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The Antioxidant Potential of Saudi Propolis Extract on Hepatorenal Toxicity in Mice

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ABSTRACT

Background: In advanced cirrhotic conditions, hepatorenal syndrome detrimentally affects renal function. Interest has grown in propolis for its cytoprotective properties against various exogenous agents. This study evaluates the efficacy of Saudi propolis extracts in mitigating hepatorenal toxicity induced by carbon tetrachloride in mice.

Methods: Thirty-two male Swiss Albino mice were divided into four groups: a Control (-) group receiving distilled water; a Control (+) group subjected to intraperitoneal CCl4 at 0.5 mL/kg (20% v/v in corn oil) on day 6; a Standard group treated daily with silymarin at 200 mg/kg and a group given an oral dose of aqueous propolis extract (APE) at 8.4 mg/kg.

Result: Histological and biochemical analyses confirm propolis extract's role in preventing hepatocyte apoptosis and reducing inflammatory infiltrates in kidney tissues, improving the histological appearance of hepatic and renal tissues with fewer fibrotic changes. The application of immunohistochemistry, along with reductions in anti-apoptotic proteins such as BCL-2 and p53, supports these findings, highlighting the antioxidant potential of Saudi propolis extracts in addressing hepatorenal toxicity.

Key words: Antioxidants, Carbon Tetrachloride, Hepatorenal toxicity, Kidney, Liver, Propolis.

INTRODUCTION

Within the realm of liver pathology, hepatorenal syndrome emerges as a critical condition marked by deteriorating renal function amidst acute liver failure or severe chronic liver disease, affecting roughly 20% of patients with liver disorders. This correlation between liver disease severity and renal dysfunction, highlighted in studies by Ginès *et al.* (2018) and Garcia-Tsao *et al*. (2008), underscores a significant public health challenge globally.

Although nephrotoxicity occurrences are rare, instances of acute renal failure and renal tubular damage have been documented in severe liver injury cases, as noted by Nauta *et al*. (2005). The pathophysiological landscape of such disorders involves the proliferation of extracellular matrix components characteristic of hepatic fibrosis and chronic liver conditions, elucidated by Song *et al*. (2005).

The risk of hepatotoxicity escalates with exposure to certain chemicals like carbon tetrachloride (CCl4), thioacetamide and paracetamol, whether through industrial exposure, laboratory use, or metabolic processes. Such exposure fosters excessive free radical production, posing significant liver damage and extending to the respiratory, central nervous and gastrointestinal systems, including the kidneys, as discussed by Mahli *et al.* (2015) and (El-Hadar and Hassanien, 2016). The oxidative damage ensuing from such exposure disrupts the oxidant-antioxidant equilibrium, promoting neutrophil infiltration and inflammatory responses, a phenomenon explored by Muniz *et al*. (2008).

Propolis, a complex resinous substance crafted by honey bees, is renowned for its broad cytoprotective actions against various exogenous agents. Characterized by a composition rich in resins, waxes and bioactive compounds like terpenes, phenolic acids, essential oils and minerals, propolis's medicinal utility spans anti-inflammatory, antibacterial, antifungal, antipyretic, antioxidant and immunostimulatory effects, with documented non-toxicity. Its historical use in folk medicine and demonstrated hepatoprotective efficacy, particularly against liver and kidney toxicities induced by diverse agents, are well-documented in literature by Cole *et al.* (2010) and Jadhav *et al*. (2013).

This investigation primarily aims to delineate the hepatorenal protective potential of Saudi propolis extracts in mitigating CCl4-induced hepatic and renal toxicity within a murine model, offering insights into its therapeutic applications in addressing hepatorenal disorders.

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MATERIALS AND METHODS

Collection and preparation of propolis samples

The propolis specimens were obtained from the Baljurashi district, situated in the south-western region of Saudi Arabia, specifically within the geographical coordinates approximately at 19°51′40″N latitude and 41°33′ 40″E longitude, during the summer of 2021. The Propolis extract

was meticulously prepared following the procedure described by (Oršolić and Bašić, 2005). The resulting supernatant was carefully harvested and subsequently stored at a frigid temperature of -20°C, preserving its integrity until it was employed for subsequent use.

