Galangin attenuates chlorpyrifos-induced kidney injury by mitigating oxidative stress and inflammation and upregulating Nrf2 and farnesoid-X-receptor in rats

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Abstract

Chlorpyrifos (CPF) is a highly toxic commonly used pesticide and can seriously harm human health. This study assessed the potential of galangin (GAL), an antioxidant flavonoid, to attenuate oxidative stress, inflammation and kidney injury caused by CPF, emphasizing the role of farnesoid-x-receptor (FXR) and Nrf2. Rats were supplemented with CPF and GAL for 28 days. CPF increased serum creatinine, urea and Kim-1, provoked several tissue alterations, and increased kidney ROS, malondialdehyde (MDA), NF-κB p65, TNF-α, iNOS, and caspase-3. GAL effectively ameliorated serum kidney injury markers, ROS, MDA, and TNF-α, suppressed NF-κB p65, iNOS, and caspase-3, and enhanced antioxidants. GAL suppressed Keap1 and upregulated FXR, Nrf2, HO-1 and NQO-1 in CPF-administered rats. GAL exhibited binding affinity with Keap1, FXR, caspase-3, iNOS, HO-1, and NF-κB. In conclusion, GAL is effective in preventing CPF nephrotoxicity by attenuating oxidative stress and inflammation. This protection is linked to upregulation of antioxidants, Nrf2/HO-1 signaling and FXR.

Keywords: Pesticides; Nephrotoxicity; Flavonoids; Farnesoid-x-receptor; Oxidative stress.

1. Introduction

Pesticides are chemical compounds widely employed in agriculture for the purpose of controlling weeds and insects, along with other domestic and veterinary applications. Organophosphorus (OP) chemicals are among the widely used pesticides for pest control and crop yield improvement [1]. The use of pesticides, including OP compounds is on the rise in many parts of the world and therefore human exposure to these chemicals increases [1]. Skin contact, inhalation and ingestion are the most common exposure routes of humans to pesticides which may occur during the mix, application, or cleaning processes. The overuse of OP pesticides is linked to increased risk of morbidity and mortality, and acute and chronic illnesses, and is on the rise in many parts of the world [2]. Chlorpyrifos (CPF) is a widely used OP

pesticide to control pests and insects and increase crop yield. It has a broad-spectrum action on various pathogens and insects and hence widely employed in indoor and agricultural fields [3]. Acetylcholinesterase (AChE) inhibition is the main mechanism underlying CPF pesticidal and insecticidal activities. Inhibition of this enzyme disrupts the function of the nervous system, leading to death of the affects insects [4]. AChE functions at the cholinergic synapses and neuromuscular junctions and is therefore non-selective towards insects and its toxic effects can extend different organisms, including invertebrates and fish [4]. In addition, CPF can reach animals and humans via direct exposure or consumption of contaminated food along with the skin adsorption or inhalation [3, 5]. Accordingly, CPF residues in amounts above the acute reference range have been detected on the surface of vegetables, grains, and fruits [6, 7]. Exposure to CPF via different routes was associated with reproductive, hepatorenal, and hematological abnormalities as well as the commonly reported neurotoxic effects and [3, 8-10]. Although the precise mechanism(s) are not fully elucidated, the toxicity of CPF and its metabolites was explained by triggered increase in ROS and inflammatory mediators, rendering the implication of oxidative stress (OS) and inflammation [11, 12].

Kidney injury is among the hazardous consequences of CPF, and previous studies highlighted the implication of OS and inflammation in CPF-induced kidney damage in animals [13-15]. In the same context, our previous study demonstrated elevated kidney ROS and inflammatory mediators and declined antioxidants following exposure to CPF [10]. Therefore, mitigation of OS and inflammation can confer protection against CPF nephrotoxicity. The activation of Nrf2 and FXR can confer effective protection against chemical-, drug-, and metabolic derangements-related OS and inflammation [16-19]. Nrf2 controls several antioxidant and cytoprotective genes and is a pharmacological target for the treatment of a wide range of OS-linked diseases [16, 20]. It is sequestered by Keap1 in the cytosol and get liberated upon exposure to ROS or electrophiles to bind to DNA and activate the transcription of many genes,

including heme oxygenase-1 (HO-1) and NQO-1 [21]. FXR is another TF that is widely expressed in the kidney [22], and can protect against kidney OS provoked by under ischemia/reperfusion (I/R) [23] and metabolic alterations [16, 24, 25]. Both Nrf2 and FXR can orchestrate protection of the kidney against I/R, hyperglycemia, and hyperglycemia [16, 23], as well as protecting the liver against cholestatic injury [26].

There is a considerable evidence suggesting that flavonoids could be effective in conferring protection against drug/chemical-induced toxicity, including kidney injury [27]. Galangin (GAL) is a flavonoid with various biological and pharmacological activities, including anti-inflammatory, antioxidant and anti-microbial [28-32]. GAL is abundant in propolis, galangal and honey and its beneficial effects against several disease has recently attracted more attention [31]. Other sources of GAL include *Plantago major, Crocus sativus, Zingiber officinale* and some other edible herbs [33]. GAL showed protective effects against OS and inflammation in the context of several disorders. In vitro studies revealed its suppressive effects on ROS, NF-κB and inflammatory response in (LPS)-activated macrophages [34], and uric acid-challenged tubular epithelial cells [35]. In a murine model of hypertension, GAL downregulated NF-κB, tumor necrosis factor receptor 1 (TNF-R1), and adhesion molecules in the aorta [36]. GAL conferred protection against chemotherapy hepatotoxicity [29], and hepatic stellate cells activation and fibrosis [37], effects mediated via ROS and inflammation mitigation. In the context of kidney disorders, GAL effectively suppressed inflammatory mediators and attenuated OS [38]. In mice challenged with cisplatin (CIS), GAL inhibited NF-κB and attenuated OS and cell apoptosis in the kidney [39]. A similar study by Tomar et al [40] revealed the ameliorative effect of GAL on CIS-mediated NF-κB and kidney injury. A recent study by Salama et al demonstrated its potent effect on cadmium nephrotoxicity where it activated Nrf2 and inhibited NF-κB nuclear translocation [41]. Although the beneficial effects of GAL in several disorders have been reported, its potential to protect the kidney against the toxic effect

