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Pleurospermum candollei Methanolic Extract Ameliorates CCl_4-Induced Liver Injury by Modulating Oxidative Stress, Inflammatory, and Apoptotic Markers in Rats

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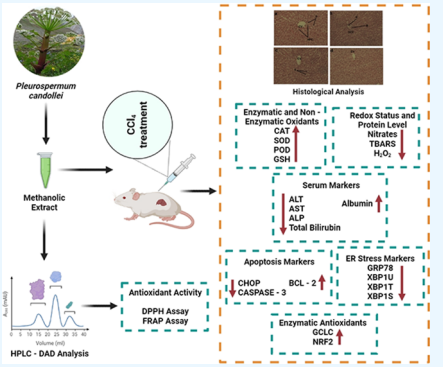
ABSTRACT: The main objective of this study was to investigate the hepatoprotective potency of the *Pleurospermum candollei* methanol extract against CCl_4-induced liver damage in rats. HPLC technique was used to estimate the presence of polyphenols in the methanol extract of *P. candollei* (PCM), while proximate analysis revealed the presence of carbohydrates, lipids, and moisture in the extract. The antioxidant potential of PCM was evaluated by 2,2-diphenylpicrylhydrazyl (DPPH) and reducing power assay, which showed a high percentage of inhibition against free radicals. Hepatotoxicity was induced by carbon tetrachloride (CCl_4). CCl_4 administration reduced the activity of endogenous antioxidants, whereas it increased the production of nitrites and hydrogen peroxide (H_2O_2) in rats. Furthermore, the level of hepatic markers in serum was also elevated after CCl_4 administration. Moreover, the expression of stress-related markers, proinflammatory mediators, and apoptotic genes was enhanced in CCl_4-treated rats. Coadministration of PCM along with CCl_4 in rats reduced the levels of free radicals and the above genes to normal levels. CCl_4 administration caused histopathological alterations in liver tissues, while cotreatment with PCM mitigated liver injuries. These findings suggest that the methanol extract of *P. candollei* possesses antioxidant and anti-inflammatory properties and can prevent liver injury. Further pharmacological research will be helpful in determining the effectiveness of *P. candollei* in humans. Development of FDA-approved plant-based anti-inflammatory drugs can help treat patients and reduce the chances of toxicity.



1. INTRODUCTION

Liver is involved in the metabolism of fatty acids and nutrients; synthesis of proteins; sugar storage; detoxification of chemicals, drugs, and xenobiotics; and other physiological functions.[1] Liver is susceptible to injury caused by various factors, such as drugs, viruses, xenobiotics, alcohol, and nutritional supplements, through various pathways triggered by toxic metabolites. These toxic substances and drugs are responsible for almost 50% of acute liver damages. It is well recognized that the combination of mechanisms including apoptosis, oxidative stress, and inflammation plays a role in the development of acute liver injury.[2,3] During aerobic respiration in the mitochondrial membrane through the electron transport chain, reactive oxygen species (ROS) are constantly generated as byproducts. Liver is very rich in mitochondria and has a high rate of oxygen consumption during metabolic processes, which increases its exposure to ROS.[4]

Various xenobiotics including vinyl chloride, arsenic, and carbon tetrachloride (CCl_4) have been recognized as hepatotoxins.[5,6] ROS and toxic metabolites may damage immune-mediated membrane or may cause disruption in intracellular processes to cause cell injury in the hepatocytes of both human and rats.[7] A cytochrome P-450 enzymatic system, such as cytochrome P-450 2E1 (CYP2E1), generates highly reactive species including the trichloromethyl radical (CCl_3) through the metabolic activation of CCl_4 in hepatocytes. This reactive intermediate interacts with molecular oxygen (O_2) to produce a highly reactive species, trichloromethyl peroxyl radical (CCl3OO*). This radical covalently binds to biological molecules, leading to the degradation of proteins and lipids, oxidative stress, cellular damage, apoptosis, and necrosis of hepatocytes.[8]

Natural antioxidants (e.g., vitamin C, α-tocopherol, quercetin, curcumin, phenolics, flavonoid, and tannin) have been studied for their potency against liver diseases,[9−11] and certain plant-derived antioxidants have shown promising

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Table 1. Signal Wavelength, Regression Analysis, and Retention Time of Reference Polyphenolics for the *P. candollei* Methanol Extract

reference polyphenolics	signal wavelength	retention time (min)	regression analysis	R^2	concentration (μg/mg) of extracts
vanillic acid	257	8.367	$y = 7.311x + 19.83$	0.9959	2.23
rutin	257	12.413	$y = 8.103x - 25.12$	0.9812	1.42
gallic acid	279	3.538	$y = 16.712x + 34.12$	0.9926	0.94
catechin	279	6.489	$y = 22.003x + 7.364$	0.9915	8.07
coumaric acid	279	13.567	$y = 5.933x + 38.183$	0.9978	0.4
emodin	279	27.289	$y = 8.384x + 19.32$	0.9829	12.12
caffeic acid	325	8.368	$y = 11.343x + 21.45$	0.9899	1.22
cinnamic acid	325	12.454	$y = 13.317x + 36.71$	0.9845	2.31
apigenin	325	20.607	$y = 17.221x + 56.38$	0.9993	0.40
myricetin	368	15.143	$y = 8.673x + 42.01$	0.9918	1.18
kaempferol	368	21.549	$y = 4.991x + 19.21$	0.9849	2.82

results in treating liver disorders in animal models.[12] Nuclear factor kappa-B (NF-κB) regulates inflammatory responses, and the inhibition of the NF-κB pathway reduces the inflammation and severity of liver injury. CCl_4 activates the NF-κB pathway, and this activation results in an increased expression of NF-κB p65 protein in liver, which in turns triggers the production and overexpression of inflammatory mediators and proinflammatory cytokines (such as tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), and interleukin-6 (IL-6)), leading to chronic inflammation. Therefore, several natural compounds have been investigated for their potential to target inflammation and oxidative stress as it can be an effective therapeutic approach for CCl4-induced liver injury.[2]

Pleurospermum candollei (DC) CB Clarke belongs to the family Apiaceae, commonly known as shabdun in the Himalayan and Karakoram zones. The herb is commercially available in this region and used as vegetable and for different ailments.[13] The whole plant is beneficial for the treatment of stomach problems and abdominal issues. It also helps to reduce the cholesterol level and blood pressure in the body and shows a protective response to heart problems. Moreover, the stem powder of the plant is used to cure joint and back pain in Gilgit-Baltistan.[14,15] *P. candollei* was found effective against unconsciousness and respiratory and cerebral disorders.[16] The powder of this plant is also locally used to cure headache and fever.[17] It is also used for the treatment of diarrhea in animals. A previous study has reported the isolation of bioactive compounds, which have anti-inflammatory activities, from *P. candollei*. The medicinal and pharmaceutical values of Pleurospermum genus is highly appreciated which is due to the presence of natural antioxidants and their biological properties.[18,19] *P. candollei*, despite its traditional use against health issues, has not been thoroughly investigated for its biological and pharmacological activities. Hence, the current study was planned to explore the antioxidant and anti-inflammatory potential of plants against stress, inflammatory, apoptotic, and fibrotic markers for pharmaceutical applications.

2. RESULTS

2.1. HPLC profile of *P. candollei*. Regression analysis, quantification, and retention time of standard polyphenolics for *P. candollei* are depicted in Table 1, while the HPLC chromatogram of *P. candollei* is illustrated in Figure 1. The HPLC-DAD profile indicated that the highest concentrations of emodin and catechin were found, followed by kaempferol, cinnamic acid, and catechin. Rutin, gallic acid, coumaric acid, caffeic acid, apigenin, and myricetin were the least abundant compounds.

2.2. Chemical Profile of *P. candollei*. A 164 g portion of methanolic extract was obtained from 1000 g of the powdered weight of *P. candollei* (16.4% w/w). The proximate analysis of the plant extract revealed a high percentage of carbohydrate (30.05%) and crude fiber (22.71%). Moreover, significant amounts of protein (8.71%), lipids (4.11%), ash content (13.28%), and moisture (18.43%) were also present in the extract (Table 2).

2.3. Quantitative Analysis of *P. candollei*. The quantitative phytochemical analysis of the *P. candollei* methanol extract revealed the presence of phenols and condensed tannins as 72.72 ± 0.73 mg GAE/g and 34.65 ± 0.59 mg CE/g, respectively.

2.4. Antioxidant Activities. The antioxidant activity of the *P. candollei* methanol extract was examined using DPPH and FRAP assays (Figure 2). Data indicated that the increased concentration of the plant showed a higher inhibition ability of the extract against free radicals. The DPPH assay showed an IC_{50} value for PCM (60.75 ± 2.01 μg/mg) which was significantly higher than the IC_{50} value of ascorbic acid (20.12 ± 0.48 μg/mg), while the FRAP assay showed an IC_{50} value for PCM (114.34 ± 1.16 μg/mg) against ascorbic acid (62.92 ± 0.93 μg/mg).

2.5. Acute Toxicity Assay. The clinical examination and observation of rats showed that the *P. candollei* methanol extract was nontoxic, and signs of stress or behavioral changes were not found in rats. The hematological study of rats showed significant ($p < 0.05$) changes in the count of platelets (PLT) and white blood cells (WBCs) at 2000 mg/kg bw and 3000 mg/kg bw (Table 3). In contrast, lymphocytes (LYM) and red blood cells (RBCs) did not show significant changes among groups. Furthermore, PCM significantly ($p < 0.05$) increased the concentration of neutrophils, hemoglobin, mean corpuscular volume (MCV), hemoglobin (HGB), and corpuscular hemoglobin concentration (MCHC) at 2000 and 3000 mg/kg.