Induced hepatorenal injury

A comprehensive study involved 32 male Swiss Albino mice, averaging 27.5±2.5 grams, obtained from the King Fahd Medical Research Center (KFMRC). These were systematically divided into five distinct groups, with eight mice in each. Held under stringent environmental conditions, they were kept at 20±2°C, with a 12-hour light/dark cycle and 65% humidity. In line with protocols from Al-Sayed *et al.* (2015) and Thomas *et al.* (1997), with the Guidelines for Animal experimentation approved by Al-Baha University, Experimental Ethics Committee in Fourth session at 17/1/2022.

The Control (-) group, serving as the primary negative control, was given distilled water orally. Conversely, the Control (+) group received a sublethal intraperitoneal dose of CCl4, mixed at 20% v/v in corn oil, on the experiment's final day, day 6. The Standard group was treated orally with silymarin at 200 mg/kg body weight daily, while another group received an aqueous propolis extract (APE) at a daily oral dose of 8.4 mg/kg.

Treatments were administered orally to all groups for five consecutive days. On day 6, all animals except the normal group received a 20% CCl4 v/v in corn oil injection intraperitoneally at a dose of 0.5 mL/kg to induce oxidative effects. Blood samples were then collected from the orbital venous plexus to assess biochemical parameters. The liver and kidney organs were isolated and fixed in 10% buffered formalin for histological examination.

Biochemical evaluations were performed using a Human kit from Germany as per the manufacturer's instructions. IL-6 and TNFα levels were quantified using ELISA kits from Abcam, Cambridge, MA, USA, strictly adhering to the provided protocols. Subsequently, liver and kidney tissues were preserved in 10% neutral formalin and examined microscopically as described by Presnell *et al.* (1997). Bcl-2 and P53 immunoreactivity within these tissues were assessed on paraffin-embedded sections, utilizing protocols from the Vectastain Elite ABC Kit. Data were represented as mean ± standard error of the mean (SEM) from triplicate assays. Statistical analyses were conducted using one-way ANOVA, followed by Tukey's post hoc test for intergroup comparisons, employing SPSS software (version 17, SPSS Inc, Chicago, IL, USA). Statistical significance was determined at $p<0.05$, ensuring rigorous evaluation of the therapeutic impacts on hepatorenal function.

RESULTS AND DISCUSSION

The study investigates the hepatorenal protective efficacy of propolis and silymarin in the context of carbon tetrachloride (CCl4)-induced toxicity, uncovering critical insights into liver and kidney biochemical dynamics. Exposure to CCl4 significantly elevated serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), markers indicative of hepatocellular injury. This contrasts with the therapeutic groups, where both propolis extract and silymarin treatments manifested a significant reversal of these enzymatic elevations, highlighting their potent hepatoprotective capabilities. The findings, delineated in Fig 1A -1C, resonate with existing literature on the subject, particularly emphasizing CCl4's propensity for renal over hepatic distribution and toxicity, as underscored by Sanzgiri *et al.* (1997). Alabbad *et al.* (2023) have implicated the involvement of oxidative pro-inflammation and apoptosis in CCl4-induced hepatic pathologies and leads to increased liver enzymes, including AST and ALT, which were quantitatively estimated in serum.