of CPF hasn’t been explored. This study investigated the efficacy of GAL against CPF-induced OS, inflammation, cell death in rat kidney, emphasizing the involvement of Nrf2 and FXR.

2. Materials and methods

2.1. Experimental design

Male Wistar rats weighing 190 ± 10 g were housed under standard temperature (23 ± 2 °C) and humidity (50-60%) on a 12h dark/light cycle. The rats were given free access to food and water and allocated into five groups (n = 6). To investigate the nephroprotective effect of GAL, it has been concurrently administered with CPF. GAL (Sigma, USA) and CPF (Agro Chem, Egypt) were dissolved in 0.5% carboxymethyl cellulose (CMC) and corn oil as vehicles, respectively, and supplemented for 28 days orally. Group I (Control) was supplemented with vehicles and Group II (GAL) received corn oil and 50 mg/kg GAL [42]. Groups III (CPF), IV (CPF + 25 mg/kg GAL), and V (CPF + 50 mg/kg GAL) were supplemented with 10 mg/kg CPF [43] and 0.5% CMC (Group III), 25 mg/kg GAL [42] (Group IV), and 50 mg/kg GAL [42] (Group V). The study was approved by the ethics committee at Al-Azhar University (Assiut, Egypt) (AZ-AS/PH-REC/35/24).

One day following the last treatment, blood for serum separation was collected via cardiac puncture under anesthesia using ketamine/xylazine. After scarification and immediate dissection, the kidneys were removed. Pieces were collected on 10% neutral buffered formalin (NBF) and other tissue pieces on RNALater. Tris-HCl buffer (pH = 7.4) was the liquid in which other pieces homogenized.

2.2. Biochemical investigations

Levels of creatinine and urea in serum were assayed using Biodignostic kits (Egypt). Serum Kim-1 and kidney NF-κB p65, caspase-3, and TNF-α were measured with ELISA kist (Cusabio, China). Kidney MDA, nitric oxide (NO), SOD, glutathione (GSH), and catalase were

measured using Biodiagnostic (Egypt) kits. Levels of ROS were measured using H_2DCF-DA (Sigma, USA) [44]., and activities of HO-1 and NQO1 were assayed according to the methods of Abraham et al [45] and Ernster and Navazio [46], respectively.

2.3. Histopathological and immunohistochemical (IHC) examinations

The kidney samples, fixed in 10% NBF for 24 h, were dehydrated, cleared, and embedded in paraffin wax. Sections (5 μm) were cut and stained with hematoxylin/eosin (H&E). The changes in FXR were evaluated as recently reported [16]. Briefly, paraffin was removed and the sections were treated with citrate buffer (50 mM, pH = 6.8) followed by 0.3% H_2O_2 and protein block. The sections were incubated for 24 h at 4 °C with anti-FXR (Biospes, China) followed by washing and incubation with the secondary antibody (Biospes, China). 3,3′-Diaminobenzidine (DAB) was employed to develop color and counterstaining with Mayer's hematoxylin was performed. Intensity of the color was measured (6 images/rat) using ImageJ (NIH, USA).

2.4. qRT-PCR

The effects of CPF and GAL on mRNA levels of different parameters were determined using qRT-PCR. To isolate RNA from kidney samples, RNA Purification Kit (Thermo Scientific, USA) was used. RNA samples with A260/A280 ≥ 1.8 were reverse transcribed into cDNA. Amplification of cDNA was performed using SYBR Green master mix and primers listed in Table 1. β-actin was used as a housekeeping gene and Ct values were analyzed via the $2^{-\Delta\Delta Ct}$ method [47].

2.5. *In silico* molecular docking

The affinity of GAL towards FXR, NF-κB p65, iNOS, caspase-3, HO-1, and Keap1 (PDB ID: 7D42, 5U01, 3EAI, 1NME, 1DVE, and 5CGJ, respectively) was explored using PyRx virtual screening software (version 0.8) [48]. To prepare the protein targets, Autodock Tools (ADT; v1.5.6) was used. The binding affinity of GAL, tropifexor (FXR agonist), and RA839 (Nrf2

activator) was investigated, PyMOL (v2.3.2) and LigPlot (v2.2.8) [49] were employed for visualization of binding mode and protein-ligand interactions, respectively.

2.6. Statistical analysis

The data are expressed as means ± SEM, and comparisons between groups was determined by one-way ANOVA followed by Tukey's post-hoc test using GraphPad Prism 7 software. $P < 0.05$ was considered significant.