2.6. Effect of *P. candollei* on the Body and Liver Weight. A notable ($p < 0.05$) reduction in the body weight of rats was observed after CCl_4 treatment, while a remarkable ($p < 0.05$) increase in the absolute weight of liver was noted (Table 4). Administration of PCM (300 and 600 mg/kg) along with CCl_4 reduced the toxic effects of CCl_4 and significantly ($p < 0.05$) increased the body weight of rats. Furthermore, administration of PCM (300 and 600 mg/kg) in CCl_4-intoxicated rats also reduced the absolute liver weight of the respective rats. However, administration of PCM (150 mg/

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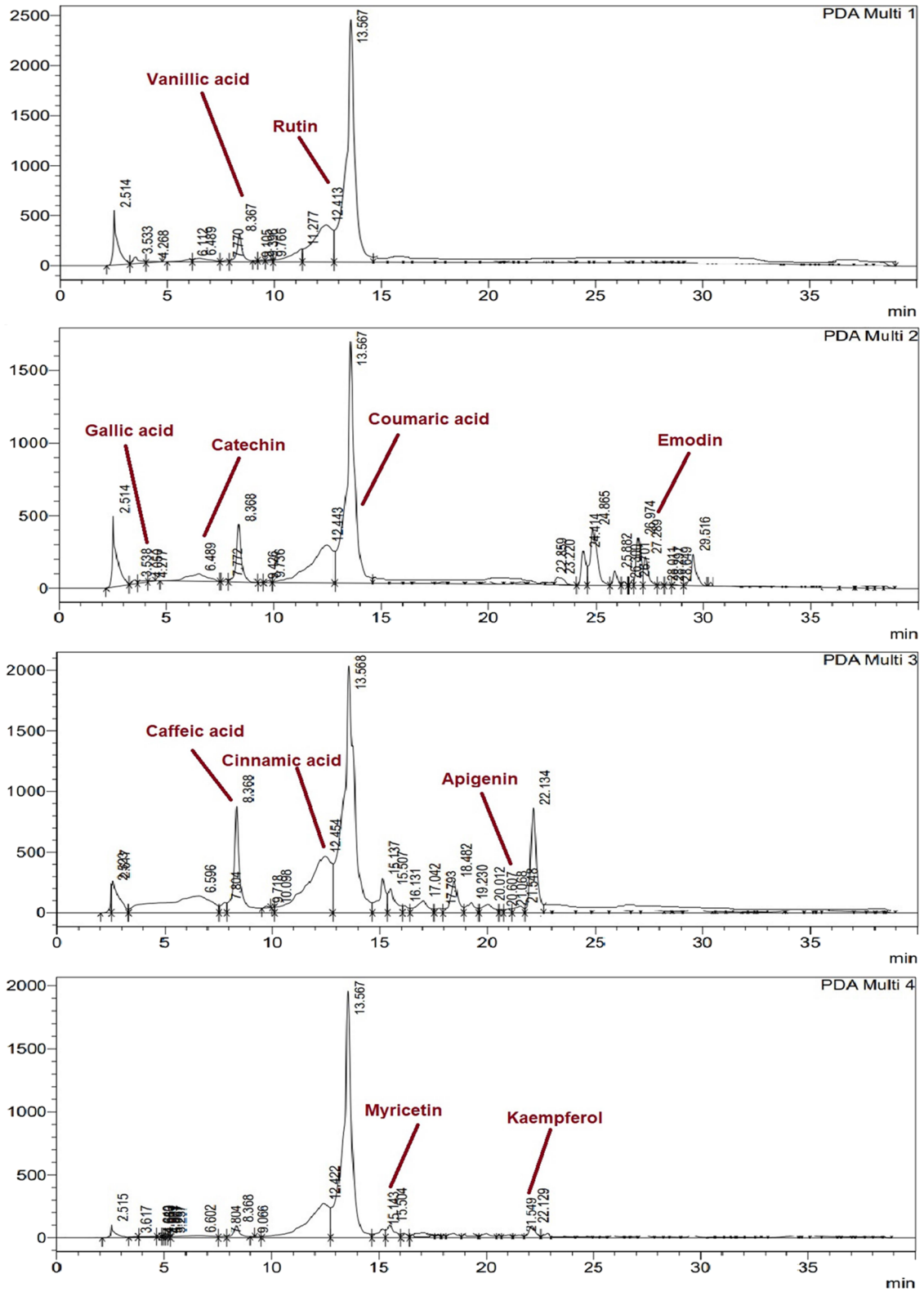

Figure 1. HPLC-DAD analysis of the *P. candollei* methanol extract at signal 1:257 λ, signal 2:279 λ, signal 3:325 λ, and signal 4:368 λ.

kg) along with CCl_4 did not show significant change in the body and absolute liver weight. Indeed, the protective effect of PCM (600 mg/kg) was comparable to that of the silymarin-treated group.

2.7. Effect of *P. candollei* on Serum Markers. CCl_4 treatment significantly elevated the concentration of liver serum markers such as ALT, AST, ALP, and total bilirubin compared to the control rats (Table 5). However, a notable decrease was obvious in the levels of albumin after CCl_4 treatment. On the other hand, rats administered with *P. candollei* (150, 300, and 600 mg/kg) along with CCl_4 showed a significantly less pronounced elevation in the levels of serum

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Table 2. Proximate Analysis of the *P. candollei* Methanol Extract[a]

	P. candollei methanol extract (PCM)
moisture (%)	18.43 ± 0.42
ash content (%)	13.28 ± 0.28
crude protein (%)	8.71 ± 0.08
lipids (%)	4.11 ± 0.10
crude fiber (%)	22.71 ± 0.33
carbohydrates (%)	30.05 ± 0.36

[a]Values are represented as mean ± SEM.

function markers, while increased levels of albumin were recorded. The hepatoprotective effect of *P. candollei* (300 and 600 mg/kg) was found to be similar to that of silymarin. However, treatment with PCM (150, 300, and 600 mg/kg) alone showed no significant ($P > 0.05$) changes in the level of ALT, AST, ALP, albumin, and bilirubin as compared to the control group.

2.8. Effect of *P. candollei* on Enzymatic and Non-enzymatic Antioxidants. To assess the antioxidant effect of *P. candollei*, enzymatic and nonenzymatic antioxidant molecules (CAT, SOD, POD, and GSH) were examined. CCl_4

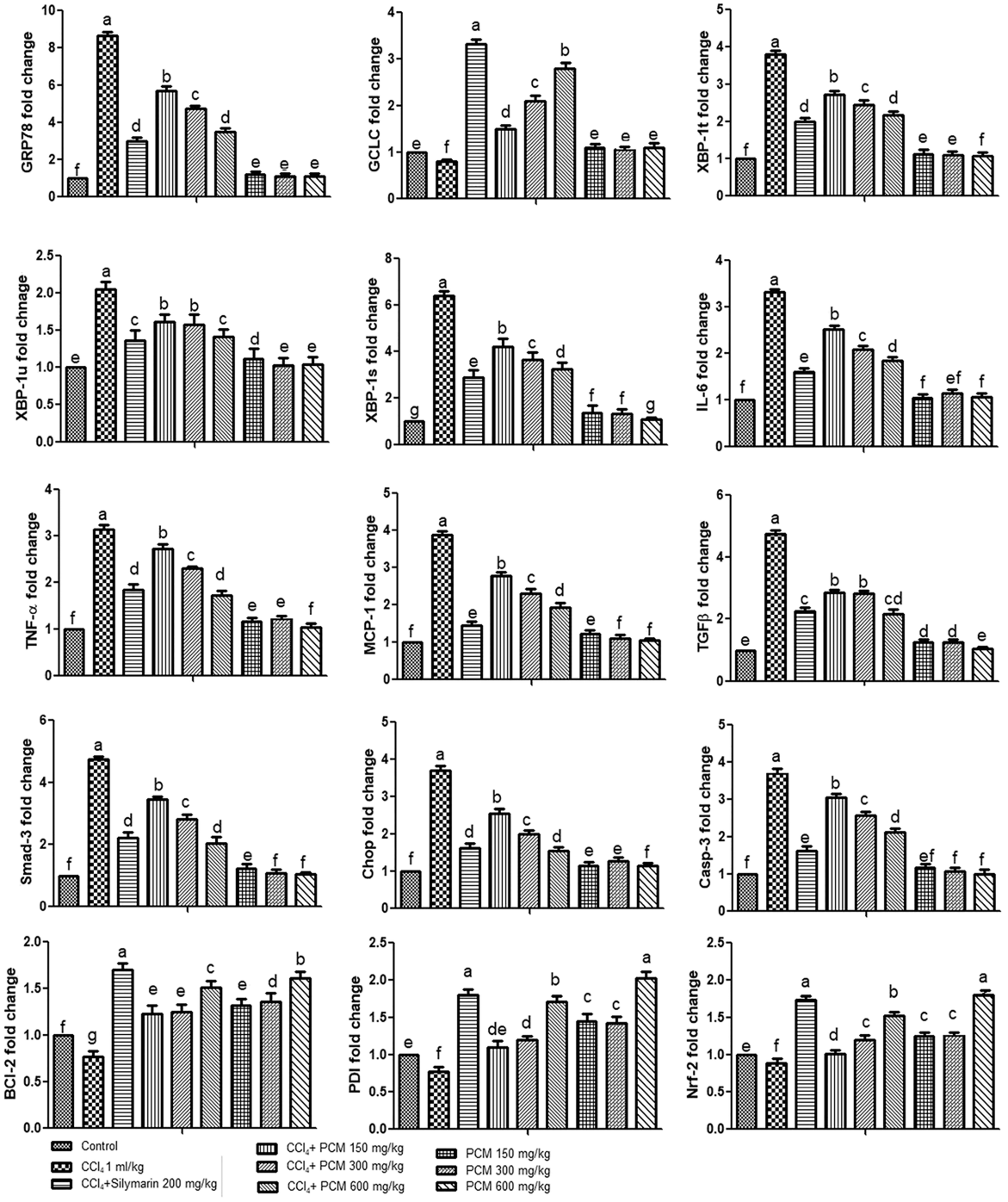

Figure 2. Graphical representation of treatment effects of *P. candollei* on the fold change of genes involved in ER stress, inflammation, apoptosis, and fibrosis. The superscripts (a−g) specify significance at $p < 0.05$.