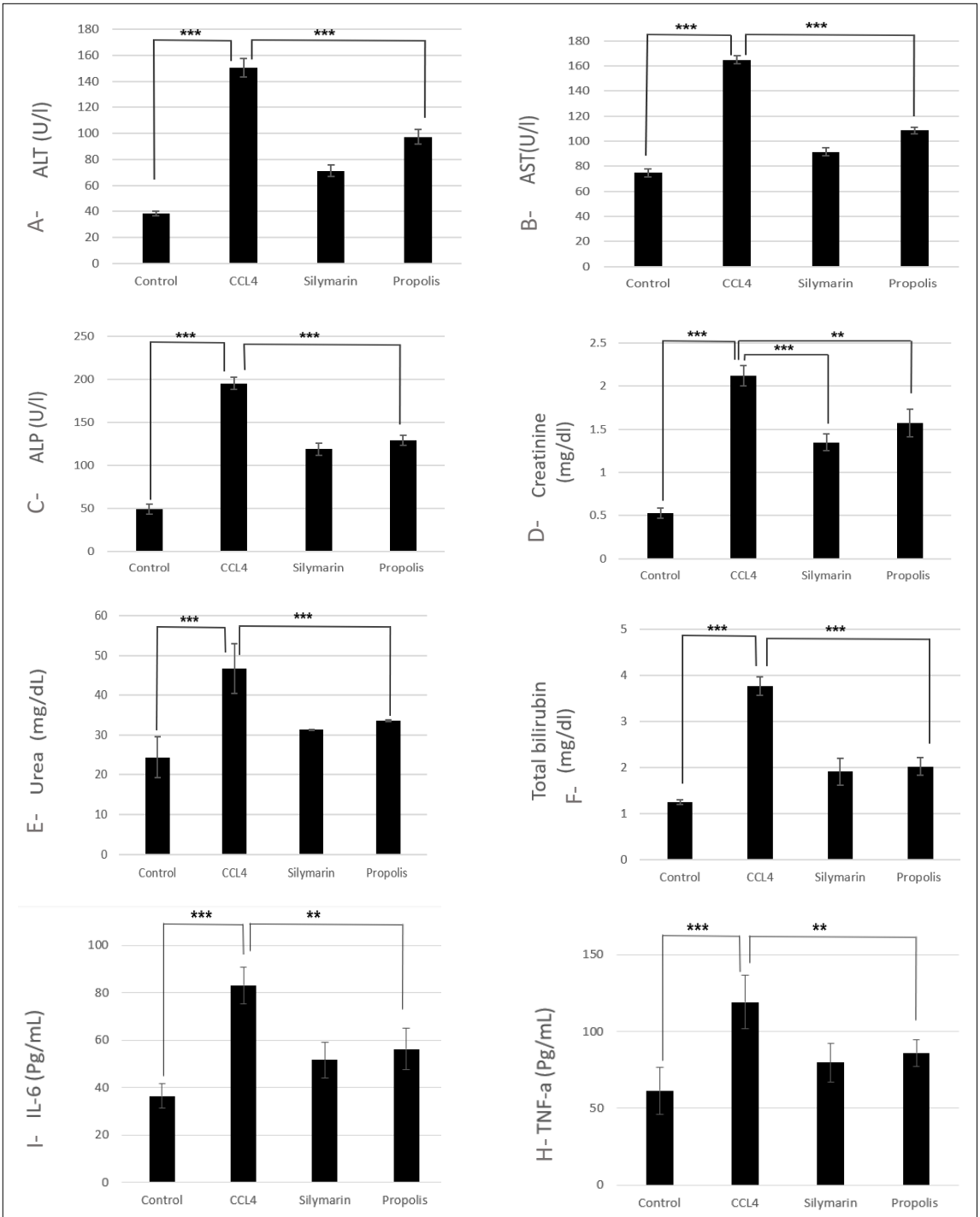
Further analyses extend into renal function parameters-creatinine, urea and total bilirubin-where CCl4 administration elicited pronounced disturbances. Conversely, interventions with silymarin and aqueous propolis extract (APE) showcased marked improvements in these kidney function markers (Fig 1D-F), delineating a clear therapeutic demarcation from the CCl4 group. This differential toxicokinetic profile of CCl4, favoring renal dissemination and injury, underpins the study's focus on elucidating the underlying mechanisms of hepatorenal injury attributed to necrotic disruptions in hepatocytes and nephrotic cells, which notably influence serum enzyme levels-a phenomenon substantiated by Achliya *et al.* (2004). Atasever *et al.* (2020) reports that lipid peroxidation brought on by CCl4's hazardous metabolites may be the cause of CCl4-dependent hepatotoxicity and the resulting histological alterations. The liver's vulnerability to CCl4-induced toxicity, as revealed, hinges on the intracellular generation of reactive oxygen species (ROS), with hydrogen peroxide (H_2O_2) implicated in mediating oxidative stress pathways, corroborating the findings by Abraham *et al.* (1999) regarding CCl4's renal affinity.