3. Results

3.1. GAL attenuates CPF-induced renal injury

The toxic effect of CPF and the protective role of GAL were evaluated by biochemical and histopathological investigations (Fig. 1). CPF increased circulating creatinine, urea, and Kim-1 ($P < 0.001$), whereas GAL ameliorated the levels of these markers (Fig. 1A-C). The 50 mg/kg dose of GAL decreased creatinine ($P < 0.001$) and Kim-1 ($P < 0.01$) significantly as compared to the lower one. Microscopic examination of H&E-stained section (Fig. 1D) showed normal renal tubules and glomeruli in control and GAL-treated rats. CPF induced glomerular damage, tubular degenerative changes, hemorrhage, and inflammatory cells infiltrations. GAL prevented CPF-induced tissue injury with some degree of injury still seen in the group that received the low dose whereas the high dose-treated rats showed nearly normal tissue architecture.

3.2. GAL mitigates CPF-induced kidney OS in rats

Rats challenged with CPF showed significantly high levels of ROS (Fig. 2A) and MDA (Fig. 2B) ($P < 0.001$). GSH, SOD, and catalase were decreased in the kidney of CPF-administered rats (Fig. 2C-E). GAL effectively and dose-dependently ameliorated kidney ROS, MDA, and antioxidants in CPF-administered rats, while exerted no effect when supplemented to normal rats.

3.3. GAL suppresses CPF-induced kidney inflammation in rats

Assessment of the mRNA abundance (Fig. 3A) and protein levels (Fig. 3B) of NF-κB p65 in the kidney of CPF-treated rats revealed significant upregulation ($P < 0.001$). TNF-α, iNOS mRNA and NO levels were elevated in CPF-administered rats ($P < 0.001$; Fig. 3C-E). Both doses of GAL decreased NO and its effect on NF-κB p65, TNF-α, and iNOS was dose-dependent.

The affinities of GAL to bind NF-κB and iNOS are represented in Fig. 4A-B and Table 2. GAL exhibited lowest binding energies (LBE) of -7.3 and -9.7 kcal/mol with NF-κB and iNOS, respectively. Two polar bonds eight hydrophobic interactions between GAL and NF-κB and one polar bond and seven hydrophobic interactions between GAL and iNOS were observed.

3.4. GAL downregulates kidney caspase-3 in CPF-induced rats

Changes in caspase-3 RNA and protein levels revealed remarkable ($P < 0.001$) increase in the kidney of CPF-treated rats as represented in Fig. 5A and 5B, respectively. GAL dose dependently decreased kidney caspase-3 when administered to CPF-challenged rats. Molecular docking revealed eight hydrophobic interactions and three polar bonds between GAL and caspase-3 with -7.6 kcal/mol as the observed LBE (Fig. 5C and Table 2).

3.5. GAL upregulates kidney Nrf2/HO-1 signalling in CPF-administered rats

Keap1 was upregulated (Fig. 6A) whereas Nrf2, HO-1, and NQO-1 mRNA (Fig. 6B-D) were decreased in the kidney of rats following CPF administration ($P < 0.001$). Similarly, CPF decreased HO-1 and NQO-1 activities in the kidney of rats ($P < 0.001$; Fig. 6E-F). Both GAL doses effectively reversed the expression pattern of Keap1, Nrf2, HO-1, and NQO-1 as well as the activities of HO-1 and NQO-1.

In silico examinations showed comparable binding energy of GAL with Keap1 (-9.3 kcal/mol) to the Keap1 inhibitor RA839 (-9.5 kcal/mol) as depicted in Fig. 7A and Table 2. Intestinally, out of the 13 amino acid residues involved in GAL interaction with Keap1, 9 were seen in the binding interaction of RA839 with Keap1. In addition, GAL showed a -8.4 kcal/mol binding energy with HO-1 and exhibited 13 hydrophobic interactions and one polar bond (Fig. 7B and Table 2).

3.6. GAL upregulates FXR in the kidney of CPF-administered rats

The effect of CPF and/or GAL on kidney FXR was determined using qRT-PCR (Fig. 8A) and IHC (Fig. 8B-C), and the affinity of GAL towards FXR was studied *in silico* (Fig. 9). CPF decreased FXR mRNA and protein levels significantly ($P < 0.001$), effects prevented by GAL. GAL showed dose-dependent upregulation of FXR ($P < 0.05$).

Two and ten amino acid residues were involved in polar bonding and hydrophobic interactions, respectively, of GAL with FXR (Fig. 9A). Nine of the amino acid residues involved in GAL interaction with FXR were also shown in the interaction of FXR with its agonist tropifexor (Fig. 9B and Table 2), and the reported LBE were -9.0 and -10.9 kcal/mol for GAL and tropifexor, respectively.

4. Discussion

The wide applications of CPF for best control in domestic and agricultural fields can increase the exposure of animals and humans to the toxic effects of this pesticide [3]. Multiple organ injuries, including kidney damage, have been reported as hazardous effects of CPF exposure. Inflammation and OS are central in the toxicity of CPF and the development of strategies to prevent these key processes can confer protection against kidney damage. We demonstrated the protective role of GAL against CPF-induced kidney injury, pinpointing to the involvement of Nrf2 and FXR in mitigating OS and inflammation.

In this study, rats exposed to CPF have been used to study the nephroprotective effect of GAL. CPF caused kidney injury supported by increased serum creatinine and urea as previously indicated in studies that employed rodents exposed to CPF [14, 15]. Besides these commonly used markers, serum Kim-1 was elevated in CPF-exposed rats. Kim-1 is used as a marker of tubular epithelium damage and its upregulation is to prevent cell death and promote reepithelization [50]. Histopathological results added supported the nephrotoxic effect of CPF and the findings coincided with previous studies showing degenerative changes, tubular epithelium damage, atrophied glomeruli, necrosis, and inflammatory cell infiltration [10, 14]. GAL showed potent nephroprotective efficacy manifested by the ameliorated circulating creatinine, urea, and Kim-1 levels as well as attenuation of histopathological alterations in CPF-administered rats. These data supported studies showing that GAL can protect the kidney against CIS [39, 40] and cadmium [41].

