Introduction

Flavonoids form a large class of phenolic substances widely distributed throughout the plant kingdom and can be detected in 3000 varieties [1]. Silymarin (SM) is extracted from seeds of milk thistle [2]. Milk thistle (Silibum marianum) is the most ancient and extensively used medicinal plant for its beneficial effects on liver and other organs [3]. SM, a mixture of three isomeric flavonolignans,
contains silybin, silydianin, and silychristin as its major components [4]. SM is usually used as hepatoprotective medicine. Clinical studies have indicated that SM is therapeutically effective against chronic hepatitis, various liver damages, early hepatic cirrhosis, and other liver-specific diseases [5]. Furthermore, in vitro, in vivo, and clinical studies have demonstrated the antioxidant and hepatoprotective effects of SM and its major active constituent silibinin (silybin), a polyphenolic molecule, in animal and human models of alcoholic and nonalcoholic chronic liver diseases [6]. The membrane-stabilizing and antioxidant properties of SM are believed to be the major protective mechanisms. Meanwhile, SM has been reported to possess many pharmacological properties such as anti-inflammatory, antitumor, and antibacterial effects, and to positively influence some risk factors of atherosclerosis [7]. Silibinin, a constituent of SM, was reported to suppress tumor growth in vivo, suggesting the potential of SM against renal carcinogenesis [8]. As a therapeutic agent, SM is well tolerated and largely free of adverse effects. The essential activity of SM is an antioxidant effect of its flavonolignans and of other polyphenolic constituents, which is attributable to the radical scavenging ability of both free radicals and reactive oxygen species [9]. SM is a potent inhibitor of NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) activation. NF-κB protects cells from undergoing apoptosis. Furthermore, activators of NF-κB are also known to induce apoptosis. The activation of NF-κB and kinases in most cases is dependent on the production of reactive oxygen species [10].

Cisplatin (cis-diammine-dichloro-platinum) is an inorganic platinum compound with broad-spectrum antineoplastic activity against different types of human tumors [11]. Cisplatin is widely used for the treatment of germ cells (e.g. testicular), head and neck cancer, and cervical cancer [12]. Nephrotoxicity is a common complication of cisplatin chemotherapy that limits its clinical use [13]. Kidney proximal tubule cells are especially sensitive to cisplatin [14]. Forty-eight to 72 h after cisplatin administration, there is impaired proximal and distal tubular reabsorption and increased vascular resistance [15]. However, the mechanism by which cisplatin causes kidney cell death is unclear. Multiple mechanisms have been implicated in cisplatin-induced nephrotoxicity, which results in tubular cell death by apoptosis and necrosis [16].

The aim of this study was to evaluate the effect of SM on cisplatin-induced renal tubular injury in male rabbits.

Materials and methods

In this study, 20 adult male rabbits of average weight 2000–2500 g were used. The animals were housed in special cages and maintained under standard laboratory conditions. They were fed tap water and a commercial diet ad libitum.

Cisplatin vials of 50 mg/50 ml were obtained from Mylan S.A.S Inc. (Saint-Priest, France), and SM (legalon) capsules of 140 mg were obtained from Chemical Industries Development Pharmaceutical Co. (CID, Cairo, Egypt). Animals were injected intraperitoneally with cisplatin at a dose of 5 mg/kg body weight [17]. SM powder was dissolved in sterile water and given orally (by means of an orogastric tube) at a dose of 52 mg/kg body weight [18].

Twenty rabbits were divided into four groups (n=5 rabbits) as follows:

In group I (the control group), animals were injected with a single intraperitoneal dose (10 ml) of saline and were given oral sterile water daily through an oral tube for 3 days. These animals served as controls. In group II (the cisplatin group), animals were injected with a single intraperitoneal dose of cisplatin (5 mg/kg body weight) and served as the affected group. In group III (the prophylactic silymarin group), animals were given a daily oral dose (for 3 days) of SM (52 mg/kg body weight) through an oral tube, after which a single intraperitoneal dose of cisplatin was injected (5 mg/kg body weight). In group IV (the curative silymarin group), animals were given a single intraperitoneal dose of cisplatin (5 mg/kg body weight), and after 2 days they were given a daily oral dose of SM (52 mg/kg body weight) for 3 days.

