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EFFECT OF SILYMARIN ON CARBON-TETRA CHLORIDE INDUCED PAROTID AND SUBMANDIBULAR SALIVARY GLANDS DAMAGE IN RATS

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ABSTRACT

Introduction: Herbal extracts have shown protective efficacy on organs toxicity. Carbon-tetra chloride (CCl4) induce the production of free radical. Silymarin has potent cytoprotecting antioxidant effects.

Aim: was to investigate the role of Silymarin in lowering the cytotoxic effect of CCL4 on the salivary glands of rats**.**

Materials and methods: Thirty-six rats were randomly assigned into three equal groups: Group I (Control) received (1 ml) saline orally for 21 days. Group II: (CCl4) received saline orally for 15 days followed by CCl4 (1.5 mg/kg, i.p.) twice per week until day 21. Group III: (Silymarin+CCl4) received silymarin (25 mg/kg, oral) orally for 15 days followed by CCl4 (1.5 mg/kg, i.p.) till day 21. Rats were euthanized after 21 days; after dissecting the salivary glands, they were prepared for routine H&E and immunohistochemical evaluation for caspase-3. Lastly, the antioxidant marker Reduced glutathione (GSH), the oxidative stress markers Malondialdehyde (MDA), and Nitric Oxide (NO) were assessed.

Results: The histological results revealed acinar and ductal apoptotic changes in the CCl4 group. In group III, the degenerative changes induced by CCl4 were reduced. CCl4 in group II significantly increased NO and MDA levels as well as decreased GSH levels. Silymarin significantly decreased NO and MDA, while increased GSH compared to group II. Caspase-3 immunoexpression was lower in Group III compared to CCl4 group**.**

Conclusions: Silymarin has a protective antioxidant activity on the salivary glands that reduces the toxic effects produced by CCl4.

KEYWORDS: Carbon-Tetra Chloride, Silymarin, Protective, Parotid Salivary Gland, Submandibular Salivary Gland.

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INTRODUCTION

Carbon tetrachloride (CCl4) is an organic compound that has the formula of CCl4. It is a colorless, volatile liquid with a non-inflammable nature. It is produced by mixing chlorine with chloroform in the presence of light. Previously, it was widely used as a cleaning agent, in refrigerants and fire extinguishers but due to its harmful effect, its use nowadays is limited to some industrial applications [1,2]. However, low level exposure of CCl4 can still occur mostly by inhalation from the surrounding atmosphere [2].

The toxic effect of CCl4 mostly arises from the activation of short-lived reactive intermediates and the release of free radicals, mainly CCl3 during its metabolism [3]. CCl4 induced cell damage results from bonding of these reactive intermediates to cell parts or from the destruction of lipids caused by lipid peroxidation by the free radicals. Phospholipids destruction will damage the plasma membrane as well as intracellular membranes. The breakdown products will then lead to further cell damage and eventually cell death [4].

At the molecular level, CCl4 has various actions. It activates some factors that causes self-destruction or fibrosis of the cells and tissues. These factors include nitric oxide (NO), transforming growth factors (TGF)-α and -ß. Another factor that is also activated by CCl4 is tumor necrosis factor (TNF)α which works by inducing apoptosis [5,6].

Apoptosis is the process of programmed cell death, which helps remove premalignant as well as damaged tissue. It has two pathways intrinsic and extrinsic pathways. The extrinsic pathway is through the activation of the initiating caspases that further activate the effector caspases 3,6,7 [7,8].

The organs mostly affected by CCl4 toxicity are the liver, kidneys, and lungs [4,5]. Many reported about the biochemical and structural damage in various organs because of CCl4, nevertheless, salivary glands were rarely mentioned [9, 10]. However, one important organ that could be affected by environmental toxins are the salivary glands.

Salivary glands have crucial role in the oral cavity, they form and secrete saliva which has essential roles. Among the functions of saliva are: to protect and lubricate the oral tissues, start the digestion of food in the mouth, antibacterial effects, tissue repair, and buffering action[11]. Impairment in the functions of the salivary glands can trigger many side effects. Salivary gland dysfunction alters the amount and type of saliva, which interferes with oral homeostasis. This in turn can cause disorders, such as xerostomia and periodontal disease or even aggravate them, which will eventually worsen the patients' quality of life [12-14].

Like humans, rodents have three pairs of major salivary glands which are the: parotid, submandibular, and sublingual salivary glands. They synthesize and secrete three types of saliva: serous, mucous, or mixed saliva. Each gland has a main excretory duct that connects and opens into to the oral cavity and through it saliva is excreted [15].

Parotid glands in both humans and rodents are comprised of pure serous acini. The acinus is a secretory unit formed of cells that contains many secretory granules in their cytoplasm. Serous acinar cells contain a well-developed rough endoplasmic reticulum and are surrounded by myoepithelial cells. The intercalated ducts and striated ducts in parotid glands are prominent in both species. However, the adipocytes are not observable in the rodent parotid gland compared to the human one. Regarding the rodent submandibular gland, it has only serous acinar cell type that shows no demilunes or mucous cells, unlike the corresponding gland in humans which is a mixed type. Both intercalated ducts and striated ducts are well developed, with the serous acini and the intercalated ducts being covered by myoepithelium [12,15]. Moreover, granular convoluted tubules are characteristic structures in only the submandibular salivary glands of rodents, absent in humans [15].

