuppfer cells and histopathological injury scores. Treatment with *Silybum marianum* infusion showed slightly preventive effect on CCl_4 induced liver damage by biochemically and histologically.

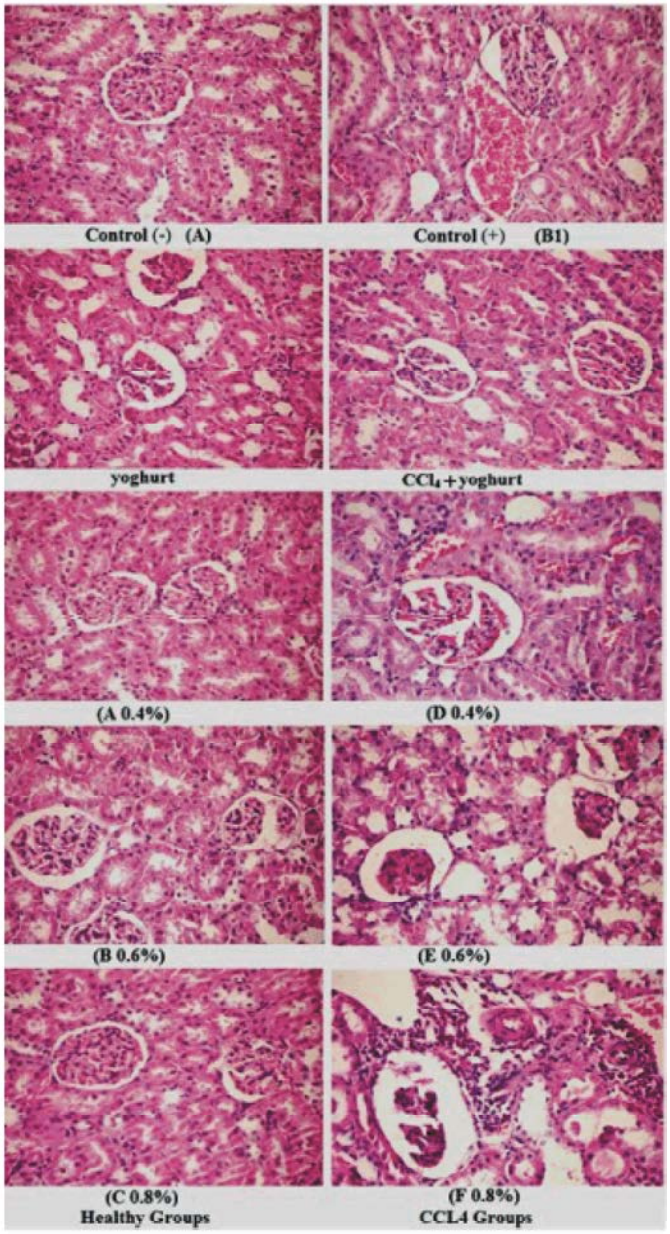

Fig. 2: Microscopical images the histological changes in the kidney of the different rats groups fed on stirred yoghurt (H & E x400). Control: stirred yoghurt without any silymarin, T1: stirred yoghurt supplemented with 0.4% silymarin, T2: stirred yoghurt supplemented with 0.6% silymarin; T3: stirred yoghurt supplemented with 0.8%. silymarin

Kidney: The control negative rats showed the normal histological structure of renal parenchyma (Fig. 2). While, control positive (+) (CCl_4) animals revealed congestion of renal blood vessel and vacuolation of epithelial lining renal tubules observed in the kidney of these animals injected with CCl_4 alone. Furthermore, kidney of animals fed on stirred yoghurt and animals affected by CCl_4 plus fed on stirred yoghurt showed normal histopathological changes. As seen in Fig. (2) from A to F the animals fed (stirred yoghurt supplemented with silymarin 0.4, 0.6 and 0.8%), showed normal histopathological changes. In contrast, animals affected by CCl_4 and fed (stirred yoghurt supplemented with silymarin 0.4% (Fig. 2A) showed focal renal hemorrhage and congestion of glomerular tuft and intertubular blood capillaries). However, 0.6% and injected by CCl_4 showed atrophy of glomerular tufts, distension of