Vascular perfusion fixation through the left ventricle with 1% glutaraldehyde was performed, and kidney samples were taken 5 days after cisplatin injection. Paraffin sections were prepared for LM examination, H&E study [19], and immunohistochemical detection of caspase-3 using a standard avidin–biotin peroxidase complex system according to the kit used Neomarkers. (Thermo Fisher Scientific Inc., Rockford, Illinois, USA), followed by diaminobenzidine visualization. Sections were counterstained with hematoxylin [20]. For electron microscopic preparation, kidney samples were processed and ultrathin sections were prepared [21] and examined in Tanta EM unit. The mean area percentage of caspase-3 expression was quantified in 10 images for each group using Image-Pro Plus program version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA). Differences in caspase-3 expression between the silymarin groups (III and IV) and the affected group (II) were assessed using the F-test, with P<0.05 as the level of statistical significance. Statistical analyses were carried out using Microsoft excel 2010 (Microsoft Egypt, Cairo, Egypt).

Results

H&E study

The control group showed normal renal corpuscles and proximal and distal convoluted tubules (Fig. 1). The cisplatin group (the affected group) showed marked vacuolization of the cell cytoplasm of renal tubules with tubular dilatation (Fig. 2). The silymarin prophylactic group showed minimal vacuolization of the tubular cell cytoplasm (Fig. 3). The silymarin curative group showed cytoplasmatic vacuolization of renal tubular cells with slight tubular dilatation (Fig. 4).
Immunohistochemical study
Positive immunohistochemical staining of caspase-3 demonstrated brown cytoplasmic staining (index for the degree of nuclear apoptosis). Negative cytoplasmic staining for caspase-3 was found in the control group (Fig. 5). In the affected group, caspase-3 was highly expressed in the cytoplasm of the cell lining the proximal and distal tubules (Fig. 6). In the silymarin prophylactic group, caspase-3 was minimally expressed in the cytoplasm of tubular cells (Fig. 7). In the silymarin curative group, caspase-3 was moderately expressed in the cytoplasm of tubular cells (Fig. 8).

The mean area percentage of caspase-3 expression for all groups is presented in Table 1 and Histogram 1. There was a significant decrease ($P < 0.05$) in caspase-3 expression in groups III and IV (the silymarin prophylactic and curative groups) compared with group II (the cisplatin group).

Electron microscopic study
In the control group, cells lining the proximal tubule showed numerous long apical microvilli, narrow intercellular space, oval nucleus, and multiple mitochondria (Fig. 9). Cells lining the distal tubule showed basal infoldings containing basal elongated mitochondria (Fig. 10). In the cisplatin group (the affected group), cells lining the proximal tubule showed loss of apical microvilli, widened intercellular space, few atrophic mitochondria, and condensed nucleus (Fig. 11). Cells lining the distal tubule showed disorganization of basal infoldings with dispersed mitochondria, cytoplasmic vacuolization, and dark condensed nucleus (Fig. 12). In the silymarin prophylactic group, cells lining the proximal tubule showed long apical microvilli, narrow intercellular spaces, multiple mitochondria, and little cytoplasmic vacuolization (Fig. 13). Cells lining the distal tubule showed basal infoldings, normal mitochondria, and little cytoplasmic vacuolization (Fig. 14). In the silymarin curative group, cells lining the proximal tubule showed few microvilli, narrow intercellular spaces, degenerated mitochondria, condensed nucleus, and cytoplasmic vacuolization (Fig. 15). Cells lining the distal tubule showed disorganization of basal infoldings, dispersed and degenerated mitochondria, narrow intercellular spaces, cytoplasmic vacuolization, and condensed nucleus (Fig. 16).
Figure 5. A photomicrograph of a section of kidney from the control group showing negative caspase-3 immunostaining of the tubular cell cytoplasm. Caspase-3 immunostaining, × 200.

Figure 6. A photomicrograph of a section of kidney from the cisplatin group showing highly expressed caspase-3 reaction in the cytoplasm (C) of tubular cells. Caspase-3 immunostaining, × 200.

Figure 7. A photomicrograph of a section of kidney of silymarin prophylactic group showing minimally expressed caspase-3 reaction in cytoplasm of tubular cells (C). Caspase-3 immunostaining, × 200.