Because of the protective effects of herbal drugs on different types of organ toxicity, their widespread use has been shown in several studies particularly silymarin [16-21]. Silymarin is a herbal drug extracted from the seeds of the milk thistle *Silybum marianum*. In 1952, scientists discovered that silymarin contains various flavonolignans and its mode of action was revealed in the 1960s [22, 23].

Traditionally, silymarin has been introduced as therapy for many diseases including hepatitis, cirrhosis, osteoarthritis, type 2 diabetes, and diabetic nephropathy. There is evidence that silymarin exerts a protecting action against liver and kidney toxicity particularly conditions that are induced by chemical and environmental toxins [16-18, 21]. Other evidence for a cardioprotective effect of silymarin was revealed in case of cardiac tissue injury induced by certain drugs [21]. Moreover, Studies suggested the role of Silymarin as an alternative treatment for salivary gland cancer [24].

The mode of action of silymarin was attributed to its prominent antioxidant effect, particularly by suppressing lipid peroxidation and acting as a free radical scavenger [25]. Moreover, it exerts an anti-inflammatory, antifibrotic, immunomodulatory, and membrane-stabilizing effects [26].

The degree of free radical and lipid peroxidation can be analyzed by several markers such as: Reduced glutathione (GSH), Malondialdehyde (MDA), and Nitric Oxide (NO)[27]. Reduced glutathione (GSH) is involved in antioxidant defense, detoxification and in clearing ROS [28]. MDA is an important product of lipid peroxidation that promote adverse effects inside the cell by altering ion permeability and enzymatic activity [29]. NO, one of the free radicals is the most important factor causing oxidative stress which is contributing factor for many detrimental diseases [29].

Little is known about the protective effect of silymarin on CCl4 induced damage of salivary glands. We intended to demonstrate the adverse effect of CCl4 on salivary glands as well as the protective effects of silymarin. In this study, we analyzed the apoptosis inducing effects of CCl4 alone and combined with Silymarin in the parotid and submandibular salivary glands by examining the immunohistochemical expression for caspase 3 antibody.

MATERIAL AND METHODS

An experimental animal study was performed on 36 adults male Wistar albino rats aged 2 months (100-150 grams in weight). Sample size was estimated assuming 5% alpha error and 80% study power. The sample size was based on Rosner's method [30] calculated by G*Power 3.1.9.7. The mean (SD) hepatic reduced glutathione (GSH) was 37.98 (1.07) µmol/g for normal control and 36.53 (0.89) µmol/g for silymarin group[18]. Based on the difference between independent means using the highest SD= 1.07 to ensure power, sample size was calculated to be 10 rats per group. This was increased to 12 rats to make up for processing errors. A total of 36 rats. Animals were purchased from the animal house colony, Science Park, European countryside, Giza, Egypt. This experiment was approved by the Alexandria University Ethics Committee for Animal Experimentation and all procedures followed the institutional guidelines. Rats were housed in wire mesh bottom cages, four per cage, controlled laboratory conditions were maintained of temperature (22-25°C), good ventilation, and light/dark cycle (12/12 h) with free access to soft standard food and water. The diet regimen was refilled on a daily basis all through the duration of the experiment. Specifically, rats previously included in research purposes or having any disorders or lesions were excluded[31]. Animals were randomly allocated (using computer-generated random numbers) into one of three groups as follows:

Group I: (Normal Control group) (n=12), rats received (1 ml) of saline orally for 21 days as a vehicle.

Group II: (CCl4 group) (n=12), rats were administered (1 ml) saline orally for 15 days followed by CCl4 (1.5 mg/kg, i.p.) twice weekly until day 21.

Group III: (Silymarin+CCl4 group) (n=12), rats were administered silymarin (25 mg/kg) orally for 15 days followed by CCl4 (1.5 mg/kg, i.p.) twice a week until day 21.

Administration of Silymarin:

Rats in group III received silymarin (Sigma-Aldrich, St Louis, MO, USA) orally using gastric gavage for the first 15 days of the experiment, to investigate its protective role against salivary glands damage by CCl4 [32].

Administration of Carbon tetrachloride (CCl4):

CCl4-induced salivary gland damage was performed according to the method of (Suja *et al*., 2004)[33]. Rats of groups II and III were administered a freshly prepared intraperitoneal injection of CCl4 (Sigma-Aldrich, St Louis, MO, USA) twice weekly until day 21 at a dose of 1.5 mg/kg after 15 days of saline or silymarin administration.

Measurement of Blood antioxidant Levels

At the end of the experimental period, blood samples were obtained from the retro-orbital vein plexuses, under ether anesthesia. Blood antioxidant levels were measured using Biodiagnostic kits (Biodiagnostic Co., Dokki, Giza, Egypt) for the detection of antioxidant levels as Reduced glutathione (GSH), and the oxidative stress markers level Malondialdehyde (MDA), and Nitric Oxide (NO). Tests were carried out in the biological section of the Pharmacology Department, National Research Center, Giza, Egypt, by serial sub-culturing.

1. **Determination of reduced glutathione (GSH)** level according to the method described by Beutler et al.[34].
2. **Determination of malondialdehyde (MDA)** according to the method described by Pradeep et al.[35].
3. **Determination of hepatic Nitric Oxide (NO)** according to the method described by Bryan & Grisham (2007)[36].

Euthanasia of experimental animals:

Rats were euthanized after 21 days by an overdose of diethyl ether [37]. The parotid and submandibular salivary glands on both sides of each rat were dissected. The glands were labelled then prepared for light microscopic examination and immunohistochemical examination.