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Table 3. Treatment Effects of *P. candollei* on the Hematological Profile[a]

	WBC (10^3/mm^3)	RBC (10^6/mm^3)	neutrophil (10^3/mm^3)	HGB (g/dL)	MCV (fl)	PLT (10^3/mL)	LYM (10^3/mm^3)	MCHC (g/dL)
control	9.26 ± 0.12^c	7.01 ± 0.11^a	15.34 ± 0.17^c	11.13 ± 0.13^c	62.81 ± 0.34^c	221.46 ± 1.26^c	3.94 ± 0.11^a	34.17 ± 0.29^b
PCM (2000 mg/kg bw)	11.72 ± 0.09^b	6.91 ± 0.07^a	18.92 ± 0.15^a	12.86 ± 0.11^b	59.67 ± 0.25^b	261.61 ± 1.10^b	4.36 ± 0.13^a	31.74 ± 0.22^c
PCM (3000 mg/kg bw)	12.84 ± 0.17^a	7.23 ± 0.09^a	20.04 ± 0.25^b	13.74 ± 0.14^a	70.10 ± 0.23^a	283.22 ± 1.55^a	4.26 ± 0.10^a	39.29 ± 0.29^a

[a]Mean ± SEM (n = 6). Means having different superscripts show significance at $p < 0.05$. PCM, *Pleurospermum candollei* methanol extract.

Table 4. Treatment Effects of *P. candollei* on the Body and Organ Weight[a]

groups	initial body weight (g)	final body weight (g)	% increase	absolute liver weight (g)	relative liver weight (mg/g)
control	130 ± 0.53	213 ± 0.65	63.84 ± 2.3^a	7.42 ± 0.31^f	34.83 ± 1.21^e
CCl_4 (1 mL/kg)	136 ± 0.65	174 ± 0.86	27.94 ± 1.8^f	9.31 ± 0.42^a	53.51 ± 1.10^a
CCl_4 + silymarin (200 mg/kg)	134 ± 0.49	208 ± 0.36	55.22 ± 0.65^b	7.81 ± 0.08^d	37.54 ± 0.33^d
CCl_4 + PCM (150 mg/kg)	133 ± 0.53	182 ± 0.53	36.84 ± 0.57^e	8.81 ± 0.15^b	48.41 ± 0.39^b
CCl_4 + PCM (300 mg/kg)	140 ± 0.61	201 ± 0.82	43.57 ± 0.90^d	8.82 ± 0.07^b	43.88 ± 0.21^c
CCl_4 + PCM (600 mg/kg)	138 ± 0.57	214 ± 0.32	55.07 ± 1.02^b	8.24 ± 0.11^c	38.50 ± 0.19^d
PCM (150 mg/kg)	143 ± 0.73	219 ± 0.77	53.14 ± 0.82^c	7.91 ± 0.06^d	36.11 ± 0.26^d
PCM (300 mg/kg)	137 ± 0.61	216 ± 0.45	57.66 ± 0.53^b	7.76 ± 0.14^e	35.93 ± 0.24^{d,e}
PCM (600 mg/kg)	144 ± 0.73	231 ± 0.82	60.41 ± 0.78^{a,b}	7.88 ± 0.11^d	34.11 ± 0.30^e

[a]Mean ± SEM (n = 6). The superscripts (a–f) specify significance at $p < 0.05$. PCM, *Pleurospermum candollei* methanol extract; CCl_4, carbon tetrachloride.

Table 5. Treatment Effects of *P. candollei* on Liver Serum Markers[a]

group	ALT (U/L)	ALP (U/L)	AST (U/L)	albumin (mg/dL)	bilirubin (mg/dL)
control	46.11 ± 0.75^f	62.19 ± 1.11^g	53.05 ± 0.32^g	4.17 ± 0.06^a	0.51 ± 0.02^g
CCl_4 (1 mL/kg)	151.34 ± 0.83^a	142.30 ± 0.95^a	158.41 ± 0.49^a	1.63 ± 0.04^g	1.73 ± 0.03^a
CCl_4 + silymarin (200 mg/kg)	56.71 ± 0.43^d	70.39 ± 0.70^{e,f}	66.39 ± 0.54^e	3.82 ± 0.08^c	0.65 ± 0.04^e
CCl_4 + PCM (150 mg/kg)	115.51 ± 0.59^b	129.75 ± 0.79^b	134.65 ± 0.50^b	1.92 ± 0.07^f	1.42 ± 0.04^b
CCl_4 + PCM (300 mg/kg)	87.47 ± 0.57^c	101.43 ± 0.84^c	103.90 ± 0.68^c	2.67 ± 0.04^e	1.05 ± 0.02^c
CCl_4 + PCM (600 mg/kg)	74.23 ± 0.68^{c,d}	81.37 ± 0.67^d	83.81 ± 0.60^d	3.59 ± 0.05^d	0.72 ± 0.03^d
PCM (150 mg/kg)	53.18 ± 0.56^d	72.18 ± 0.44^e	62.93 ± 0.38^f	3.89 ± 0.03^c	0.62 ± 0.02^e
PCM (300 mg/kg)	48.85 ± 0.86^e	68.26 ± 0.67^f	59.04 ± 0.15^f	3.96 ± 0.41^b	0.59 ± 0.01^f
PCM (600 mg/kg)	46.39 ± 0.60^e	63.18 ± 0.59^g	55.21 ± 0.47^g	4.11 ± 0.07^a	0.52 ± 0.02^g

[a]Mean ± SEM (n = 6). The superscripts (a–g) specify significance at $p < 0.05$. PCM, *Pleurospermum candollei* methanol extract; CCl_4, carbon tetrachloride.

Table 6. Treatment Effects of *P. candollei* on Liver Tissue Antioxidants[a]

groups	CAT (U/min)	POD (U/min)	SOD (U/mg protein)	GSH (μmol/mg)
control	11.90 ± 0.33^a	9.83 ± 0.20^a	6.78 ± 0.26^a	15.57 ± 0.35^a
CCl_4 (1 mL/kg)	2.18 ± 0.13^g	3.01 ± 0.22^g	1.79 ± 0.11^e	4.65 ± 0.34^g
CCl_4 + silymarin (200 mg/kg)	10.17 ± 0.38^c	8.94 ± 0.34^d	5.92 ± 0.13^b	12.43 ± 0.24^{b,c}
CCl_4 + PCM (150 mg/kg)	4.39 ± 0.22^f	5.04 ± 0.25^f	2.40 ± 0.07^d	6.98 ± 0.44^f
CCl_4 + PCM (300 mg/kg)	6.61 ± 0.29^e	6.73 ± 0.15^e	3.66 ± 0.15^c	9.44 ± 0.26^e
CCl_4 + PCM (600 mg/kg)	9.83 ± 0.40^d	8.84 ± 0.28^d	5.80 ± 0.17^b	11.75 ± 0.32^d
PCM (150 mg/kg)	10.47 ± 0.35^{b,c}	9.07 ± 0.16^c	6.03 ± 0.04^{a,b}	13.71 ± 0.30^c
PCM (300 mg/kg)	10.91 ± 0.26$_b$	9.41 ± 0.19^{b,c}	6.36 ± 0.23^a	14.43 ± 0.39^b
PCM (600 mg/kg)	11.53 ± 0.36^a	9.86 ± 0.21^a	6.61 ± 0.19^a	15.40 ± 0.34^a

[a]Mean ± SEM (n = 6). The superscripts (a–g) specify significance at $p < 0.05$. PCM, *Pleurospermum candollei* methanol extract; CCl4, carbon tetrachloride.

treatment significantly ($p < 0.05$) inhibited the activities of antioxidant molecules such as CAT, SOD, POD, and GSH. However, *P. candollei* treatment significantly reversed the toxicity caused by CCl_4 and elevated the levels of antioxidants in the liver (Table 6). Silymarin administration along with CCl_4 also showed protective effects by increasing the activity levels of these antioxidants. Administration of *P. candollei* (150, 300, and 600 mg/kg) alone did not show any significant changes in antioxidants compared with normal rats.

2.9. Effect of *P. candollei* on the Redox Status and Protein Level in Liver. Administration of CCl_4 significantly ($p < 0.05$) increased the concentration of nitrites, TBARS, and H_2O_2 but reduced the total soluble protein compared to the control group, thus causing a disturbance in the redox status of hepatocytes (Table 7). Coadministration of *P. candollei* (150,

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Table 7. Treatment Effects of *P. candollei* on the Redox Status and the Protein of Liver[a]

groups	protein (μg/mg tissue)	TBARS (nM/min/mg protein)	H_2O_2 (nM/min/mg tissue)	nitrite content (μM/mL)
control	11.71 ± 0.75^{a}	51.30 ± 1.51^{g}	5.93 ± 0.21^{e}	53.37 ± 0.92^{g}
CCl_4 (1 mL/kg)	5.70 ± 0.42^{e}	113.16 ± 3.01^{a}	11.10 ± 0.48^{a}	82.05 ± 1.50^{a}
CCl_4 + silymarin (200 mg/kg)	10.97 ± 0.69^{b}	63.49 ± 2.68^{e}	6.27 ± 0.36^{d}	58.28 ± 1.03^{e}
CCl_4 + PCM (150 mg/kg)	6.77 ± 0.35^{d}	98.12 ± 2.16^{b}	8.37 ± 0.24^{b}	72.60 ± 2.14^{b}
CCl_4 + PCM (300 mg/kg)	8.34 ± 0.40^{c}	82.17 ± 2.31^{c}	7.33 ± 0.40^{c}	67.33 ± 1.82^{c}
CCl_4 + PCM (600 mg/kg)	10.83 ± 0.51^{b}	70.73 ± 1.99^{d}	6.37 ± 0.27^{d}	60.31 ± 1.17^{d}
PCM (150 mg/kg)	11.18 ± 0.37^{a,b}	61.24 ± 1.17^{e}	6.11 ± 0.15^{d,e}	56.83 ± 0.94^{f}
PCM (300 mg/kg)	11.46 ± 0.57^{a,b}	55.67 ± 1.32^{f}	5.96 ± 0.11^{e}	53.64 ± 1.06^{g}
PCM (600 mg/kg)	11.81 ± 0.64^{a}	52.18 ± 1.98^{g}	5.81 ± 0.13^{f}	53.49 ± 1.24^{g}