Given the implication of OS and inflammation in mediating the toxic effects of CPF [13-15], it is noteworthy postulating that the nephroprotective role of GAL was exerted via its dual effect against these processes [39-41]. Increased ROS and MDA, and decreased GSH and antioxidant enzymes demonstrated OS in the kidney of CPF-treated rats as shown previously [10, 14]. CPF induces surplus ROS production and subsequently induced signaling mediators of cell dysfunction, inflammation and death, including NF-κB [51]. Exposure to CPF was linked to elevated ROS in Neuro-2a cells [51] and microglial cells *in vitro* [12] and in rat kidney [10]. Excess ROS can provoke oxidation of cellular macromolecules, thereby leading to increased LPO, DNA damage, and protein oxidation that might inactivate multiple proteins, including antioxidant enzymes, transport proteins and other proteins vital to cell function [52, 53]. These events will ultimately lead to cell death that is orchestrated not only be elevated ROS, but also the inflammatory mediators. Here, CPF elicited an inflammatory response in rat kidney as shown by the upregulated NF-κB p65, TNF-α and iNOS. NF-κB is activated in

response to elevated ROS, leading to the release of multiple mediators involved in inflammation, such as TNF-α and iNOS accompanied with elevated NO levels produced by iNOS [54, 55]. Accordingly, CPF administration was shown to upregulate hepatic [56] and renal iNOS in rats [10], explaining the reported increase in NO. Upon reaction with superoxide, NO produces peroxynitrite, a versatile oxidant that breaks DNA and causes cell death [57]. Moreover, excess cytokines and ROS negatively impact the mitochondrial permeability with subsequent release of cytochrome c into the cytoplasm and caspase-3 activation. In this study, CPF upregulated caspase-3 that is known to elicit cell death by provoking DNA fragmentation and degradation of the cytoskeleton [58, 59].

The renoprotective efficacy of GAL against CPF was associated with effective attenuation of OS, inflammation and apoptosis mediated via suppression of ROS, MDA, NF-κB, TNF-α, iNOS, NO, and caspase-3 along with enhanced antioxidant defenses. These findings demonstrated the potent antioxidant, cytoprotective and anti-inflammatory efficacies of GAL. In accordance, GAL has shown protective effects against OS and its consequences in various disease models. For instance, GAL showed *in vitro* ROS scavenging and LPO inhibitory activity [60], and prevented high fructose- [61] and CIS-induced OS, LPO and kidney injury [39]. Moreover, GAL inhibited NF-κB and attenuated OS and renal cell death in CIS-challenged mice [39, 40], and rats with cadmium nephrotoxicity [41]. Therefore, attenuation of OS and inflammation by GAL resulted in protection against CPF-induced apoptosis and kidney injury. The role of GAL in suppressing NF-κB and caspase-3 were further supported by in silico investigations. Molecular docking showed GAL's affinity towards NF-κB p65 and caspase-3. Binding of GAL with NF-κB p65 might play a role in the suppression of inflammation by preventing p65 subunit binding to DNA. Also, binding with caspase-3 could be of significant value in preventing apoptotic cell death.

The nephronprotective effect of GAL was further explored by investigating changes in Nrf2 and FXR. CPF-challenged rats exhibited upregulation of Keap1, downregulation of Nrf2, HO-1 and NQO-1 mRNA, and suppressed HO-1 and NQO-1 activities. These data demonstrated downregulation of the Nrf2/HO-1 pathway in agreement decreased antioxidants and surplus ROS in the kidney of CPF-treated rats. Despite activated by ROS, literature has shown suppressed Nrf2/HO-1 signaling under conditions of prolonged excessive ROS generation [29, 62-64]. Deficiency of Nrf2 can increase the vulnerability to kidney damage [65], and CPF negatively impacted Nrf2 signaling in the fruit fly [66], human neuroblastoma cells [67], and rodent liver [56]. Similar to Nrf2, CPF administration resulted in downregulation of FXR in rat kidney. FXR is a TR that forms a heterodimer with RXR and controls many genes involved in essential metabolic functions. Therefore, FXR alterations as implicated in inflammation and other disorders, including diabetes and cholestasis [16, 68, 69]. Accelerated progression of kidney disease in diabetes was associated with FXR deficiency [70]. Recently, activation of FXR has been demonstrated to significantly reduce LPO by upregulating ferroptosis gatekeepers, including GPX4 and PPARα [71]. Activation of Nrf2 and FXR can therefore represent an effective strategy to suppress OS and inflammation provoked by FXR. Treatment with GAL downregulated Keap1 and enhances Nrf2, HO-1, NQO-1, and FXR, demonstrating the possible role of FXR and Nrf2 in its protection against CPF nephrotoxicity. The positive effect of GAL on Nrf2 signaling was demonstrated in rat models of cadmium nephrotoxicity [41], and chemotherapy hepatotoxicity [29]. GAL reduced inflammation and OS by activating SIRT1/Nrf2/HO-1 signaling and decreasing the levels of IL-1β and TNF-α [41]. Moreover, GAL-mediated activation of Nrf2 and consequent induction of HO-1 attenuated cyclophosphamide hepatotoxicity in rats [29]. In addition to Nrf2/HO- upregulation, this study introduced novel information on the ability of GAL to upregulate FXR and mitigate CPF-induced kidney injury. Activation of FXR has shown beneficial effects in protecting the kidney