Light microscope procedures

After labelling, specimens were fixed in 10% neutral buffered formalin. After washing, specimens were dehydrated in ascending concentrations of ethanol solution, cleared with xylene, infiltrated, and embedded in paraffin wax. Using a rotary microtome, 5μm thick sections were cut followed by staining with Hematoxylin & Eosin stains (H&E) for histological evaluation[38].

Analysis of IHC staining

Several sections from the parotid and submandibular gland were used for Immunohistochemical (IHC) staining. The staining was done using a caspase 3 polyclonal antibody (cell signaling technology, USA; catalog number #9662).The prepared tissue sections were stained at a dilution ratio of 1:50 of rabbit polyclonal anti- antibody). At the beginning the tissues were fixed by 10% neutral buffered formalin for 24 h at room temperature. Then dehydrated in ethanol and embedded in paraffin. Immunohistochemical staining was achieved by the labeled streptavidin-biotin method[39]. Antigen retrieval was done by being microwaved to unmask the epitope. Endogenous peroxidase blocking was performed by placing the sections with 0.3% hydrogen peroxide in phosphate-buffered solution (PBS) (100 μl) for 10 min at room temperature. The sections were incubated with caspase 3 for 1 hour at room temperature. Further incubation with biotinylated secondary antibody in PBS for 30 min. Finally,

with streptavidin–peroxidase conjugate. Diaminobenzidine hydrochloride was the chromogen of choice used to visualize the activity of peroxidase. The sections were counterstained using Mayer's Hematoxylin[40]. Evaluation was done by two pathologists in randomly selected microscopic fields at a magnification of 400´ to determine the intensity of the immune stain as a mean area percentage. Data were analyzed quantitatively using ImageJ 1.46 r software. Moreover, positive cells were counted in five different microscopic fields that demonstrated more intense staining.

Statistical analysis:

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). For continuous data, Shapiro-Wilk test was used to test the normality The Quantitative data were expressed as range (minimum and maximum), mean, standard deviation and median for normally distributed quantitative variables. ANOVA was used for comparing the different studied groups and followed by Post Hoc test (Tukey) for pairwise comparison. Significance of the obtained results was judged at the 5% level.

RESULTS

Histological evaluation

The parotid salivary gland:

Examination of the parotid glandular tissue showed in the control group normal structure and organization. The serous acini showed a regular rounded outline, basophilic cytoplasm, and basal round basophilic nuclei (Fig. 1a,b). The duct system also showed normal structure, and intralobular striated ducts showed basal striations. The interlobular duct showed pseudostratified lining (Fig. 1c,d).

Group II (CCl4) showed areas of acini that appeared as an amalgamated mass with an irregular outline, faintly stained cytoplasm and deeply stained nuclei. Thinning of the ductal lining was also noticed (Fig.2a,b). The interlobular striated ducts showed loss of basal striations. Vacuolated nuclei and cytoplasm of acinar and duct cells were also revealed (Fig. 2c,d).

In group III (Silymarin+CCl4) the normal structure of the gland was restored. The acini appeared with normal outline and organization. Round secretory end portions with basophilic cytoplasm were revealed (Fig. 3a,b). The basal

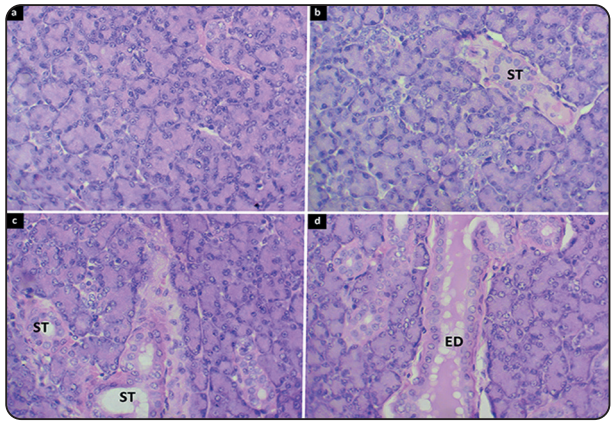

Fig. (1): Light micrograph (LM) of the parotid salivary glands of the control group showing (a,b) rounded serous acini with basophilic cytoplasm and basal round nuclei; (c,d) intralobular striated ducts (ST) with basal striations and interlobular duct excretory (ED) with pseudostratified lining. (H&E X400)

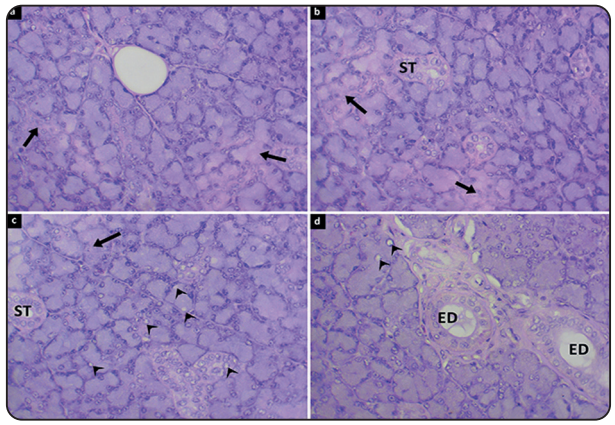

Fig. (2): LM of the parotid salivary glands of group II (CCl4) showing: (a,b) irregular acini that appeared as an amalgamated mass (arrows) with a faintly stained cytoplasm; (c,d) striated ducts (ST) with loss of basal striations and vacuolated nuclei and cytoplasm of acinar and duct cells (arrow heads), ED: excretory duct. (H&E X400)

striations of striated ducts were evident. The ductal lumen appeared filled with secretion. Less acinar and ductal vacuolization were revealed (Fig. 3c,d).