[a]Mean ± SD (n = 6). Means with different superscripts (a–g) specify significance at $p < 0.05$. PCM, *Pleurospermum candollei* methanol extract; CCl_4, carbon tetrachloride

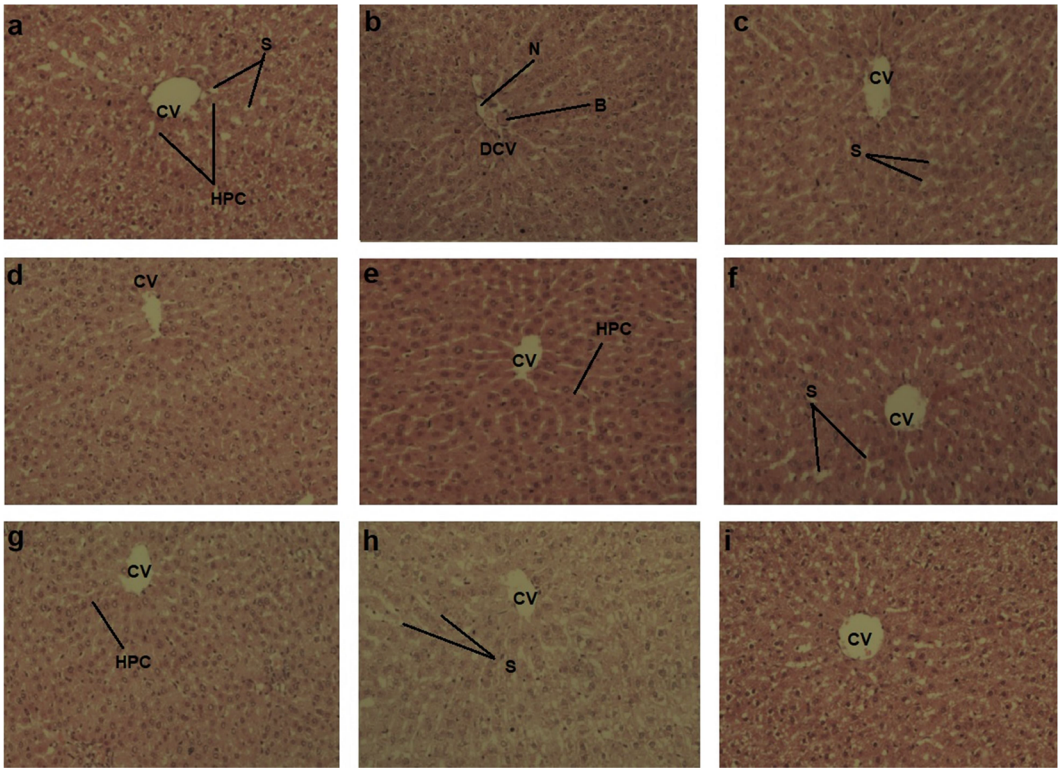

Figure 3. Protective effects of *P. candollei* treatment on the liver histopathology of rats (40× magnification with H&E stain). (a) Control group, (b) CCl_4 (1 mL/kg bw), (c) CCl_4 + silymarin (200 mg/kg), (d) CCl_4 + PCM (150 mg/kg), (e) CCl_4 + PCM (300 mg/kg), (f) CCl_4 + PCM (600 mg/kg), (g) PCM (150 mg/kg), (h) PCM (300 mg/kg), and (i) PCM (600 mg/kg). CV, central vein; DCV, damaged central vein; HPC, hepatocytes; N, necrosis; and B, ballooning of hepatocytes.

300, and 600 mg/kg) restored the levels of biochemical parameters and the total protein toward the normal rats and reduced the disturbance in the redox status. Silymarin also revealed protective effects against oxidative stress by restoring the levels of free radicals and protein in the liver homogenate.

2.10. Effect of *P. candollei* on the ER Stress Marker. The study also examined the expression of ER stress markers, namely, GRP-78, XBP-1u, XBP-1t, and XBP-1s. CCl_4 treatment significantly ($P < 0.05$) induced an increase in the mRNA expression of these markers compared to the control group (Figure 2). In contrast, administration of *P. candollei* with CCl_4 reduced the CCl_4-induced cellular detoxification by suppressing the expression of ER stress markers. Similarly, silymarin treatment restored the levels of stress markers, thus protecting the hepatocytes from CCl_4-mediated toxicity.

2.11. Effect of *P. candollei* on Inflammatory Mediators. After CCl_4 administration to rats, inflammation was initiated and propagated through an elevation in the expression of inflammatory cytokines including IL-6, TNF-α, and chemokine MCP-1. Administration of *P. candollei* or silymarin to CCl_4-treated rats, however, inhibited the overexpression of proinflammatory mediators, suggesting that *P. candollei* exhibits an anti-inflammatory effect (Figure 2). However, treatment with *P. candollei* alone revealed a nonsignificant ($P > 0.05$) change in the expression of the abovementioned markers compared to that in normal rats.

2.12. Effect of *P. candollei* on Apoptotic Markers. In CCl_4-treated rats, the mRNA expression of apoptotic markers Chop and caspase-3 was significantly ($P < 0.05$) enhanced as compared with normal rats, while that of the antiapoptotic marker, namely, BCl-2 was downregulated (Figure 2).

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However, treatment with *P. candollei* (150, 300, and 600 mg/kg) along with CCl_4 significantly enhanced the levels of these antiapoptotic genes toward normal levels, indicating the protective effect of *P. candollei* against cell death. mRNA expression of Chop and caspase-3 was inhibited by the administration of *P. candollei* to CCl_4-intoxicated rats. Furthermore, treatment of rats with *P. candollei* (300 and 600 mg/kg) alone did not illustrate significant alterations in the levels of these markers compared with that of normal rats.

2.13. Effect of *P. candollei* on Enzymatic Antioxidants. In the CCl_4-intoxicated group, the mRNA expression of antioxidant markers GCLC and Nrf-2 was significantly reduced after 4 weeks of treatment as compared with the control group (Figure 2). On the contrary, treatment with *P. candollei* (150, 300, and 600 mg/kg) along with CCl_4 significantly enhanced the levels of these antioxidant enzymes toward the normal group, indicating the protective effect of *P. candollei* on the hepatocytes. Silymarin also exhibited this protective effect, indicated by the increased levels of these enzymatic antioxidants in the relevant group. Furthermore, treatments of rats with *P. candollei* (300 and 600 mg/kg) alone also enhanced the levels of these antioxidant enzymes compared with that of normal rats.

2.14. Histopathological Findings. The histological analysis of the sample tissues of the control group and rats treated with *P. candollei* (150, 300, and 600 mg/kg) alone revealed narrow sinusoids, normal hepatic cords radiating from the central vein, and prominent nuclei. On the contrary, the liver sections of CCl_4-treated groups showed hepatocyte ballooning, infiltration of inflammatory cells, and disruption in the architecture of hepatic lobules (Figure 3). However, the rats with the coadministration of *P. candollei* (300 and 600 mg/kg) with CCl_4 displayed less severe injuries than CCl_4-intoxicated rats, with minimal apoptotic hepatocytes. Rats treated with silymarin (200 mg/kg) showed much healthier liver tissues than the CCl_4-treated rats.

3. DISCUSSION

CCl_4 belongs to the class of hepatotoxins that require metabolic activation to induce oxidative damage and hepatic injuries. When CCl_4 is metabolized by hepatocytes, it generates free radicals that can cause impairments to the hepatocyte cell membrane and organelles, such as mitochondria. Acute liver injury is related to the liver oxidative stress.[20] This oxidative stress leads to peroxidation, which induces detrimental effects on liver, including inflammation (leukocyte infiltration), central vasodilation, hepatocellular fibrosis and necrosis, vascular degeneration, bile duct proliferation, vascular occlusion, cellular hypertrophy, and increased collage depositions.[21−23] The mechanism of hepatotoxicity due to oxidative stress involves the imbalance of oxidation and antioxidant systems; thus, liver exposure to CCl_4 increases the production of ROS and reduces the antioxidant capacity.[24] The current study indicated that treatment with CCl_4 on alternate days for a period of 4 weeks led to severe hepatic injury and inflammation in rats. The findings of this study are similar to those of previous studies.[25]

Natural products, which are derived from plants, animals, and microorganisms, have been studied for their potential to protect the liver from oxidative stress, reduce inflammation, and act as novel agents against injuries. Natural medicinal plants are valuable sources of bioactive compounds for the development of natural medicines such as vitexin, salvianolic acid, and polysaccharides, and Dendrobium nobile Lindl. alkaloids (DNLA) have been studied for their hepatoprotective effects.[2] The plants of Apiaceae family and Pleurospermum genus have been recognized for their medicinal, nutraceutical, cosmeceutical, and traditional uses; furthermore, antioxidants found in these plants have the potential to prevent and treat diseases.[18,19] *P. candollei* has been found to be used as a traditional medicine to treat various diseases; its antioxidant property and biological potential have not been thoroughly explored.[26] Therefore, this study was designed to investigate the phytochemical profile through HPLC, antioxidant activities, anti-inflammatory activities in biological systems, and the effect on stress, fibrosis, and inflammatory markers through molecular studies to unfold the pharmaceutical applications of *P. candollei*.