Silymarin's deployment as a benchmark treatment unveiled its significant ameliorative impact on CCl4-induced liver and kidney biochemical alterations, affirming its esteemed therapeutic stature. The silymarin treatment group, particularly at higher doses, demonstrated a conspicuous normalization of liver enzymes, echoing the therapeutic observations noted in hepatocellular carcinoma research by Demiroren *et al.* (2018). The alleviation of elevated ALT levels, a clinical hallmark of liver distress across various pathological spectrums Rodrigues Jr *et al.* (1995), underscores silymarin's comprehensive hepatoprotective effect.

Introducing propolis extracts into the therapeutic milieu catalyzed significant enhancements across liver and kidney enzyme profiles, concurrently restoring histological liver integrity. This protective hypothesis of propolis against

cellular perturbations aligns with the investigative narrative of Wen *et al.* (2012). The study also ventures into cytokine modulation, with treatments substantially diminishing proinflammatory cytokines IL-6 and TNFα levels, hence spotlighting the anti-inflammatory and antioxidative prowess of propolis and silymarin Ibrahim *et al.* (2019).

Venturing further, the discourse encapsulates the intricate interplay between oxidative stress and



Statistically significant differences between the compared values are denoted as (** p<0.01), while instances of highly significant distinctions are represented as (*** p<0.01).

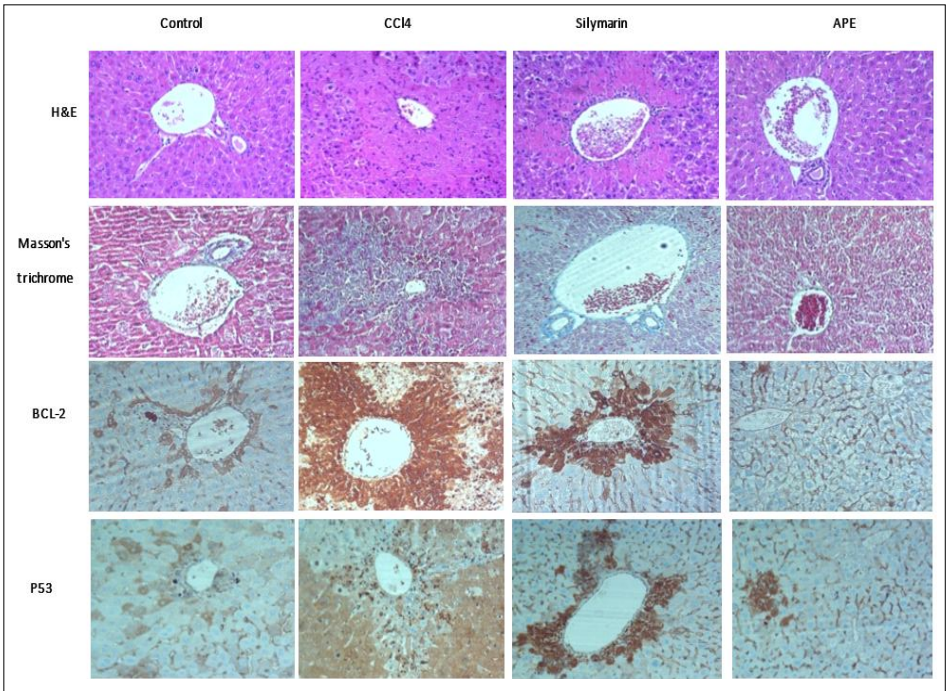
Fig 1: illustrates the impact of aqueous propolis extract (APE) on the development of hepatorenal toxicity in relation to liver and kidney functions.

inflammatory cytokines in the genesis of chemically induced acute renal damage. The observed cytokine attenuation post-treatment elucidates the antioxidative and anti-inflammatory mechanisms underlying propolis and silymarin's therapeutic action, reinforcing the complex oxidative-inflammation nexus in hepatorenal toxicity detailed by (Elmarakby and Sullivan, 2012) and Gomaa *et al*. (2019).