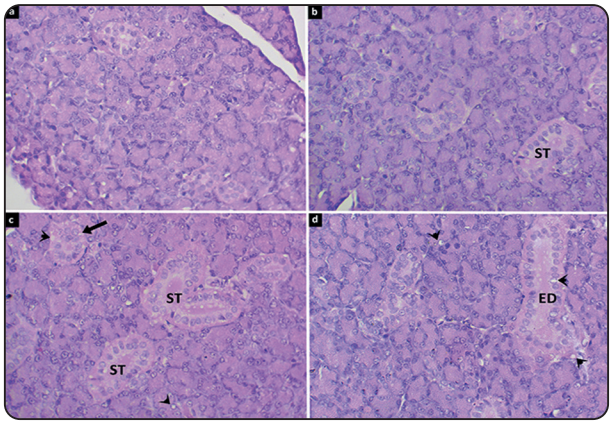

Fig. (3): LM of the parotid salivary glands of group III Silymarin+CCl4 showing: (a,b) normal acinar outline and organization with basophilic cytoplasm; (c,d) striated ducts (ST) with evident basal striations (arrow) and less acinar and ductal vacuolation (arrow heads) were revealed, note excretory duct (ED) filled with secretion. (H&E X400)

The submandibular salivary gland

Examination of the submandibular salivary gland revealed in the control group the normal structure of the epithelial and connective tissue constituents. The acini showed a basophilic cytoplasm and round basally located nuclei (Fig. 4a). The intralobular ducts represented by the intercalated and striated ducts were evident and showed normal structure (Fig. 4b,c). The granular convoluted tubules were also revealed with their acidophilic granules. The excretory duct was also noticed with a pseudostratified wall (Fig. 4d).

In the CCl4 group, the acini showed cytoplasmic vacuolations and shrunken crescent shaped nuclei (Fig. 5a). Degeneration affected the intralobular ductal linings. The intercalated ducts were shrunken (Fig. 5a,b). The striated ducts showed loss of basal striations (Fig. 5b). The granular convoluted tubules were collapsed with reduced acidophilic content (Fig. 5b,c).The excretory ducts did not show major changes (Fig. 5d).

The submandibular gland in the Silymarin+CCl4 group showed preserved acini and ducts, vacuoles were seldomly noted (Fig. 6 a,b). The secretory acini showed regular intact outline and basophilic cytoplasm. The intercalated ducts showed normal structure (Fig. 6a) and the striated ducts showed evident basal striations (Fig. 6b). the Granular convoluted tubules were also noted with granular content (Fig. 6c). The excretory ducts were noted surrounded by vascularity and fibrous stroma (Fig. 6d).

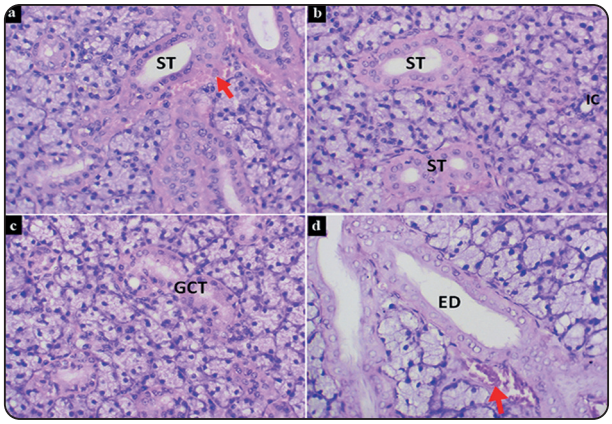

Fig. (4): LM of the submandibular salivary glands of the control group showing (a) acini with basophilic cytoplasm and round basally located nuclei. (b) The intercalated ducts (IC) and secretory striated ducts (ST) of normal structure. (c) Granular convoluted tubules (GCT) with their acidophilic granules. (d) Excretory duct (ED) with a pseudostratified wall and surrounded by vascularity (red arrow). (H&E X400)

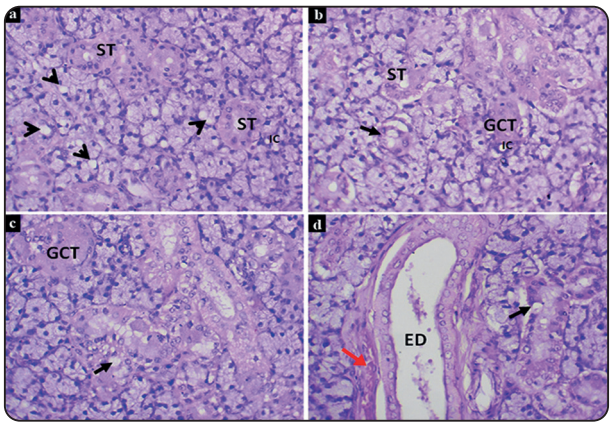

Fig. (5): LM of the submandibular salivary glands of the CCL4 group showing (a) acini with cytoplasmic vacuolations (arrowheads) and shrunken crescent shaped nuclei, shrunken intercalated ducts (IC). (b,c) striated ducts (ST) loss of basal striations and vacuolated lining (arrows); collapsed (arrow) Granular convoluted tubules (GCT). (d) The excretory duct (ED) with a pseudostratified wall surrounded by fibrous tissue and blood vessel (red arrow). (H&E X400)