Plants from the Apiaceae family are commonly used in everyday nutrition, particularly as spices. These plants are rich sources of dietary supplements due to their low calorie content and high levels of protein, fixed oils, fibers, carbohydrates, and essential oils. Additionally, seeds of Apiaceae family contain a variety of water-soluble glycosides, aromatic compounds, phospholipids, flavonoids, terpenoids, sugars, lactones, quinones, and carotenoids.[27] In our study, the proximate analysis of the methanol extract of *P. candollei* revealed significant levels of proteins, carbohydrates, fibers, lipids, and moisture. Thus, this plant can also be a rich source of active compounds and nutrients.

Phenolic compounds have a functional hydroxy group attached to an aromatic ring that acts as an electron donor. This functional group transfers electrons to a free radical, neutralizing its activity and preventing it from damaging proteins and DNA. The presence of phenolic compounds in natural products is important because they can help to reduce the risk of various diseases associated with oxidative stress. The HPLC-DAD analysis confirmed high amounts of flavonoids and phenols in the methanol extract of *P. candollei*. Same results were reported by Al-Dalahmeh et al.[28] Reference polyphenols were selected on the basis of their medicinal properties; for example, rutin possesses antioxidant, antiviral, and hepatoprotective effects,[29] while gallic acid, caffeic acid, and catechin have antioxidant and anticancer properties.[30,31] Myricetin is famous for its antioxidant and chelation properties against free radicals.[32] Apigenin is famous for having antioxidant, antiviral, anti-inflammatory, and anticancer properties.[33] Therefore, the presence of gallic acid, rutin, apigenin, myricetin, and catechin in *P. candollei* accounts for its antioxidant, antiapoptotic, anti-inflammatory, and protective properties. The presence of phytochemicals such as phenolics and vanillin in plant extracts is associated with their high antioxidant activities. Studies have found that plant extracts with high TPC and TFC levels have the potency to quench free radicals including DPPH and exhibit reducing power activities.[34] Moreover, Apiaceae extracts typically contain polyphenolic compounds, including flavonoids, tannins, and phenolic acids. These compounds have been shown to possess anti-inflammatory properties, which is shown by the inhibition of the production or action of proinflammatory mediators. As a result, they can be considered as a potential alternative to anti-inflammatory drugs. Essential oils extracted from these plants show stronger antioxidant activities due to the presence of high content of flavonoids and phenols.[27] In this study, TPC and HPLC-DAD analysis of *P. candollei* revealed the presence of polyphenols in the *P. candollei* extract, which provides a

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rationale for the anti-inflammatory and antioxidant properties of the *P. candollei* methanol extract.

Any injury to liver cells can lead to a process called degeneration, where cells lose their normal structure and begin to breakdown. ALT, ALP, and AST are found inside the cells, but when liver cells undergo degeneration, these enzymes leak out of the cells and enter into the blood. Therefore, the levels of ALT, ALP, and AST in the serum can be used to evaluate the liver injury. In the current study, elevated levels of these enzymes and bilirubin in the serum after CCl_4 administration is a sign of liver disease. Same results were found in the previous research of Unsal et al.[35] However, our study confirmed that the *P. candollei* treatment resulted in the reduction in the level of these enzymes and bilirubin in serum. These findings suggested that the high polyphenolic content and antioxidant properties of *P. candollei* may be responsible for its significant protective effects against hepatic damage.

Antioxidant supplementation is an attractive strategy to reduce the risk of oxidative stress and various diseases induced by free radicals. Enzymatic defense systems of the body protect cells from degeneration and damage. SOD transfers highly reactive superoxide radicals into less reactive H_2O_2, while CAT and GSH neutralize H_2O_2 to protect the liver from ROS. POD also helps to maintain the hemostasis of cells by breaking down H_2O_2. In our study, CCl_4 exposure decreased the activity of CAT, SOD, and POD in the hepatocytes of rats, indicating a decline in the activity of the defense system, which is in agreement with the previous studies.[36−38] On the other hand, treatment of rats with the *P. candollei* methanol extract increased the levels of antioxidant enzymatic molecules, especially at a high dose of 600 mg/kg bw, suggesting that *P. candollei* has the potential to protect the liver against oxidative stress by degrading the free radicals generated by CCl_4.

CCl_4 injection induced oxidative stress, which was validated by the increase in the concentration of lipid peroxidation, H_2O_2, and nitric oxide, additionally, with a reduction in GSH and total protein levels in the liver. Increased production of nitrites after CCl_4 treatment occurs through the activation of the inducible nitric oxide synthase (iNOS) enzyme. Depletion of GSH levels is associated with the weakened antioxidant defense system of the body, while the reduction in total protein levels is likely due to the increased production of ROS, which inhibits protein synthesis and causes damage to the protein.[39] In our current study, silymarin and *P. candollei* treatment mitigated the oxidative stress by inhibiting the production of ROS and normalizing the production of proteins and GSH levels. Thus, *P. candollei* can reduce oxidative stress in the liver by neutralizing the effects of ROS and activating the antioxidant system.

Studies have revealed that CCl_4 activates Kupffer cells, which upregulate proinflammatory cytokines such as TNF-α and IL-6. These upregulated proinflammatory cytokines can disrupt cellular signaling pathways, leading to tissue damage, inflammation, and apoptosis. ROS can trigger the NF-κB pathway by various mechanisms, including the degradation of the inhibitor of kappa B-alpha (IκB-α) and phosphorylation of NF-κB p65/p50 subunits, in response to the inflammatory cytokines. NF-κB stimulates the production of inflammatory cytokines, which in turn initiate inflammatory response, through a series of cascades.[2] Our study also showed that the levels of these cytokines (TNF-α, IL-6, and MCP-1) were upregulated in CCl_4 intoxicated rats, while the expression of these markers was restored in PCM-treated rats, especially at a high dose of 600 mg/kg. This finding is similar to the previous study of Naz et al.[34]

The TGF-β1/Smad signaling pathway is a key indicator of liver fibrosis. Overexpression of TGF-β1 is an attribute of damage to liver cells and liver fibrosis. TGF-β1 binds to the cell surface receptor of the Smad pathway and activates the Smad protein, which leads to cell apoptosis. In our current study, CCl_4 treatment increased the expression of TGF-β1 and Smad in liver tissues. Our results are consistent with that of previous study which also reported the overexpression of these fibrosis markers during liver injury.[40] Inhibition of TGF-β1 expression can prevent liver fibrosis and can be employed as a therapeutic strategy. *P. candollei* treatment significantly downregulated the mRNA expression of these TGF-β1 and Smad.

Apoptosis is regulated by the activation of caspases, a family of cysteine proteases that cleave various proteins within the cells. One of the key regulator of apoptosis is BCl-2, an antiapoptotic protein, which inhibits the proapoptotic protein BAX.[41] The disturbance in balance between BCl-2 and BAX leads to various diseases, such as cancer. Our current study revealed that CCl_4 treatment downregulated the expression of BCl-2 in comparison to the control group but upregulated the mRNA expression of caspase-3. Our studies are consistent with that of previous studies, reporting reduction in the activity of BCl-2 and elevation in the levels of caspase-3 as an indication of liver cell apoptosis.[38] On the contrary, *P. candollei* showed antiapoptotic effects against CCl_4 by restoring the normal levels of caspase-3 and BCl-2 in the liver of rats.

The Nrf-2 signaling pathway helps to modulate cellular redox homeostasis by activating the antioxidant genes. Numerous natural products, including phytochemicals, have the potency to activate the Nrf-2 pathway, thus providing protective effects. However, PDI is involved in the normal folding of newly synthesized proteins and preventing them from aggregation, thus regulating the ER stress response. Additionally, GCLC, an antioxidant enzyme regulator, plays a crucial role in the biosynthesis of the glutathione enzyme. Elevated expression of GCLC in cells is associated with increased glutathione levels in response to oxidative stress. In our current results, lower expression levels of Nrf2, PDI, and GCLC were found in the liver homogenate after CCl_4 treatment, indicating liver injuries, ER stress, and hepatotoxicity. Our results are similar to those of previous studies.[34,39] However, significant increases in the expression levels of these antioxidant markers were found after *P. candollei* administration. Furthermore, prior to clinical trials and dose selection for humans, the determination of safety and toxicity profile of extract in humans is mandatory. An equivalent human dose of extract can be calculated by the body surface area (BSA) scaling method. The following equation is used for this calculation:

$$\text{Human dose} = \text{Animal dose} \times (\text{Animal weight}/3)^{0.33} \times (\text{HumanBSA}/\text{AnimalBSA})$$

The value of 3.0 is the average weight of rats in toxicology studies. Once the equivalent human dose is calculated, it can be compared to typical human doses used in clinical studies or the estimated safe levels of exposure.[42]

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4. CONCLUSIONS

The current study has found that *P. candollei* exhibits strong antioxidant, anti-inflammatory, and antiapoptotic properties. The findings illustrated the inhibition of ROS, which may be attributed to the presence of polyphenols in *P. candollei*. The results also revealed that natural bioactive compounds provide amelioration effects against inflammation and fibrosis. Our results confirmed that this plant can restore the antioxidant enzymes of the biological system and can also normalize the mRNA expression of ER stress markers, apoptotic mediators, and inflammatory and fibrosis genes. Thus, *P. candollei* could be a promising anti-inflammatory and antifibrotic drug for the treatment of liver diseases.

5. MATERIALS AND METHODS

5.1. Plant Collection. The collection of aerial parts of *P. candollei* was carried out from the region of Skardu, Gilgit-Baltistan. The collected plant was identified by Dr. Mushtaq Ahmed. A voucher specimen no. 132015 was deposited at Pakistan Herbarium, Quaid-i-Azam University, Pakistan.