Figure 8. A photomicrograph of a section of kidney from the silymarin curative group showing moderately expressed caspase-3 reaction in the cytoplasm of tubular cells (C). Caspase-3 immunostaining, × 200.

Figure 9. An electron micrograph of a proximal tubule from the control group showing long apical microvilli (double arrows), narrow intercellular spaces (arrow), nucleus (N), and multiple mitochondria (M). EM, × 5000.

Figure 10. An electron micrograph of a distal tubule from the control group showing nucleus (N) and numerous basal infoldings (F) containing basal mitochondria (M). EM, × 5000.
Figure 11. An electron micrograph of a proximal tubule from the cisplatin group showing loss of apical microvilli (double arrows), widened intercellular spaces (arrow), few atrophic mitochondria (M), and condensed nucleus (N), Marked vacuolization (V) in the cytoplasm is noticed. EM, × 5000.

Figure 12. An electron micrograph of a distal tubule from the cisplatin group showing disorganization of basal infoldings (F) with dispersed and degenerated mitochondria (M), cytoplasmic vacuolization (V), and dark condensed nucleus (N). EM, × 5000.

Figure 13. An electron micrograph of a proximal tubule from the silymarin prophylactic group showing long apical microvilli (double arrows), narrow intercellular spaces (arrow), multiple mitochondria (M), nucleus (N), and minimal cytoplasmic vacuolization (V). EM, × 5000.

Figure 14. An electron micrograph of a distal tubule from the silymarin prophylactic group showing basal infoldings (F), multiple mitochondria (M), few cytoplasmic vacuolization (V), and nucleus (N). EM, × 5000.

Figure 15. An electron micrograph of a proximal tubule in silymarin curative group showing few apical microvilli (double arrows), narrow intercellular space (arrow), degenerated mitochondria (M), condensed nucleus (N) and cytoplasmic vacuolization (V). EM, × 5000.

Figure 16. An electron micrograph of a distal tubule from the silymarin curative group showing disorganized basal infoldings (F), dispersed and degenerated mitochondria (M), narrow intercellular spaces (arrow), cytoplasmic vacuolization (V), and condensed nucleus (N). EM, × 5000.
Cisplatin cytotoxicity is likely caused by a combination of multiple mechanisms, involving DNA damage, caspase activation, mitochondrial dysfunction, and formation of reactive oxygen species. However, the exact molecular and cellular mechanisms by which cisplatin induces nephrotoxicity remain unclear [33].

SM has received attention because of its antihepatotoxic and antioxidant (radical scavenging) effects, leading to alteration of DNA expression by suppression of nuclear factor NF-κB [34].

SM was used as a daily oral dose of 100mg/kg/day in rats [18]. In the present study, the doses for rabbits were calculated according to Paget’s formula [35].

In the present study, SM when used for prophylaxis (daily oral dose 3 days before cisplatin injection) markedly prevented the tubular damage induced by cisplatin, whereas when it was used as a curative (daily oral dose 2 days after cisplatin for 3 days) SM slightly reduced the nephrotoxicity of cisplatin. This agreed with the results of some authors [36–38], who reported that pretreatment with SM significantly ameliorated the tubular damage induced by cisplatin. Also, others have reported that induction of nephrotoxicity by cisplatin is assumed to be a rapid process involving reaction with proteins in the renal tubules. It is important that the protective agent is present in renal tissue before damage occurs. This might explain why complete protection did not result when SM or its extract was given after administration of cisplatin [4]. On the contrary, other studies [39] have reported that pretreatment with flavonoids including silybin was ineffective in improving the survival rate of renal cells. It was also stated that pretreatment with curcumin did not reduce the nephrotoxicity induced by cisplatin in renal tissue. A slight but not significant reduction was observed in animals treated with SM plus cisplatin when compared with the cisplatin group [40], whereas other researchers [41] have reported that in the kidney cells damaged by cisplatin, administration of silybin before or after the chemical-induced injury can reduce or avoid nephrotoxic effects.

The protective effect of SM in cisplatin nephrotoxicity was explained by some investigators [4,42], who reported that the protective effect of SM was associated with its antioxidant properties, as it acts as a free-radical scavenger and lipid peroxidation inhibitor, increasing the intracellular concentration of glutathione and superoxide anions, thereby inactivating nitric oxide (NO).