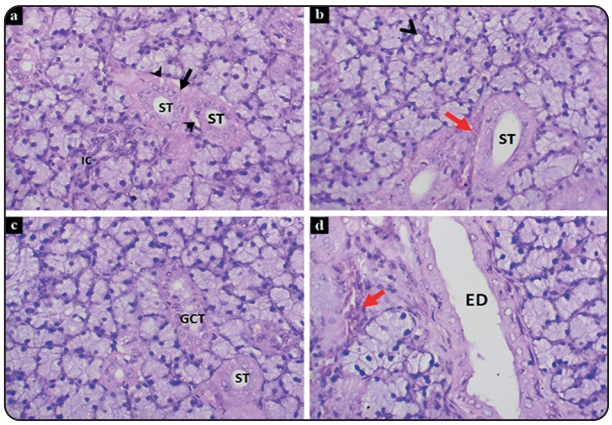

Fig. (6): LM of the submandibular salivary glands of the Silymarin+CCl4 showing (a) acini with regular intact outline and basophilic cytoplasm and normal intercalated ducts (IC) and striated ducts (ST) with basal striation (arrow) and mild vacuoles (arrowheads). (b) Striated ducts (ST) with basal striations and associated blood capillary (red arrow), note acinar cytoplasmic vacuole (arrowhead). (c) Granular convoluted tubules (GCT) with their granular content. (d) Excretory duct (ED) surrounded by vascularity (red arrow) and fibrous stroma. (H&E X400)

Measurement of Blood antioxidant Levels

The assessment of antioxidant activity of silymarin was based on measurements of blood sample malondialdehyde (MDA), glutathione (GSH), and nitric oxide (NO) (Fig. 7, 8 & 9). Table (1) showed that CCl4 in group II significantly decreased (GSH) levels and increased (NO) and (MDA) levels as compared to normal control in group I (mean values in group II= 0.46, 2.78, and 79.5, Group I= 1.55, 0.48 and 22.5 respectively). On the other hand, Silymarin coverage in group III significantly increased (GSH) levels as well as decreased (NO) and (MDA) levels as compared to CCl4 in group II (mean values in Group III= 1.3, 0.81 and 25 respectively).

Immunohistochemical Results

Immunohistochemical analysis for the three studied groups showed variable results regarding caspase 3 expression in the parotid and submandibular glands (Tables 2,3) (Fig. 10,11&12). In the CCl4-treated rats the histopathological changes were marked. In group I, parotid and submandibular gland very few acinar cells showed caspase 3 staining, which it is marker of apoptosis. The reaction was mainly limited to the acinar cytoplasm (Fig. 10 a,d). While caspase 3 immunosignals were markedly enhanced in response to CCl4-treatment reflecting the cytotoxic effect of the CCl4 (Fig 10 b,e). The ductal and acinar cells showed positive immunosignals and higher expression by comparing it to the control group. Silymarin treated rats showed significantly less caspase 3 positive ducal cells indicating reduced salivary gland damage (Fig 10 c,f). Although the differences in caspase 3 expression showed significant results in submandibular gland. On the other hand, it did not reach the level of significance in the parotid gland. They all point to that silymarin has protective effect on the salivary glands.

TABLE (1) NO, MDA, GSH activity in CCl_4 levels among groups

	GSH (μg/ml)	MDA (nmol/ml)	NO (Um/ml)
	Mean ± SE	**Mean ± SE**	**Mean ± SE**
Control (saline)	1.55 ± 0.15	0.48 ± 0.05	22.5 ± 2.5
CCL4 group	0.46^{a} ± 0.01	2.78^{a} ± 0.07	79.5^{a} ± 4.5
Silymarin+ CCL4	1.3^{b} ± 0.10	0.81^{ab} ±0.02	25^{b} ± 2
F (p)	**29.957* (0.010*)**	**624.528* (<0.001*)**	**102.057* (0.002*)**

SE: Standard error of mean
F: F for One way ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)
p: p value for comparing between the studied groups ****: Statistically significant at p ≤ 0.05***
a: Significant with Control (saline) ***b: Significant with CCL4 group***

TABLE (2) Immunoexpression for Caspase 3 in the three studied groups in the partoid gland

	Normal control (saline) (n = 13)	CCl4 group (n = 13)	Silymarin+ CCl4 (n = 13)	F	p
Parotid caspase 3					
Mean ± SD.	48.1 ± 9.6	95[a] ± 19.7	79.6[ab] ± 10.4	37.968*	<0.001*
Median (Min. – Max.)	45.1 (30.7 – 67.5)	91 (66.9 – 129.9)	81.1 (58.8 – 96.5)		
		p_1<0.001* p_2<0.001*,p_3=0.021*			

SD: Standard deviation

F: F for One way ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)

p1: p value for comparing between Normal control and CCL4 group

p2: p value for comparing between Normal control and Silymarin+ CCl4

p3: p value for comparing between CCl4 group and Silymarin+ CCl4

****: Statistically significant at p ≤ 0.05*** ***a: Significant with Normal control group*** ***b: Significant with CCl4 group***

TABLE (3) Immunoexpression for caspase-3 in three studied groups in the submandibular gland

	Normal control (saline) (n = 11)	CCl4 group (n = 11)	Silymarin+ CCl4 (n = 11)	F	p
Submandibular caspase 3					
Mean ± SD.	41 ± 11.4	115.4[a] ± 35.8	59.1[b] ± 12.3	31.879*	<0.001*
Median (Min. – Max.)	37.8 (27.7 – 59)	110.6(78.7–193.1)	65.5 (36.3 – 74.5)		
		p_1<0.001* p_2=0.168,p_3<0.001*			