Histopathological examination of liver samples underscored distinct contrasts between groups. The control group's liver architecture showcased standard morphology with intact liver lobules, central veins and portal triads. In stark contrast, CCl4-exposed mice exhibited significant hepatic disruption, marked by neutrophilic infiltrates, hepatocyte degeneration, necrosis and biliary abnormalities, underscoring the compound's toxic impact. However, silymarin treatment post-CCl4 exposure mitigated these effects, restoring liver architecture towards normalcy with healthy hepatocytes and Kupffer cells, alongside enhanced hepatocyte regeneration. The APE similarly evidenced improved liver structure with decreased inflammation and a robust presence of hepatocytes and Kupffer cells (Fig 2, H and E × 100).

Kidney tissue analysis further highlighted the protective effects of silymarin and APE against CCl4-induced renal damage. While CCl4 treatment led to significant kidney alterations-distorted Bowman's capsule, tubular necrosis, inflammation and glomerular and tubular distress-the silymarin group showed marked structural recovery, particularly in Bowman's capsule and tubular dimensions. APE treatment further emphasized this therapeutic trend, revealing substantial renal recovery with lessened glomerular and tubular abnormalities, reduced cellular hyperactivity and minimized inflammation (Fig 3, H and E × 100). These findings collectively underscore the cytoprotective capacity of silymarin and propolis extract against CCl4-induced hepatorenal toxicity, offering promising insights into their therapeutic potential.

Masson's trichrome staining of liver sections from the control group revealed normal fibrotic architecture, whereas liver tissues from the CCl4-exposed mice displayed significant fibrosis, characterized by a dense accumulation of fibrotic tissue. This fibrotic response was mitigated in mice treated with silymarin, showing a notable improvement in liver structure and a reduction in collagen fiber deposition.



CCl4-treated group displayed significant hepatic injury, including coagulative necrosis of hepatocytes and mononuclear inflammatory infiltration. Silymarin treatment resulted in a notable improvement in liver architecture, approaching normalcy with healthier hepatocytes and Kupffer cells. The APE treatment group exhibited improved liver structure with decreased inflammatory and fibrotic markers. Both treatments effectively mitigated the histological markers of damage induced by CCl4, including reducing hepatocyte apoptosis and fibrosis, as evidenced by decreased BCL-2 and P53 staining.

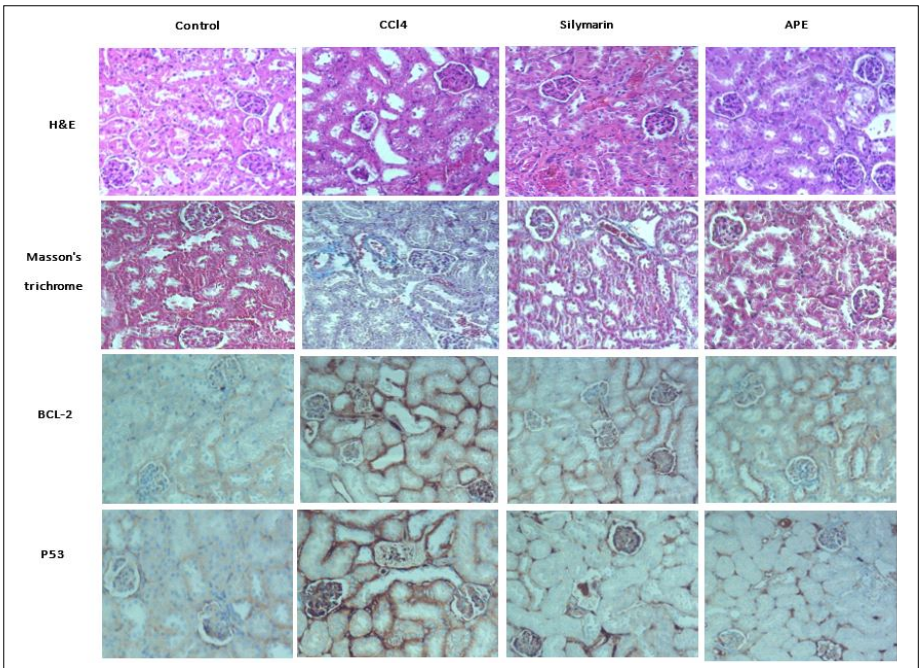
Fig 2: Histological evaluation of liver tissues, the control group exhibited pristine liver architecture, characterized by well-structured hepatocytes and portal triads, without any signs of fibrosis or apoptosis, as confirmed by Masson's trichrome and immunohistochemistry (IHC) staining for BCL-2 and P53.