### Discussion

Kidneys are dynamic organs and represent the major control system maintaining body homeostasis. They are affected by many chemicals and drugs [22]. Excretion of cisplatin is predominantly renal. Cisplatin accumulates in the renal tubular cells to approximately five times its extracellular concentration [23]. Consequently, the kidney is considered to be the primary target organ for cisplatin toxicity [24].

In the present study, cisplatin induced extensive tubular necrosis with tubular dilatation, cell apoptosis (highly expressed caspase-3 reaction), loss of apical microvilli with widened intercellular space of proximal tubules, and disorganization of basal infoldings with dispersed mitochondria in the distal tubules 5 days after intraperitoneal single injection. This agreed with the results of some researchers [25], who reported that cisplatin, administered as a single dose, caused kidney dysfunction in rats by day 3, which further increased by day 5. In addition, other researchers [26,27] reported that morphological alterations in the kidney 5 days after cisplatin injection included a marked necrosis in the proximal tubules and hyaline casts in the tubular lumen. Some studies [28] have reported that cisplatin can induce apoptosis in renal tubular cells both in animal models and in cell culture systems and that the apoptosis is evident within 3 days of cisplatin injection. Further, it was reported that cisplatin induced alterations and serious damage to the mitochondrial structure [29–31]. Others [32] reported that morphological changes in the kidney after 5mg/kg cisplatin treatment included widespread tubular necrosis and degeneration of the proximal tubules on day 3.

In the present study, cisplatin induced kidney tubular degeneration with preservation of the glomeruli, and this agreed with studies reporting that cisplatin nephrotoxicity primarily causes tubular lesions, whereas glomeruli undergo no obvious morphologic changes [15]. On the contrary, others stated that sections of kidney that were treated with cisplatin showed local cellular alterations in glomerular and tubular cells [11].

### Table 1. Showing the mean area percentage of caspase-3 expression and SD in groups I, II, III, and IV

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean area %</th>
<th>SD ±</th>
<th>P-value</th>
<th>Significance</th>
</tr>
</thead>
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<tr>
<td>Group I</td>
<td>0</td>
<td>11.3</td>
<td>0.0055</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>2.2</td>
<td>8.5</td>
<td>0.0197</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
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<td>0.0056</td>
<td></td>
<td>S</td>
</tr>
<tr>
<td>Group IV</td>
<td>11.3</td>
<td>2.2</td>
<td>0.0008</td>
<td>S</td>
</tr>
</tbody>
</table>

Groups III and IV compared with group II. S, significant.

Histogram 1.

Showing the mean area percentage of caspase-3 expression in groups I, II, III, and IV.
In conclusion, the present study suggests that SM when used as a prophylactic agent is more effective in protection against cisplatin nephrotoxicity than when used as a curative agent and may be considered as a potentially useful candidate in combination chemotherapy with cisplatin.

Acknowledgements
Conflicts of interest
There is no conflict of interest to declare.

References
تأثير عقار السيليمارين ضد اصابات الأنيببات الكلوية المحثة بعقار السيسبلاتين (دراسة هستولوجية و هستوكيميائية مناعية وبالميكروسكوب الإلكتروني)

محمد محمود يوسف سالم، أميمة كامل هلال، و نرمين عدلى

تستخدم مركب ينتمي إلى عائلة الشبطات بين الأحماض الأمينية، وهو عضو من العائلة الكيميائية المعقدة أثناء العلاج بمضادات السرطان، ويوفر تأثيراً مهماً في حالات تسمم الكبد. يُعتبر تسمم الأعضاء من المشاكل الطبية المعقدة أثناء العلاج بمضادات السرطان. السبيلامين هو مركب بيلانى يستعمل بفاعلية كدواء ضد السرطان، ولكن يستخدم عادة للتشخيص والعلاج. يُعتبر عقار السيبيلين من علاجات كيميائية مناعية ونتيجةً هو استخدامه كعامل علاجي. تشير النتائج إلى أن عقار السيبيلين يقلل من التأثير السام لعقار السيسبلاتين، مما يتيح استخدامه كدواء في العلاج الكيميائي لأمراض السرطان، مما يعزز من فعالية العلاج.