SD: Standard deviation

F: F for One way ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)

p: p value for comparing between the three studied groups

p1: p value for comparing between Normal control and CCl4 group

p2: p value for comparing between Normal control and Silymarin+ CCl4

p3: p value for comparing between CCl4 group and Silymarin+ CCl4 ****: Statistically significant at p ≤ 0.05***

a: Significant with Normal control group ***b: Significant with CCl4 group***

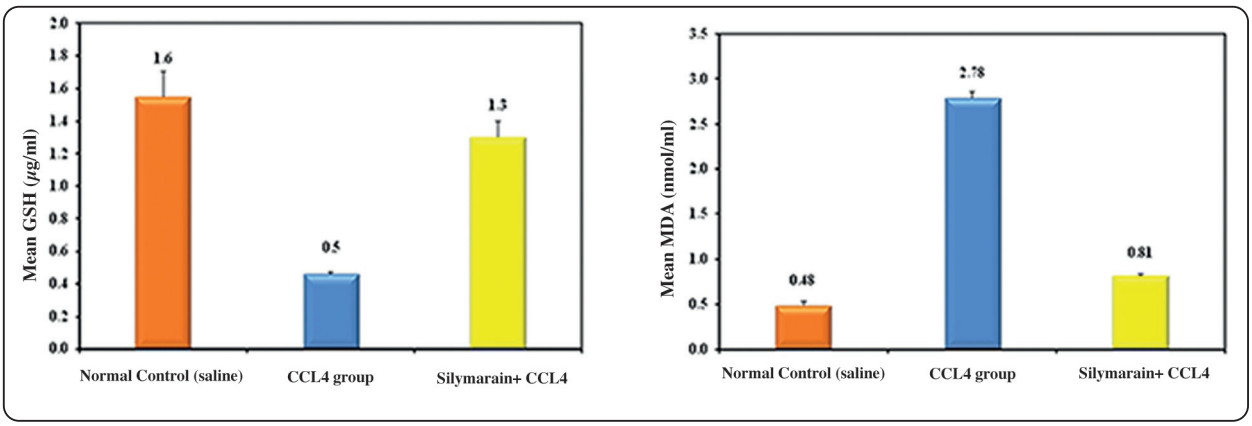

Fig. (7) Fig. (8)

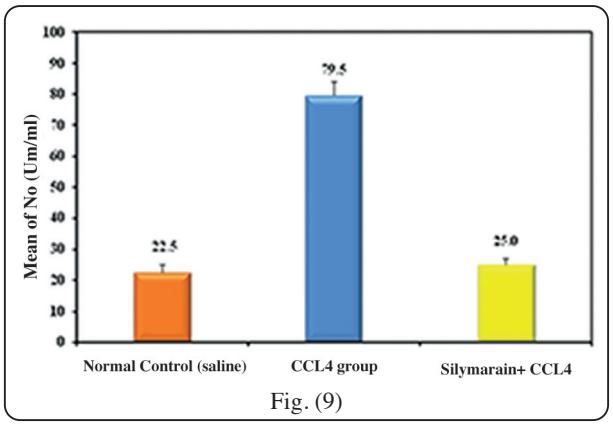

Fig. (9)

Fig. (7) Effects of oral administration of Silymarin on GSH activity in CCl4 induced salivary glands toxicity in rats

Fig. (8) Effects of oral administration of Silymarin on MDA activity in CCl4 induced salivary glands toxicity in rats

Fig. (9) Effects of oral administration of Silymarin on NO activity in CCl4 induced salivary glands toxicity in rats

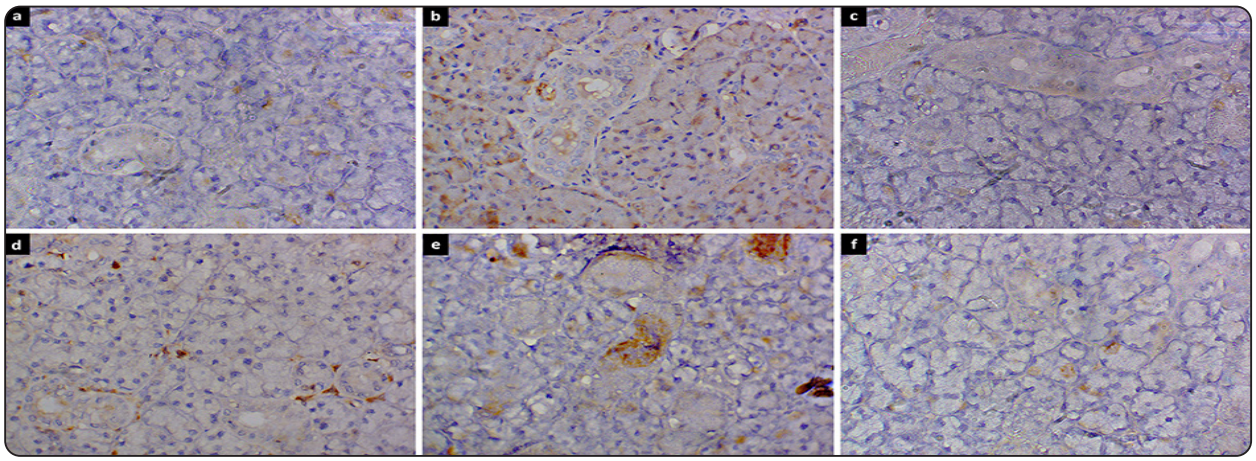

Fig. (10) (a)A photomicrograph for the parotid gland showing caspase-3 imunnoexpression in the parotid the control group showing very few positive signals in sporadic in the acinar cells (x400) (b) Caspase-3 immunoexpression in the parotid gland of CCL4 group revealing strong expression in the ductal and acinar cells (x400) (c) The CCl4+Silymarin parotid gland exhibited some positive reaction mainly in the ductal cells (x400). (d) The submandibular gland of the control group showed positive immunosignals limited mainly to basal compartments of the basal cells (x400)(e) The submandibular gland of the CCl4 treated group showed more intense immunosignals in the ductal and acinar cells (f) The CCl4+Silymarin submandibular glands showed scattered weak positive expression against caspase-3 (X400)