Table 8. Primer Sequences for Real-Time PCR

gene		primer sequence (5′−3′)	product size (bp)
GRP-78	F	GTAGCATATGGTGCCGCTGT	103
	R	GAGCAGGAGGGATTCCAGTC	
XBP-1s	F	CATGGATTCTGACGCTGTTG	110
	R	CTCTGGGAAGGACATTTGA	
XBP-1u	F	TGAAGCGCTGCGGAGGACA	114
	R	AGCTGGAGTTTCTGGTTCT	
XBP-1t	F	TGTCACCTCCCCAGAACATC	103
	R	ACAGGGTCCAACTTGTCCAG	
MCP-1	F	TGTTCACAGTTGCTGCCTGT	141
	R	CGACTCATTGGGATCATCT	
IL-6	F	GCCTGCAGAGAGATTCAATCA	140
	R	GTATCAGTGGGGGTCAGCAG	
TNF-α	F	GTCTGTGCCTCAGCCTCTTC	122
	R	GCCATGGAACTGATGAGAG	
TGF-ß	F	GCCTGCAGAGATTCAAGTCA	109
	R	GTATCAGTGGGGGTCAGCAG	
Smad-3	F	CCTCCTGGCTACCTGAGTGA	118
	R	GTTATTGTGTGCTGGGGACA	
Nrf-2	F	TCCAGACAGACACCAGTGGA	122
	R	GAATGTCTCTGCCAAAAGC	
GCLC	F	GAGAACATCAGGCTCTTTGC	106
	R	AGATGCACCTCCTTCCTCTG	
PDI	F	AGAACTCCAGGCGGTGTCT	150
	R	GCCATGGAACTGATGAGAG	
ß-actin	F	CCTCTATGCCAACACAGTGC	178
	R	CATCGTACTCCTGCTTGCTG	

5.2. Extraction. The plant material was shade dried in a nonhumid area and ground in powder by a willy mill and extracted three times with 90% methanol by refluxing for 3 h in separatory funnel. The ratio of powdered plant material to methanol was 1:5 (w/v). After filtration, solvent was allowed to evaporate in a rotatory evaporator. The *P. candollei* methanol extract (PCM) was kept under 4 °C for further studies.

5.3. HPLC Analysis. HPLC analysis of the *P. candollei* methanol extract was performed to assess flavonoids and polyphenols by using Agilent Technology-1200 Series, Germany. The HPLC column was reverse-phase Zorbex plus RSC80, with 25 mL of separation capacity and 5 μm particle size. The standards vanillic acid and rutin were eluted at 257 nm; gallic acid, emodin, catechin, and coumaric acid at 279 nm; caffeic acid, ferulic acid, cinnamic acid, and apigenin at 325 nm; and myricetin and kaempferol at 368 nm. The stock solutions of reference standards were prepared in methanol. The mobile phase was composed of acetic acid (2%; A) and acetonitrile (B). The injection volume was 10 μL while a constant flow rate of 1 mL/min was maintained. The sample solution was prepared by dissolving in HPLC-grade methanol (10 mg/mL). Calibration curves were constructed on three consecutive days by the analysis of a mixture containing each of the standard compounds, and peaks were identified against the concentration of each reference standard.

5.3.1. Recovery, Precision, Accuracy, and Limit of Detection. The accuracy of the assay was evaluated by adding standard analytes at four concentration levels (50, 100, 150, and 200 μg/mL) using the method of standard addition. Four replicates were analyzed for standards at each concentration level, and the peak area ratios of the extracted sample were compared to those of the standard solutions to calculate the percent recovery. Additionally, to assess the intraday and interday precision and accuracy, the same four concentrations of standards were spiked and analyzed. Intraday variability was evaluated by analyzing the samples within the same day, while interday precision was assessed by repeating the procedure on two nonconsecutive days. The precision was measured as the relative standard deviation (RSD %), and the accuracy was expressed as the percentage of the analyte concentration measured in each sample relative to the known amount of analyte added to the sample. The limit of detection (LOD_{approx}) was calculated by the following equation:

$$\mathrm{LODapprox} = 3(\mathrm{AS}) \times [(n-2)/(n-1)]^{1/2}$$

where n is the number of total measurements for each calibration set, and AS is the analytical sensitivity.

5.4. Proximate Analysis. The chemical profile of *P. candollei* was identified by the evaluation of the protein, moisture, fiber, carbohydrate, lipids, and ash content. The standard protocol of AOAC 1990 was followed for the analysis.[43]

5.5. Quantitative and Antioxidant Analysis of *P. candollei*. *5.5.1. Total Phenolic Content (TPC) Assay.* The plant sample (500 μL), with a concentration of 1 mg/mL, was mixed with the Folin−Ciocalteu reagent. Following this, 200 μL of 10% sodium carbonate was added to the mixture.[44] It was allowed o incubate at room temperature for 30 min, and the absorbance was measured at 765 nm. The calibration curve of gallic acid ($y = 0.012x + 0.9883$: $R^2 = 0.9272$) was used to assess the phenolic content.

5.5.2. Vanillin−HCl Assay. One milliliter of plant sample (1 mg/mL) was mixed with the same amount of vanillin solution (1% in methanol) and HCl (9 M). The mixture was allowed to stand for 20 min at 30 °C. The absorbance was measured at 500 nm.[45] The calibration curve of catechin ($y = 0.031x + 0.328$: $R^2 = 0.997$) was used to determine vanillin.

5.5.3. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay. One milliliter of plant extract (concentration, 0.1−1 mg/mL) was mixed with 1 mL of DPPH solution (0.3 mmol/L). The mixture was kept at room temperature for 10 min, and the absorbance was measured at 517 nm.[46,47] Ascorbic acid was used as a reference antioxidant. All of the experiments were run

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in triplicate. The percentage of inhibition was calculated by the following equation:

$$\%\text{decolorization} = (1 - \text{sample/control}) \times 100$$

5.5.4. Reducing Power (FRAP) Assay. A reaction mixture was prepared by mixing 500 μL of the plant extract (concentration, 0.1−1 mg/mL) with 200 μL of $FeCl_3$ (0.1%). Then, 500 μL of 0.3 mol/L solution of 2,4,6-tris(2-pyridyl)-*s*-triazine (dissolved in 0.2 mol/L sodium phosphate buffer) was added to the mixture.[48] After incubation for 30 min at 40 °C, the absorbance was measured at 700 nm. Ascorbic acid was used as a reference antioxidant.

5.6. Ethical Statement. The Ethical Committee of Quaid-i-Azam University approved the protocol of the current study. The research study was performed in accordance with the eighth edition of the NIH Guidelines for Laboratory Animal Care and Use.

5.6.1. Acute Toxicity Assay. Acute toxicity study was conducted by using a minimum dose of 50 mg/kg PCM at the earliest stage and was observed to notice any deterioration effects. This dose did not cause any signs of toxicity. Subsequently, four female rats were given 100, 500, 1000, 2000, and the maximum dose of 3000 mg/kg PCM, and the rats were examined to observe any changes in their skin, weight, fur, behavior, or respiratory system.[49] After 14 days, there were no signs of mortality, so blood was collected from the rats treated with higher doses of PCM (2000 and 3000 mg/kg), and eventually 600 mg/kg bw (1/5th), 300 mg/kg bw (1/10th), and 150 mg/kg bw (1/20th) of the highest dose were chosen to elucidate the hepatoprotective effect of PCM.

5.7. Animal Treatment. A total of 54 Sprague male rats (weighing 150 ± 4.5 g) were used for the experiment. The rats were kept under laboratory standard conditions (12 h light-dark cycle) at 25 ± 2 °C. The rats were provided with clean water and a standard diet.

5.7.1. Experimental Design. The experimental rats were exposed to a fasting period of 24 h before the commencement of the experiment. Rats were divided into nine groups (six rats in each) and treated with sample solutions on alternate days for 36 days (18 doses). Group I served as normal control and received 0.9% saline (1 mL/kg). Group II received CCl_4 intraperitoneally (i.p.) mixed with olive oil (3:7 v/v, 1 mL/kg). Group III received i.p. injection of CCl_4 and silymarin (200 mg/kg) as a reference compound. Group IV, V, and VI were administered with i.p. injection of CCl_4 and 150, 300, and 600 mg/kg of PCM, respectively. Group VII, VIII, and IX were administered with 150, 300, and 600 mg/kg of PCM alone, respectively. The rationale behind selecting the specific dose was the high pharmacological activity and nontoxicity of the extract at a higher dose. Moreover, the oral route of administration results in lower bioavailability due to the metabolism and excretion of the extract. The plant extract was dissolved in distilled water. Rats of all nine groups were weighed on the first and last days of the experiment. Forty-eight hours after the last administration of test solutions, the rats were properly anesthetized, and blood was collected in centrifuge tubes. The rats were then sacrificed, and the liver was removed, weighed, and washed with saline water. A small section was stored in formalin for histological examination, while the remaining pieces were stored in liquid nitrogen and used for biochemical and molecular studies.

5.7.2. Biochemical Parameters in Serum. Centrifugation of the collected blood was carried out at 3000 rpm for 10 min to separate the serum for the evaluation of biochemical parameters. The level of alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, and albumin in serum were assessed with an AMP diagnostic kit (Austria).

5.7.3. Preparation of Tissue Homogenate. 100 mg of liver tissue was homogenized in 50 mM potassium phosphate buffer with a glass homogenizer. The homogenate was incubated at 4 °C for 30 min, and the supernatant was collected after centrifugation at 12,000 × *g*.

5.7.4. Enzymatic Antioxidants. Enzymatic antioxidants such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were estimated according to the standard protocols described by Sun et al.[50] and Chance and Maehly,[51] respectively. CAT and POD activities were calculated as the absorbance change of 0.01 in 1 min of reaction, while all the activities were expressed as units per milligram of protein.

5.7.5. Nonenzymatic Antioxidant Molecules. Glutathione (GSH) activity of liver was determined spectrophotometrically following the protocol of Ellman.[52] The principle involved the oxidation of GSH to glutathione disulfide (GSSG) by 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) to form a yellow solution. GSH activity was expressed as μM GSH/mg protein.