from OS and inflammation in different conditions. FXR activation inhibited mitochondrial dysfunction and surplus production of ROS in kidney I/R [23], and mitigated OS, fibrosis and inflammation of the kidney in diabetes [72]. We have recently shown that upregulation of both Nrf2 and FXR protected the diabetic kidney against injury mediated via OS and inflammation [16]. The beneficial role of FXR in kidney I/R was blocked by silencing Nrf2 [23], and both Nrf2 and FXR cooperatively prevented liver injury in cholestasis [26]. In view of these studies, the protective role of GAL against CPF-induced kidney injury involved its dual ability to upregulate Nrf2 and FXR. In silico, GAL showed high affinity towards Keap1 and FXR. Interestingly, GAL exhibited similar binding patterns to RA839 (Nrf2 activator) and tropifexor (FXR agonist). The relatively low binding energy and the presence of several polar bonding and hydrophobic interactions proposed the potency of GAL to modulate Keap1, HO-1, and FXR. However, the lack of data showing the direct interaction between GAL and these proteins and the potential functional effect of this binding is considered a limitation of this study.

5. Conclusion

This study shows new information on the protective role of GAL against CPF nephrotoxicity and the involvement of FXR and Nrf2. GAL prevented kidney tissue injury, and suppressed excess ROS, LPO, NF-κB, caspase-3, inflammatory mediators, and Keap1 in CPF-intoxicated rats. GAL upregulated Nrf2 and FXR and enhanced enzymatic antioxidants. The protective effect of GAL was supported by *in silico* data showing its potent binding affinity with Keap1, FXR, HO-1, NF-κB, caspase-3, and iNOS. This study may have significant clinical implications and underscore the protective role of GAL against CPF toxicity.

Declaration of competing interest

No conflict of interest is to be declared.

Data availability

The manuscript contains all data supporting the reported results.

Acknowledgment

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Tables:

Table 1. Primers used for qRT-PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Nrf2	TTGTAGATGACCATGAGTCGC	TGTCCTGCTGTATGCTGCTT
HO-1	GTAAATGCAGTGTTGGCCCC	ATGTGCCAGGCATCTCCTTC
Keap1	TCAGCTAGAGGCGTACTGGA	TTCGGTTACCATCCTGCGAG
NQO-1	GGCCATCATTTGGGCAAGTC	TCCTTGTGGAACAAAGGCGA
FXR	CATTAACAACGCGCTCACCTG	TTCCTTAGCCGGCAATCCTG
Caspase-3	GGAGCTTGGAACGCGAAGAA	ACACAAGCCCATTTCAGGGT
iNOS	ATTCCCAGCCCAACAACACA	GCAGCTTGTCCAGGGATTCT
NF-κB p65	TTCCCTGAAGTGGAGCTAGGA	CATGTCGAGGAAGACACTGGA
β-actin	AGGAGTACGATGAGTCCGGC	CGCAGCTCAGTAACAGTCCG

Table 2. Binding affinities of GAL towards NF-κB, iNOS, caspase-3, HO-1, Keap1, and FXR.

		Lowest binding energy (kcal/mol)	**Polar interacting residues**	**Hydrophobic interacting residues**
NF-κB		-7.3	Lys56, Gln114	Thr55, Ser276, Ile24, Glu25, Thr60, Thr57, Pro275, His58
iNOS		-9.7	Trp188	Trp366, Phe363, Tyr483, Leu203, Asn364, Gly196, Gly365
Caspase-3		-7.6	Ser120, Gln161, Arg64	His 121, Glu123, Gly122, Tyr204, Cys163, Arg207, Ser205, Ala162
HO-1		-8.4	Arg136	Thr135, Phe167, Phe166, Met34, Phe207, Asn210, Leu54, Leu147, Gly139, Phe214, His25, Gly144, Gly143
Keap1	GAL	-9.3	Val512, Val606	Gly462, Arg415, Leu557, Ala556, Ile416, Val463, Gly605, Ile559, Gly367, Ala510, Gly464
	RA839	-9.5	Val604, Val418, Leu557	Val463, Ala510, Val465, Cys513, Val512, Gly419, Val420, Ile559, Gly605, Ile416, Leu365, Gly367, Ala366, Thr560, Val606, Ala607, Val608
FXR	GAL	-9.0	Tyr369, Met328	Phe329, Met450, Met365, Phe443, Leu287, Trp454, Phe284, Ser332, Phe461, Trp469
	Tropifexor	-10.9	Arg331	Phe443, Phe329, Met328, Val325, Ser332, Ile357, His447, Ile352, Phe284, Trp454, Thr288, Ala291, Tyr369, Phe461, Leu287, Ile335, Leu348, His294, Met265

Figures:

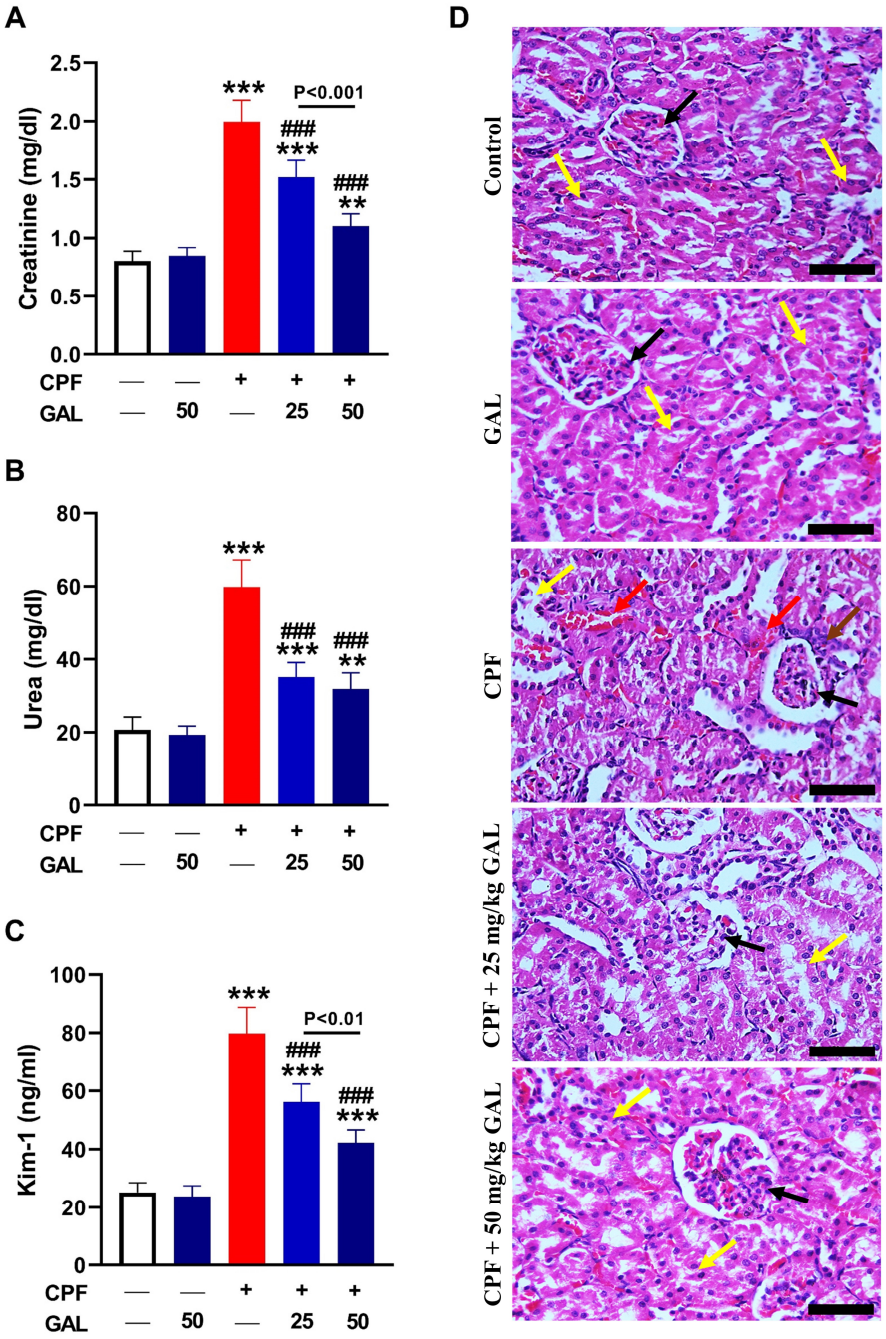

Fig. 1. GAL prevented CPF-induced kidney injury. (A-C) GAL ameliorated serum creatinine (A), urea (B) and Kim-1 (C) in CPF-administered rats. Data are mean ± SEM, (n = 6). $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* Control, and $^{###}P < 0.001$ *vs* CPF. (D) Photomicrographs of H&E-stained sections in kidney of control and GAL-treated rats showing normal glomeruli (black arrow) and tubules (yellow arrow), CPF-administered rats showing altered glomeruli (black arrow), inflammatory cells infiltration (brown arrow), degenerative changes in renal tubules (yellow arrow) and hemorrhage (red arrow), and CPF-administered rats treated with 25 and 50 mg/kg showing improvement in glomeruli (black arrow) and renal tubules (yellow arrow). (Scale bar = 50 µm).