A similar decrease in collagen accumulation was evident in the livers of mice treated with APE, indicating its fibrosis-attenuating effects (Fig 2, Masson's trichrome × 100).

Conversely, control group kidney tissues were free from fibrotic alterations, maintaining a normal fiber distribution. CCl4 exposure resulted in a pronounced increase in collagen fibers around blood vessels and in the peri-glomerular space, indicative of renal fibrosis. Silymarin treatment afforded a moderate reduction in collagen deposition compared to the CCl4 group, suggesting its renoprotective efficacy. Remarkably, the APE-treated mice exhibited a restoration of normal collagen fiber distribution within kidney tissues, as highlighted in Fig 3 (Masson's trichrome × 100).

Histological evaluation further revealed significant hepatorenal tissue damage in the CCl4 group, with HandE staining uncovering mononuclear cell infiltration, centrilobular necrosis and central vein congestion in the liver. These observations were corroborated by Masson's trichrome staining, which detailed the extensive fibrosis and septa formation, underscoring the critical role of inflammation and oxidative stress in driving liver fibrosis, as noted by (Kisseleva and Brenner, 2021) and Li *et al.* (2016). Similarly, the kidney tissues of the CCl4 group exhibited severe pathological changes, including Bowman's capsule enlargement and tubular degeneration, with Masson's trichrome staining revealing significant collagen deposition and fibrotic region formation around blood vessels. These renal alterations, consistent with findings by Al-Yahya *et al.* (2013).

The mechanistic underpinnings of CCl4 induced renal damage have been a focal point of numerous studies, with oxidative stress emerging as a pivotal factor in renal injury. The toxicological pathway of CCl4 in the kidney primarily involves the formation of trichloromethyl (CCl3-) radicals, a result of CCl4 dehalogenation mediated by the



Immunohistochemical analysis further confirmed the absence of anti-apoptotic protein expression (BCL-2) and fibrosis markers (P53), indicating a baseline of renal health. Conversely, CCl4 treatment induced notable renal pathology, including disfigured Bowman's capsule architecture, tubular necrosis and widespread inflammation (HandE) staining. Masson's trichrome stain revealed extensive fibrotic tissue development, particularly around Bowman's capsules and within the interstitial spaces in renal tissues treated with CCl4. Silymarin treatment post-CCl4 exposure showed a partial restoration of renal architecture, with lessened cellular infiltration and fibrosis, indicating its protective effect. The APE treatment that remarkably improved renal structure, reducing tubular dilatation and restoring collagen fiber distribution to near-normal levels, as observed in both HandE and Masson's trichrome stains. The reduction in apoptosis and fibrosis markers within the APE-treated group underscores its potent anti-apoptotic and anti-fibrotic effects, offering substantial protection against CCl4-induced renal damage.

Fig 3: The histological investigation of kidney. In the control group, kidney tissues presented with standard histology, showcasing intact glomeruli, interstitial tubules and vascular structures, with no evidence of fibrotic alterations observed through Masson's trichrome staining.

cytochrome P450 enzyme system. The disruption in these enzymatic defenses culminates in the production of reactive oxygen species (ROS), which are central to the pathogenesis of tubular necrosis (Wu *et al.,* 2012).

The biochemical cascade of CCl4-induced renal damage is characterized by altered renal microsomal NADPH cytochrome P450 levels, enhanced lipid peroxidation and a perturbed reduced/oxidized glutathione (GSH/GSSG) ratio (Walker *et al.,* 1996).

Kidneys' critical role in osmoregulation and ion homeostasis further exacerbates the impact of CCl4-induced disturbances. Oxidative stress triggers the release of vasoactive mediators, leading to renal vasoconstriction and a reduction in the glomerular capillary ultrafiltration coefficient, ultimately diminishing glomerular filtration rate (Garcia-Cohen *et al.,* 2000).