5.7.6. Nitrite Assay. The Griess method was followed for the determination of the level of nitrite/nitrate in the liver homogenate.[53] 50 μL of sodium nitrite was mixed with each sample, and then 50 μL of the Griess reagent was added to the reaction mixtures. The mixture was incubated at room temperature for 10 min in the dark. Absorbance was measured at 512 nm. Using the standard curve of sodium nitrite, the nitrite concentration was calculated.

5.7.7. Hydrogen Peroxide Assay. The level of hydrogen peroxide (H_2O_2) production was assessed by the ability of horseradish peroxidase to oxidize phenol red in the presence of H_2O_2. The concentration of H_2O_2 was expressed as nM H_2O_2/min/mg tissue, and the phenol red curve was generated as a standard curve.[54]

5.7.8. Lipid Peroxidation Assay. The method of Iqbal and Wright was adapted for the assessment of lipid peroxidation in liver homogenate,[55] and the levels of thiobarbituric acid (TBARS) were expressed as nM TBARS/min/mg tissue using the molar coefficient of $1.56 \times 105\ M^{-1}\ cm^{-1}$.

5.7.9. Protein Estimation. The standard protocol of Lowry, Rosebrough was applied for the total protein estimation in the tissue homogenate.[56] The absorbance was measured at 562 nm, and a standard curve of bovine serum albumin (BSA) was plotted to assess the total protein concentration.

5.8. RNA Extraction. The total RNA from the tissue of rat was isolated using the TRIzol reagent.[57] 100 mg of tissue was homogenized by adding 1 mL of TRIzol and incubated for 5 min at room temperature. Chloroform was added to the solution, and the aqueous layer containing RNA was separated after centrifugation. Purification of isolated RNA pellets was done by the ethanol precipitation method. RNase-free DNase I was used to digest any residual genomic DNA, and 20 μL of RNase-free ultrapure water was used to dissolve each pellet. Nanodrop quantification of the samples was performed.

5.8.1. Quantitative RT-PCR Analysis. First-stand complementary DNAs (cDNAs) were reverse-transcribed using a Script cDNA synthesis kit. The reaction mixture was prepared by adding the respective primers and Power SYBR Green (Invitrogen) to the cDNA samples, and qRT-PCR reaction was carried out on an Applied Biosystems 7500 Instrument.

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The cycling conditions for amplification were as follows: 95 °C for 60 s, 40 cycles at 94 °C for 15 s, and 60 °C for 90 s. Specific primers were purchased from Eurofins Genomics LLC (Kentucky, USA). Beta-actin was used as a housekeeping gene. Each reaction was carried out in duplicate to determine ΔCT. The primer sequences for beta-actin, IL-6. TNF-α, Chop, Casp-3, BCl-2, MCP-1, TGF-β, XBP 1s, XBP1u, XBP1t, Smad-3, GCLC, GRP-78, Nrf-2, and PDI are listed in Table 8.

5.9. Histological Examination. The liver tissue was fixed in 10% formaldehyde, dehydrated in ethanol, and embedded in paraffin wax. Then, slides were prepared by the segmentation of 4−5 μm section of the tissue and stained with hematoxylin and eosin (H&E) dye. A light microscope (DIALUX 20EB) was used to examine the specimens with a 40× magnification lens, and images were captured.

5.10. Statistical Analysis. Values are presented as mean ± standard deviation, and p values <0.05 were taken to be statistically significant. One-way analysis of variance (ANOVA) was performed on the data obtained from different assays. Tukey's post hoc test was applied to differentiate various groups.

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REFERENCES

(1) Jiang, M.; Huang, C.; Wu, Q.; Su, Y.; Wang, X.; Xuan, Z.; Ge, C. Sini San ameliorates CCl4-induced liver fibrosis in mice by inhibiting AKT-mediated hepatocyte apoptosis. *J. Ethnopharm.* **2023**, *303*, No. 115965.

(2) Sun, L.; Zhang, Y.; Wen, S.; Li, Q.; Chen, R.; Lai, X.; Sun, S. Extract of Jasminum grandiflorum L. alleviates CCl4-induced liver injury by decreasing inflammation, oxidative stress and hepatic CYP2E1 expression in mice. *Biomed. Pharmacol.* **2022**, *152*, No. 113255.

(3) Long, X.; Wang, P.; Zhou, Y.; Wang, Q.; Ren, L.; Li, Q.; Zhao, X. Preventive effect of Lactobacillus plantarum HFY15 on carbon tetrachloride (CCl4)-induced acute liver injury in mice. *J. Food Sci.* **2022**, *87*, 2626−2639.

(4) Wu, Z.; Sun, L.; Chen, R.; Wen, S.; Li, Q.; Lai, X.; Sun, S. Chinese tea alleviates CCl4-induced liver injury through the NF-κBorNrf2Signaling pathway in C57BL-6J mice. *Nutrients* **2022**, *14*, 972.

(5) Devaraj, V. C.; Krishna, B. G.; Viswanatha, G. L.; Kamath, J. V.; Kumar, S. Hepatoprotective activity of Hepax-a polyherbal formulation. *Asian Pac. J. Trop. Biomed.* **2011**, *1*, 142−146.

(6) Ostapowicz, G.; Fontana, R. J.; Schidt, F. V.; Larson, A.; Davern, T. J.; Han, S. H.; Mccashland, T. M.; Shakil, A. O.; Hay, J. E.; Hynan, L. Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. *Ann. Int. Med.* **2002**, *137*, 947−954.

(7) Lee, W. M. Review article: drug-induced hepatotoxicity. *Aliment Pharm. Ther.* **1993**, *7*, 477−485.

(8) Ben Hsouna, A.; Hfaiedh, M.; Ben Slima, S.; Romdhane, W. B.; Akacha, B. B.; Bouterra, M. T.; Ben Salah, R. Antioxidant and hepatoprotective effects of novel heteropolysaccharide isolated from Lobularia maritima on CCl4-induced liver injury in rats. *Food Sci. & Nutrition* **2022**, *10*, 2271−2284.

(9) Yen, F.-L.; Wu, T.-H.; Lin, L.-T.; Cham, T.-M.; Lin, C.-C. Naringenin-Loaded Nanoparticles Improve the Physicochemical Properties and the Hepatoprotective Effects of Naringenin in Orally-Administered Rats with {CCl}4-Induced Acute Liver Failure. *Pharm. Res.* **2009**, *26*, 893−902.

(10) Chávez, E.; Reyes-Gordillo, K.; Segovia, J.; Shibayama, M.; Tsutsumi, V.; Vergara, P.; Moreno, M. G.; Muriel, P. Resveratrol prevents fibrosis, NF-kappa B activation and TGF-beta increases induced by chronic CCl4 treatment in rats. *J. Appl. Toxicol.* **2008**, *28*, 35−43.

(11) Casas-Grajales, S. Antioxidants in liver health. *World J. Gastrointest. Pharmacol. Ther.* **2015**, *6*, 59.

(12) Mohammed, S. A.; Khan, R. A.; El-Readi, M. Z.; Emwas, A. H.; Sioud, S.; Poulson, B. G.; Mohammed, H. A. Suaeda vermiculata aqueous-ethanolic extract-based mitigation of ccl4-induced hepatotoxicity in rats, and hepg-2 and hepg-2/adr cell-lines-based cytotoxicity evaluations. *Plants* **2020**, *9*, 1291.

(13) Ghosh, S. K.; Sur, P. K. Sanjeevani and Bishalyakarani plants-myth or real! *Int. J. Curr. Res.* **2015**, *7*, 19964−19969.

(14) Abbas, Q.; Hussain, A.; Khan, S. W.; Hussain, A.; Shinwari, S.; Hussain, A.; Ullah, A.; Zafar, M.; Ali, K. Floristic Diversity, Ethnobotany and Traditional Recipes of Medicinal Plants of Maruk Nallah, Haramosh Valley, District Gilgit, Gilgit Baltistan: Traditional