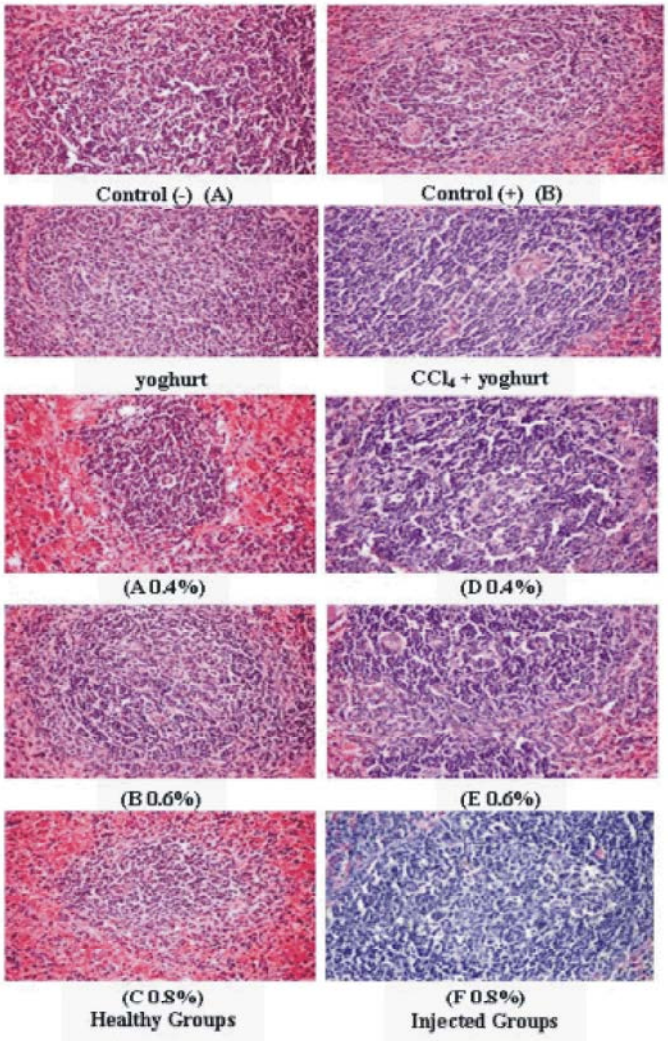

Fig. 3: Microscopical images the histological changes in the spleen of the different rats groups fed on stirred yoghurt (H & E x 400). Control: stirred yoghurt without any silymarin, T1: stirred yoghurt supplemented with 0.4% silymarin, T2: stirred yoghurt supplemented with 0.6% silymarin; T3: stirred yoghurt supplemented with 0.8%. silymarin

Bowman's space and vacuolation of renal tubular epithelium (Fig. 2E), while (stirred yoghurt supplemented with silymarin 0.8%) showing interstitial nephritis, atrophied tuft and distension of Bowman's space and presence of protein cast in the lumen of renal tubules (Fig. 2F). Soto *et al.* [60] noticed that, renal tissue from diabetic rats showed glomerular damage, cell disruption (in medulla and cortex), vacuolization and tubule lysis in epithelium zones were observed after 12 weeks of alloxan administration. Silymarin treatment blocked these changes and restored normal structure to the cortex and medulla, as well as to the glomeruli, proximal, distal tubules and vessels. Diabetes increased the mesangial matrix and caused thickening of the capillary basement membrane in the glomeruli and capsule, but silymarin treatment blocked these changes. The damage or death of hepatocytes usually due to leakage of the enzymes in the affected tissue into the blood stream [61].

Spleen: Histopathological examination of the spleen sections of control (-) group showed normal lymphoid follicle as seen in (Fig. 3A), while the rats' illustrated lymphocytic necrosis and depletion. On the other hand, spleen of animals fed on stirred yoghurt and animals injected by CCl_4 plus fed on stirred yoghurt showed normal histopathological changes as seen in Fig. 3A and B. Animals fed on stirred yoghurt plus 0.4, 0.6 and 0.8% silymarin (Fig. 3A-C), showed normal histopathological

changes. While, animals injected by CCl_4 and fed stirred yoghurt plus 0.4% silymarin (Fig. 3D) showed slight lymphocytic necrosis. Spleen of injected rat by CCl_4 and fed stirred yoghurt plus 0.6 and 0.8% silymarin showed no histopathological changes (Fig. 9E and F).

CONCLUSION

In conclusion, supplementation with yoghurt by different levels of silymarin that may increase the function properties and nutritional value of yoghurt. It could be used as a safe, effective and easily accessible source of natural antioxidants to improve the liver functions. Silymarin have a good protection of liver tissues treated with CCl_4 in experimental animals. Also some histopathological, effects have been confirmed to distinguish silymarin levels feeding but caused some minor changes observed. Successful application of silymarin in liver diseases still awaits further investigation on the optimal range to be used as fortification therapy.

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