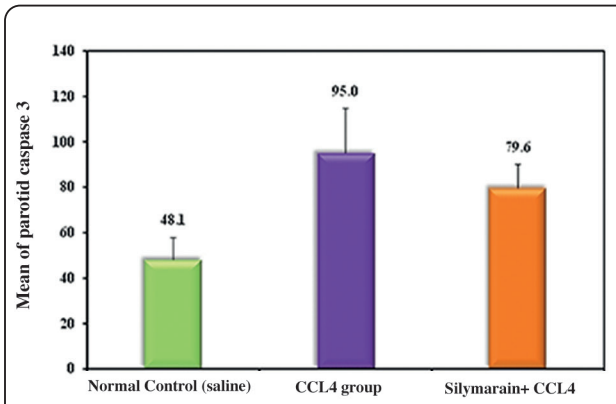

Fig. (11)

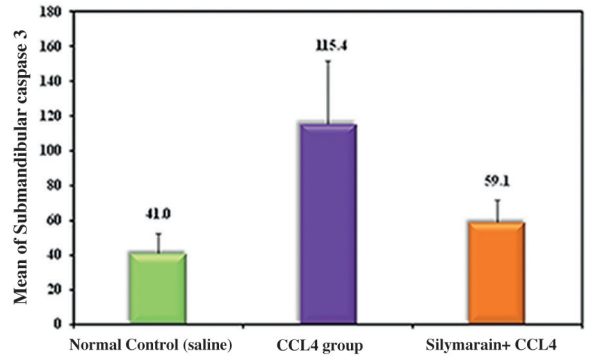

Fig. (12)

Fig. (11) Comparison between the three studied groups in terms of caspase-3 immunoexpression in the parotid gland

Fig. (12) Comparison between the three studied groups in terms of caspase-3 immunoexpression in the submandibular gland

DISCUSSION

In the present study, we used parotid and sub mandibular salivary glands of rats considering their remarkable histological similarities to human salivary glands, they are ideal model of research to be used. This was supported with the findings of the Amano et al. [15] and Roa etal [41] research understanding what is involved in this instance is necessary. Hence, the submandibular gland in humans is a mixed gland comprising serous demilunes and mucous acinar cells, although the one in rodents only has the serous type with no serous demilunes. Both human and rat parotid glands are made up of pure serous acini. Moreover, When compared to human submandibular glands, GCT parts of rodent submandibular glands are frequently misinterpreted for mucous acini or tubules [15].

Human toxicity usually happens on by intake, through cutaneous absorption after direct skin contact, or aspiration of toxic gases. It can also be intentionally ingested as a self-homicidal agent[1]. In fact, Carbon tertrachloride is frequently accompanied by an elevation in formation of free radicals and/or alteration of antioxidant defenses[42]. CCl4 is highly toxic and harmful, referring to free radicals, adversely impacting cells in multiple body organs, primarily the liver, kidneys, and lungs[4,9,10]. The production of the free radical CCl3 and other metabolites by cytochrome P450 is the cause of CCl4 toxicity rather than CCl4 itself. They eventually result in cellular harm by changing the structure of the cell through lipid peroxidation and other processes [1, 42].

The biochemical significance of natural antioxidants gained a great deal of attention in biological system against several toxic free radicals. [43,44] Any molecule found at a low concentration, inhibits the oxidative reaction cycle by eliminating the free radical precursor is called antioxidant agent. Yet both plants and animals continue to have complicated systems that contain various kinds of antioxidants[45].

Silymarin, a natural antioxidant, originates from milk thistle seeds and is a blend of flavonolignans. It has received a great notice over the latest decennary as a botanical medicine for many diseases[46]. Many research investigations have demonstrated that this mixture exhibits significant liver-protective and anti-diabetic properties [23]. The anti-cancer potential of silymarin has recently been investigated [24]. Further studies have demonstrated that silymarin could alter the equilibrium between pro-survival and pro-apoptotic signals, through the generation of cell cycle and apoptosis regulating proteins, respectively[47, 48] Thus far, the protective effects of silymarin on salivary gland toxicity with CCl4 its underlying molecular mechanisms have not been studied yet. Herein, we prove that silymarin can reduce production of CCl4 toxic free radicals and inhibit apoptosis in salivary gland, resulting in protective effect against CCl4 in salivary glands.

Our histological findings showed vacuolation and damaging of acinar cell membranes in Group II, CCL4 of both salivary glands, with presence of pyknotic nucleoli and damaged duct system this was discussed by Unsal et al[42]. The explanation by which CCl4 affects tissues is oxidative damage carried on by lipid peroxidation, which occurs after CCl4 has been converted by the cytochrome P450 enzyme to the highly hazardous trichloromethyl radicals (•CCl3) and trichloromethyl peroxyl radicals (•CCl3O2). The main sign of oxidative damage is complete breakdown of lipids (i.e., peroxidation) as in mitochondria and endoplasmic reticulum, which are structures rich in membrane phospholipids. In addition, Free radicals trigger many biological processes, such as apoptosis, necrosis, and autophagy[42].