Mammals have evolved sophisticated mechanisms to counteract ROS, with rats exemplifying the species' capacity to mitigate elevated ROS through enzymes like SOD and non-enzymatic antioxidants like GSH. These systems play crucial roles in neutralizing free radicals and are integral to the body's antioxidative defense (Wong *et al.,* 2014).

At the cellular level, the expression of the B-cell lymphoma 2 (Bcl-2) anti-apoptotic protein illustrates the biological response to oxidative stress. In the context of CCl4 exposure, a significant increase in hepatocyte apoptosis was observed, contrasting with the control group. Treatments with silymarin and APE were effective in reducing hepatocyte apoptosis, highlighting their protective effects against CCl4-induced liver damage. Additionally, histological analyses revealed fibrosis and the role of p53 in chronic liver regeneration following CCl4 exposure. Notably, silymarin and APE treatments led to a marked reduction in liver fibrosis, emphasizing their potential in mitigating fibrotic liver disease (Fig 2, P53 × 100).

The extensive exploration of CCl4's impact on renal and hepatic systems underscores the critical role of oxidative stress in mediating tissue damage. The study highlights the therapeutic potential of silymarin and APE in addressing oxidative damage and suggests a broader applicability of these agents in combating oxidative stress-related diseases.

The CCl4-treated group exhibited pronounced positive staining for apoptosis in renal tubules, highlighting significant cellular death and damage. Notably, the application of Silymarin and APE treatments demonstrated a remarkable reduction in apoptosis markers compared to the CCl4 group, as evidenced by decreased staining in Fig 3 (Bcl-2 × 100).

Immunohistochemistry of liver samples subjected to CCl4 exposure disclosed an upsurge in apoptotic markers, specifically an increase in Bcl-2 and p53 expressions, indicative of heightened apoptotic activity. Such findings underscore the engagement of the mitochondrial-mediated intrinsic apoptotic pathway, regulated by the Bcl-2 protein family, which includes antiapoptotic members (Bcl-2, Bcl-XL) and proapoptotic members (Bax, Bak). The interplay between these proteins dictates cellular fate, with Bcl-2 serving as a protective agent against oxidative stress-induced lipid peroxidation and Bax promoting apoptosis through cytochrome c release and caspase-3 activation, as detailed by (Abdel Moneim, 2016).

The CCl4 treatment led to significant fibrosis, marked by extensive positive p53 staining within renal epithelial cell nuclei, indicating cellular stress and potential fibrotic progression in liver. However, Silymarin and APE treatments effectively diminished p53 expression, highlighting their protective role against fibrosis (Fig 3, P53 × 100).

The antioxidative capacity of Silymarin was further corroborated through its impact on anti-apoptotic protein expressions, including BCL-2 and p53, alongside its ability to elevate hepatic glutathione levels, reducing hepatotoxin binding and restoring antioxidant balance within the liver, as reported by Vivekanandan *et al.* (2018). p53's pivotal role in apoptosis regulation and its involvement in the mitochondrial pathway of apoptosis elucidate its tumor-suppressive function.

Propolis extract, through its antioxidant and anti-inflammatory properties, demonstrated significant hepatorenal protective effects. Its capacity to scavenge free radicals, inhibit lipid peroxidation and enhance intracellular antioxidant defenses, including glutathione and superoxide dismutase, underscores the comprehensive protective mechanisms propolis offers against oxidative stress-induced hepatorenal damage. Furthermore, propolis's influence on hepatic enzyme activity and its anti-inflammatory action, as detailed by Nakamura *et al.* (2013).

CONCLUSION

In summary, this study conducted in a murine model of hepatorenal toxicity induced by CCl4 highlights that CCl4 administration resulted in hepatorenal toxicity, leading to impaired liver and kidney function. This manifested as increased damage to both liver and kidney tissues. Importantly, the administration of propolis extract exhibited a notable capacity to mitigate the toxic effects of CCl4, as evidenced by improvements observed at both histological and physiological levels. Therefore, the findings of this study underscore the potential of propolis extract as a promising therapeutic agent for addressing hepatorenal toxicity, emphasizing the significance of these results.

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Conflict of interest

The author declared that I have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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