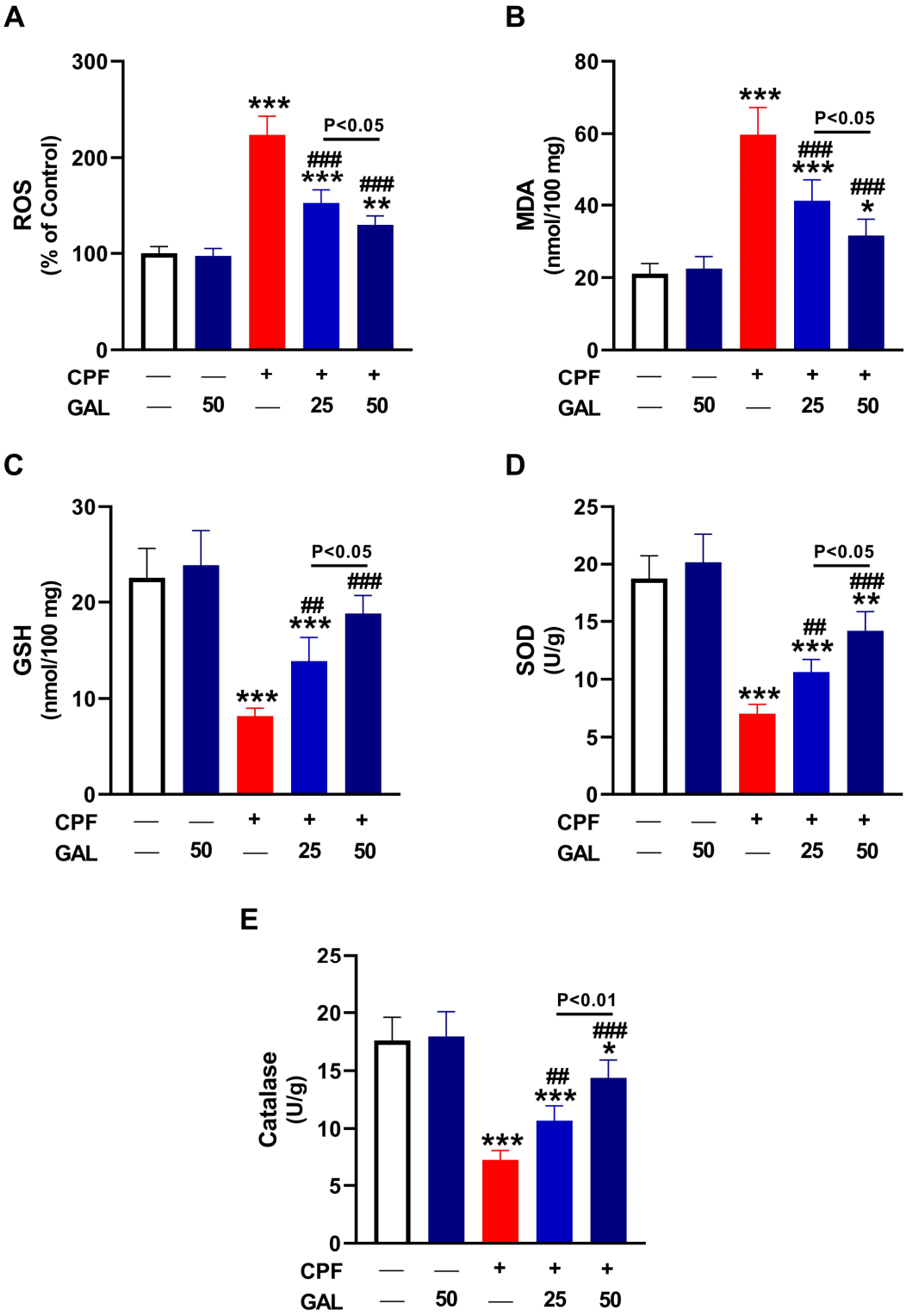

Fig. 2. GAL attenuated CPF-induced kidney oxidative stress. GAL decreased ROS (A) and MDA (B) and increased GSH (C), SOD (D) and catalase (E) in CPF-administered rats. Data are mean ± SEM, (n = 6). *P < 0.05, **P < 0.01 and ***P < 0.001 *vs* Control. ##P < 0.01 and ###P < 0.001 *vs* CPF.