recipes of Maruk Nallah, Haramosh Valley, District Gilgit. *Proc. Pak. Acad. Sci. B Life Environ. Sci.* **2019**, *56*, 97−112.
(15) Abbas, Z.; Khan, S. M.; Abbasi, A. M.; Pieroni, A.; Ullah, Z.; Iqbal, M.; Ahmad, Z. Ethnobotany of the balti community, tormik valley, karakorum range, Baltistan, Pakistan. *J. Ethnobiol. Ethnomed.* **2016**, *12*, 1−38.
(16) Ali, I.; Mu, Y.; Atif, M.; Hussain, H.; Li, J.; Li, D.; Shabbir, M.; Bankeu, J. J. K.; Cui, L.; Sajjad, S. Separation and anti-inflammatory evaluation of phytochemical constituents from Pleurospermum candollei (Apiaceae) by high-speed countercurrent chromatography with continuous sample load. *J. Sep. Sci.* **2021**, *44*, 2663−2673.
(17) Shah, A.; Bharati, K. A.; Ahmad, J.; Sharma, M. New ethnomedicinal claims from Gujjar and Bakerwals tribes of Rajouri and Poonch districts of Jammu and Kashmir, India. *J. Ethnopharmacol.* **2015**, *166*, 119−128.
(18) Thiviya, P.; Gamage, A.; Piumali, D.; Merah, O.; Madhujith, T. Apiaceae as an Important Source of Antioxidants and Their Applications. *Cosmetics* **2021**, *8*, 111.
(19) Kawarty, A. M. A.; Behçet, L.; Çakilcioğglu, U. An ethnobotanical survey of medicinal plants in Ballakayati (Erbil, North Iraq). *Turk. J. Bot.* **2020**, *44*, 345−357.
(20) Yang, K.; Zou, Z.; Wu, Y.; Hu, G. MiR-195 suppression alleviates apoptosis and oxidative stress in CCl4-induced ALI in mice by targeting Pim-1. *Experimental and Molecular Pathology* **2020**, *115*, No. 104438.
(21) Dutta, S. Amelioration of CCl4 induced liver injury in swiss albino mice by antioxidant rich leaf extract of Croton bonplandianus Baill. *PLoS One* **2018**, *13*, No. 0196411.
(22) Sahreen, S.; Khan, M. R.; Khan, R. A Ameliorating effect of various fractions of Rumex hastatus roots against hepato-and testicular toxicity caused by CCl4. *Oxid. Med. Cell Longev.* **2013**, *2013*, No. 325406.
(23) Huang, X. Extract of Averrhoacarambola L.(Oxalidaceae) roots ameliorates carbon tetrachloride-induced hepatic fibrosis in rats. *Biomed. Pharmacother.* **2020**, *121*, No. 109516.
(24) Lee, H. Y.; Lee, G. H.; Yoon, Y.; Chae, HJ. R. verniciflua and E. ulmoides Extract (ILF-RE) protects against chronic CCl4-induced liver damage by enhancing antioxidation. *Nutrients* **2019**, *11*, 382.
(25) Batool, R.; Khan, M. R.; Zai, J. A.; Ali, S.; Maryam, S.; Naz, I.; Bibi, S. Brachychiton populneus (Schott & Endl.) R. Br. ameliorate carbon tetrachloride induced oxidative stress through regulation of endoplasmic reticulum stress markers and inflammatory mediators in Sprague-Dawley male rats. *Biomed. Pharmacol.* **2018**, *107*, 1601−1610.
(26) Ahmed, M.; Khan, K. U. R.; Ahmad, S.; Aati, H. Y.; Ovatlarnporn, C.; Rehman, M. S. U.; Anwar, M. Comprehensive phytochemical profiling, biological activities, and molecular docking studies of Pleurospermum candollei: An insight into potential for natural products development. *Molecules* **2022**, *27*, 4113.
(27) Sayed-Ahmad, B.; Talou, T.; Saad, Z.; Hijazi, A.; Merah, O. The Apiaceae: Ethnomedicinal family as source for industrial uses. *Ind. Crops Prod.* **2017**, *109*, 661−671.
(28) Al-Dalahmeh, Y.; Al-Bataineh, N.; Al-Balawi, S. S.; Lahham, J. N.; Al-Momani, I. F.; Al-Sheraideh, M. S.; Mayyas, A. S.; Abu Orabi, S. T.; Al-Qudah, M. A. LC-MS/MS Screening, Total Phenolic, Flavonoid and Antioxidant Contents of Crude Extracts from Three Asclepiadaceae Species Growing in Jordan. *Molecules* **2022**, *27*, 859.
(29) Sajid, M.; Khan, M. R.; Shah, N. A.; Shah, S. A.; Ismail, H.; Younis, T.; Zahra, Z. Phytochemical, antioxidant and hepatoprotective effects of Alnus nitida bark in carbon tetrachloride challenged Sprague Dawley rats. *BMC Complement. Altern. Med.* **2016**, *16*, 268.
(30) Shahid, A.; Ali, R.; Ali, N.; Hasan, S. K.; Bernwal, P.; Afzal, S. M.; Vafa, A.; Sultana, S. Modulatory effects of catechin hydrate against genotoxicity, oxidative stress, inflammation and apoptosis induced by benzo (a) pyrene in mice. *Food Chem. Toxicol.* **2016**, *92*, 64−74.
(31) Roleira, F. M. F.; Tavares-Da-Silva, E. J.; Varela, C. L.; Costa, S. C.; Silva, T.; Garrido, J.; Borges, F. Plant derived and dietary phenolic antioxidants: Anticancer properties. *Food Chem.* **2015**, *183*, 235−258.
(32) Zhang, C.; Zhang, G.; Liao, Y.; Gong, D. Myricetin inhibits the generation of superoxide anion by reduced form of xanthine oxidase. *Food Chem.* **2017**, *221*, 1569−1577.
(33) Jeong, G. S.; Lee, S. H.; Jeong, S. N.; Kim, Y. C.; Kim, E. C. Anti-inflammatory effects of apigenin on nicotine- and lipopolysaccharide-stimulated human periodontal ligament cells via heme oxygenase-1. *Int. Immunopharmacol.* **2009**, *9*, 1374−1380.
(34) Naz, I.; Khan, M. R.; Zai, J. A.; Batool, R.; Maryam, S.; Majid, M. Indigofera linifolia ameliorated CCl4 induced endoplasmic reticulum stress in liver of rat. *J. Ethnopharma* **2022**, *285*, No. 114826.
(35) Unsal, V.; Cicek, M.; Sabancilar, İ. Toxicity of carbon tetrachloride, free radicals and role of antioxidants. *Rev. Environ. Health* **2021**, *36*, 279−295.
(36) Cui, Y. M.; Yang, X. B.; Lu, X. S.; Chen, J. W.; Zhao, Y. Protective effects of polyphenolsenriched extract from Huangshan Maofeng green tea against CCl4 -induced liver injury in mice. *Chem. Biol. Interact.* **2014**, *220*, 75−83.
(37) Sun, Y. F.; Yang, X. B.; Lu, X. S.; Wang, D. Y.; Zhao, Y. Protective effects of Keemun black tea polysaccharides on acute carbon tetrachloride-caused oxidative hepatotoxicity in mice. *Food Chem. Toxicol.* **2013**, *58*, 184−192.
(38) El-Aarag, B.; Khairy, A.; Khalifa, S. A.; El-Seedi, H. R. Protective effects of flavone from Tamarix aphylla against CCl4-induced liver injury in mice mediated by suppression of oxidative stress, apoptosis and angiogenesis. *In. J. Mol. Sci.* **2019**, *20*, 5215.
(39) Dkhil, M. A.; Abdel Moneim, A. E.; Hafez, T. A.; Mubaraki, M. A.; Mohamed, W. F.; Thagfan, F. A.; Al-Quraishy, S. Myristica fragrans kernels prevent paracetamol-induced hepatotoxicity by inducing anti-apoptotic genes and Nrf2/HO-1 pathway. *Int. J. Mol. Sci.* **2019**, *20*, 993.
(40) Niu, L.; Cui, X.; Qi, Y.; Xie, D.; Wu, Q.; Chen, X.; Ge, J.; Liu, Z. Involvement of TGF-β1/Smad3 Signaling in Carbon Tetrachloride-Induced Acute Liver Injury in Mice. *PLoS One* **2016**, *11*, No. e0156090.
(41) Lopez, J.; Tait, S. W. G. Mitochondrial apoptosis: Killing cancer using the enemy within. *Br. J. Cancer* **2015**, *112*, 957−962.
(42) Jacob, S.; Nair, A. B.; Morsy, M. A. Dose conversion between animals and humans: A practical solution. *Indian J. Pharm. Educ. Res.* **2022**, *56*, 600−607.
(43) Sambucetti, M. E.; Zuleta, A. Resistant starch in dietary fiber values measured by the AOAC method in different cereals. *Cereal Chem.* **1996**, *73*, 759−761.
(44) Seifzadeh, N.; Sahari, M. A.; Barzegar, M.; Gavlighi, H. A.; Calani, L.; Del Rio, D.; Galaverna, G. Evaluation of polyphenolic compounds in membrane concentrated pistachio hull extract. *Food Chem.* **2019**, *277*, 398−406.
(45) Herald, T. J.; Gadgil, P.; Perumal, R.; Bean, S. R.; Wilson, J. D. High-throughput micro-plate HCl-vanillin assay for screening tannin content in sorghum grain. *J. Sci. Food Agric.* **2014**, *94*, 2133−2136.
(46) Gökmen, V.; Serpen, A.; Fogliano, V. Direct measurement of the total antioxidant capacity of foods: The "QUENCHER" approach. *Trends Food Sci. Technol.* **2009**, *20*, 278−288.
(47) Goupy, P.; Dufour, C.; Loonis, M.; Dangles, O. Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *J. Agric. Food Chem.* **2003**, *51*, 615−622.
(48) Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "'antioxidant power"': The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70−76.
(49) Naz, I.; Khan, M. R.; Zai, J. A.; Batool, R.; Zahra, Z.; Tahir, A. Pilea umbrosa ameliorate CCl4 induced hepatic injuries by regulating endoplasmic reticulum stress, pro-inflammatory and fibrosis genes in rat. *Environ. Health Prevent. Med.* **2020**, *25*, 1−53.
(50) Sun, Y.; Oberley, L. W.; Li, Y. A simple method for clinical assay of superoxide dismutase. *Clin. Chem.* **1988**, *34*, 497−500.
(51) Chance, B.; Maehly, A. C. [136] Assay of catalases and peroxidases. *Meth. Enzymol.* **1955**, *2*, 764−775, DOI: 10.1016/S0076-6879(55)02300-8.

https://doi.org/10.1021/acsomega.3c02031
ACS Omega 2023, 8, 25999−26011

(52) Ellman, G. L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70−77.
(53) Green, L. C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. S.; Tannenbaum, S. R. Analysis of nitrate, nitrite, and [15n] nitrate in biological fluids. *Anal. Biochem.* **1982**, *126*, 131−138.
(54) Pick, E.; Keisari, Y. Superoxide anion and hydrogen peroxide production by chemically elicited peritoneal macrophages—induction by multiple nonphagocytic stimuli. *Cell Immunol.* **1981**, *59*, 301−318.
(55) Iqbal, M.; Wright, D. Host resistance to insecticides can confer protection to endo-larval parasitoids. *Bull. Entomol Res.* **1996**, *86*, 721−723.
(56) Lowry, O.; Rosebrough, N.; Farr, A.; Randall, R. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.
(57) Chomczynski, P.; Sacchi, N. The single-step method of RNA isolation by acid guanidinium thiocyanate−phenol−chloroform extraction: twenty-something years on. *Nat. Protoc.* **2006**, *1*, 581−585.

https://doi.org/10.1021/acsomega.3c02031
ACS Omega 2023, 8, 25999−26011