In the present study, almost full restoration of the damaged acinar cell membrane in Group III, Silymarin treated group of both salivary glands, this in agreement with Simona et al., who stated that Silymarin stabilizing the cell membrane by reducing the free radicals formation[49].

In the current research, we used antioxidant markers as MDA, GSH, and NO to assess the oxidative stress of CCl4 and the antioxidant level of Silymarin. Since the oxidative stress causes an imbalance between pro-oxidants and antioxidants, this favors free radicals[50]. Thus, the antioxidant system of the body protects against the cellular damage elevated by free radicals, including the enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), as well as the non-enzymes vitamin C, vitamin E, and carotenoids [51]. Malondialdehyde (MDA) is also produced when free radicals start to exert adverse effects on membrane phospholipids [27].

The assessment of antioxidant activity confirmed our histological results which was based on measurements of blood samples antioxidant markers malondialdehyde (MDA), glutathione (GSH), and nitric oxide (NO). In Group II (CCl4) significantly increased (NO) and (MDA) levels as well as decreased (GSH) content as compared to normal control. This finding was in accordance with Dutta et al.[52] and Raj S [53].

It has been shown that CCl4 reduces the activities of antioxidant enzymes such as GSH-Px, CAT, SOD, GST, and Glutathione to endogenous antioxidants[53] The reason could be that CCl4 elevates the protein carbonyl content, a byproduct of protein oxidation, at the level of the oxidative stress biomarker MDA [54].

Besides several studies reported that CCl4 activates TGF-21, which is a pro-fibrotic cytokine, and significantly increases the expressions of proinflammatory cytokine as IL-1β, IL-6, TNF-α, Monocyte chemoattractant Protein-1, Macrophage inflammatory protein-2, and nuclear factor-mB p65 protein in the CCl4 induced organ injury animals[52].

On the other hand, Silymarin significantly decreased blood (NO) and (MDA) levels as well as increased (GSH) levels as compared to CCl4. This was in accordance with Pradeep et al. [35]. Silymarin has been found to act as an antioxidant, decreasing free radicals and salivary glands protecting effects by stabilizing the membrane permeability through inhibiting lipid peroxidation (LPO)[35, 55]

It is well known that by Silymarin treatment, all elevated indexes of the oxidative stress caused by CCl4 were significantly decreased, and they were restored almost to the initial physiological levels, as proved by several studies [53, 56-58].

Furthermore, Silymarin had a protective effect against CCl4 induced salivary glands damage[46]. First, directly scavenging free radicals. Second, by inhibiting certain enzymes that produce free radicals so as prevent their formation. Third, by helping to maintain the cell's ideal oxidative state by triggering a variety of antioxidant enzymes and non-enzymatic antioxidants, mostly through transcription factors like NF-kB. At last, by stimulating a variety of vitagenes that are in charge of releasing protective molecules, such as heat shock proteins HSP, further protection can be provided under stressful conditions [46].

From the present study results, it can be mentioned that the natural antioxidants are the best to reduce or eliminate CCl4-induced negative effects [59].

Additionally, we examined the immunohistochemical expression of caspase-3, which was reported as a marker of apoptosis[60]. Apoptosis happens through two main pathways, The intrinsic pathway is activated as a result from the formation of apoptosome that further activates caspase-9 and caspase 3 that promotes apoptosis[61]. Several studies elaborated that silymarin interfere with the proteins needed for the expression of cell cycle and apoptosis as it regulates the equilibrium between pro-survival and pro-apoptotic signals, so expressing the apoptotic proteins is controversial according to the type of tissue and benign or malignant[24, 47].

As far as we know, this is the first research to investigate the role of silymarin in reducing the toxic effect of CCl4 on normal salivary gland tissue. Limited researches studied the effect of silymarin

on salivary gland carcinomas. Our study revealed that silymarin reduced the apoptotic changes in both normal parotid and submandibular glands compared to the CCl4 group. This goes with Choi et al. study in salivary gland carcinomas cell lines showed that silymarin had a tumoricidal evidence through increasing pro-apoptotic proteins, as a consequence it stimulates the apoptosis pathway by mitochondria [24]. This supports that silymarin show a tumoricidal and a protective effect on salivary glands.

Interestingly, the results of our study showed more caspase-3 expression in the salivary glands CCl4 treated rats than silymarin group. This effect are compatible with previous reports and have been explained due to the inflammation induction and oxidative stress caused by CCl4[60, 62].

As limited studies were done on salivary gland tissue, A recent research by Kim et al was on similar tissue to salivary glands such as glandular tissue breast carcinomas, strengthened our research since they showed that silymarin taken orally reduced growth of tumors through stimulating apoptosis without revealing and changes in the histological picture[63]. All these results concluded that Silymarin serves as a pro-apoptotic agent and promotes cell apoptosis to perform through its anti-tumor actions and lowers the toxic effects of other agents such as CCl4.

CONCLUSIONS

Hence, our research proved that silymarin is a strong natural antioxidant to protect salivary glands from CCl4 toxic effect.

RECOMMENDATIONS

Further studies are recommended to test the different doses of Silymarin and longer intervals of CCl4 ingestion on salivary glands ultrastructure and structure of saliva.

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