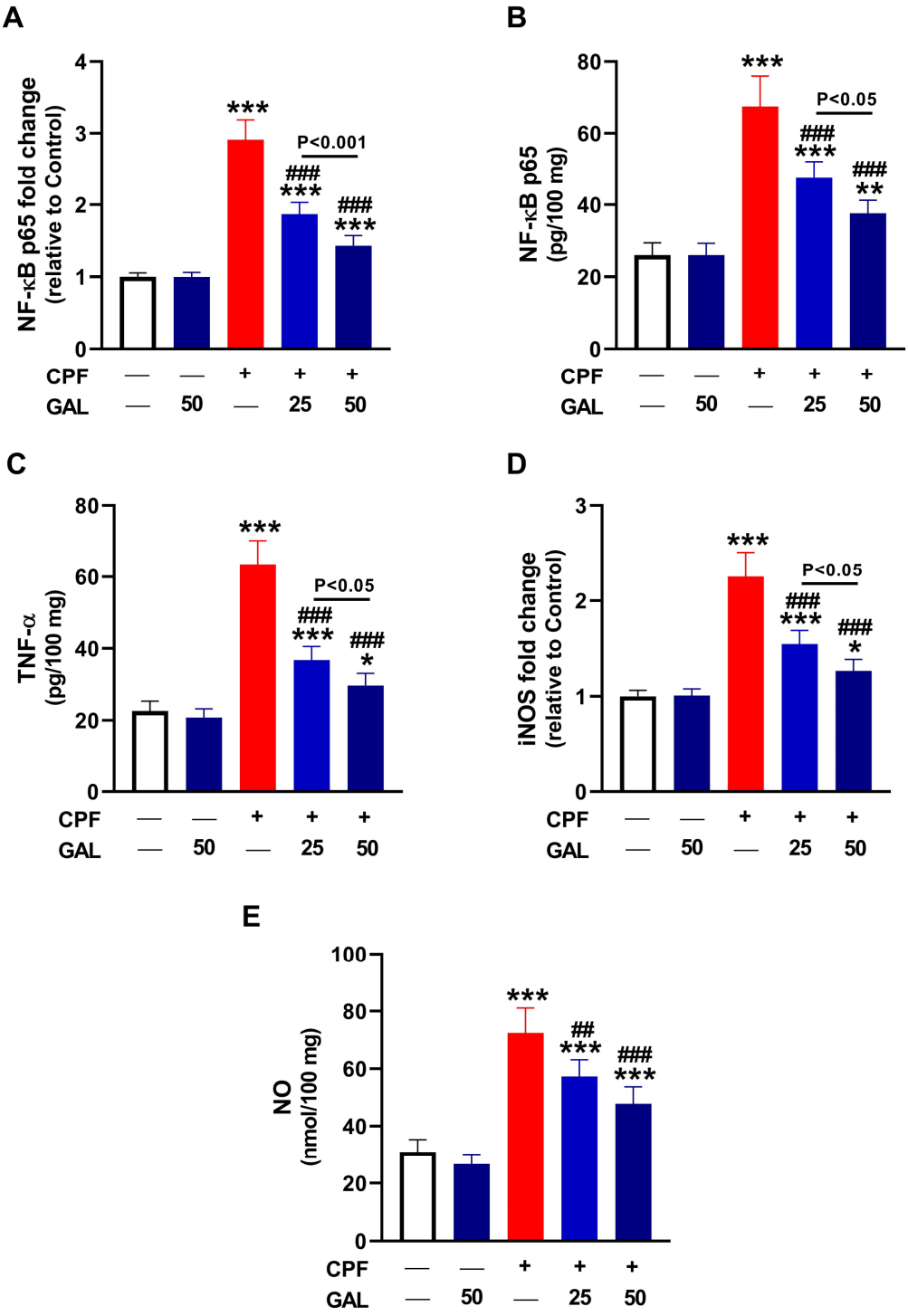

Fig. 3. GAL mitigated CPF-induced kidney inflammation. GAL downregulated NF-κB p65 mRNA (A) and protein (B), TNF-α (C), iNOS mRNA (D) and NO (E). Data are mean ± SEM, (n = 6). *P < 0.05, **P < 0.01 and ***P < 0.001 *vs* Control. ##P < 0.01 and ###P < 0.001 *vs* CPF.

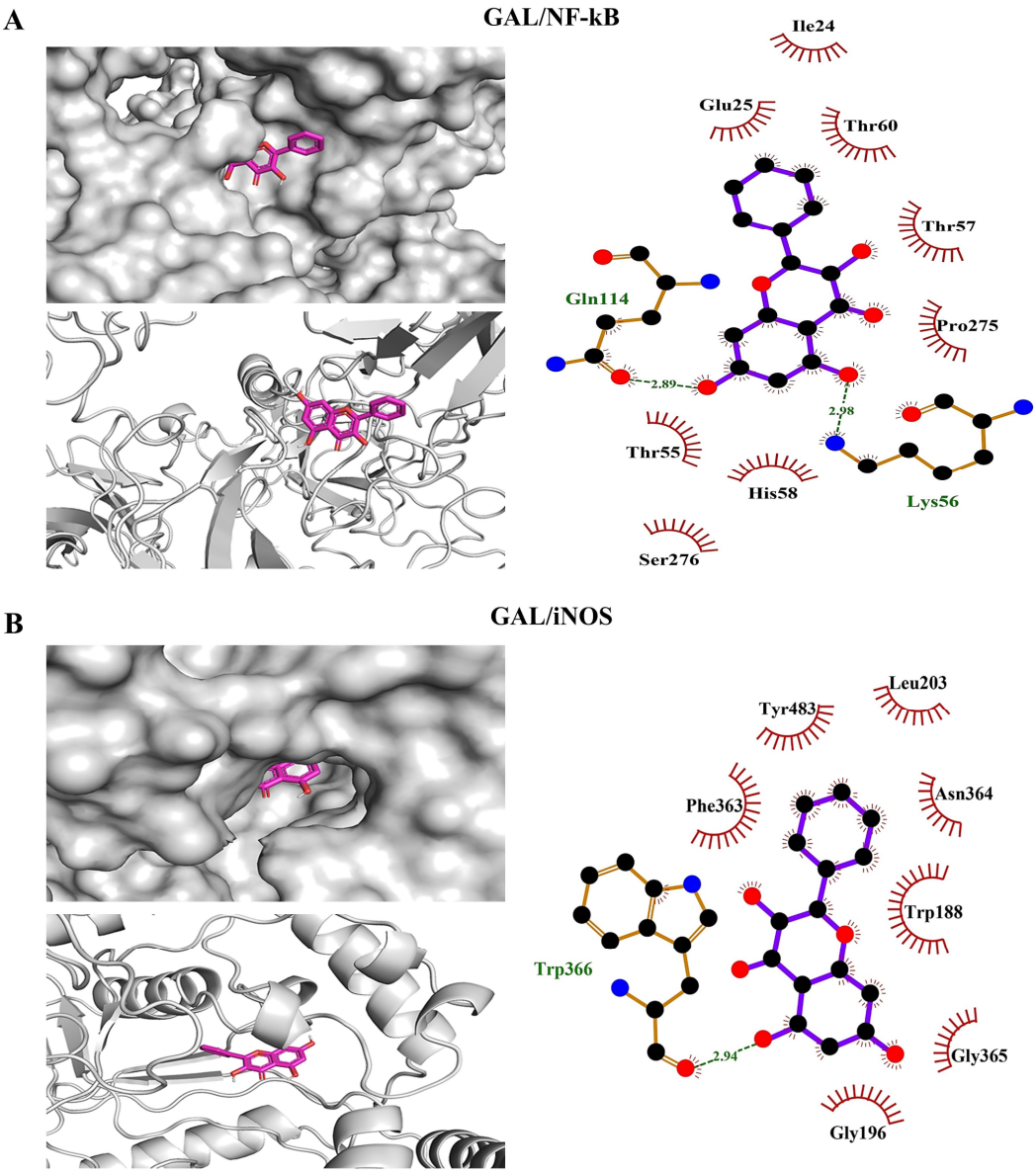

Fig. 4. Molecular docking of GAL with NF-κB (A) and iNOS (B) showing the crystal structure and amino acid residues involved in polar bonding and hydrophobic interactions.

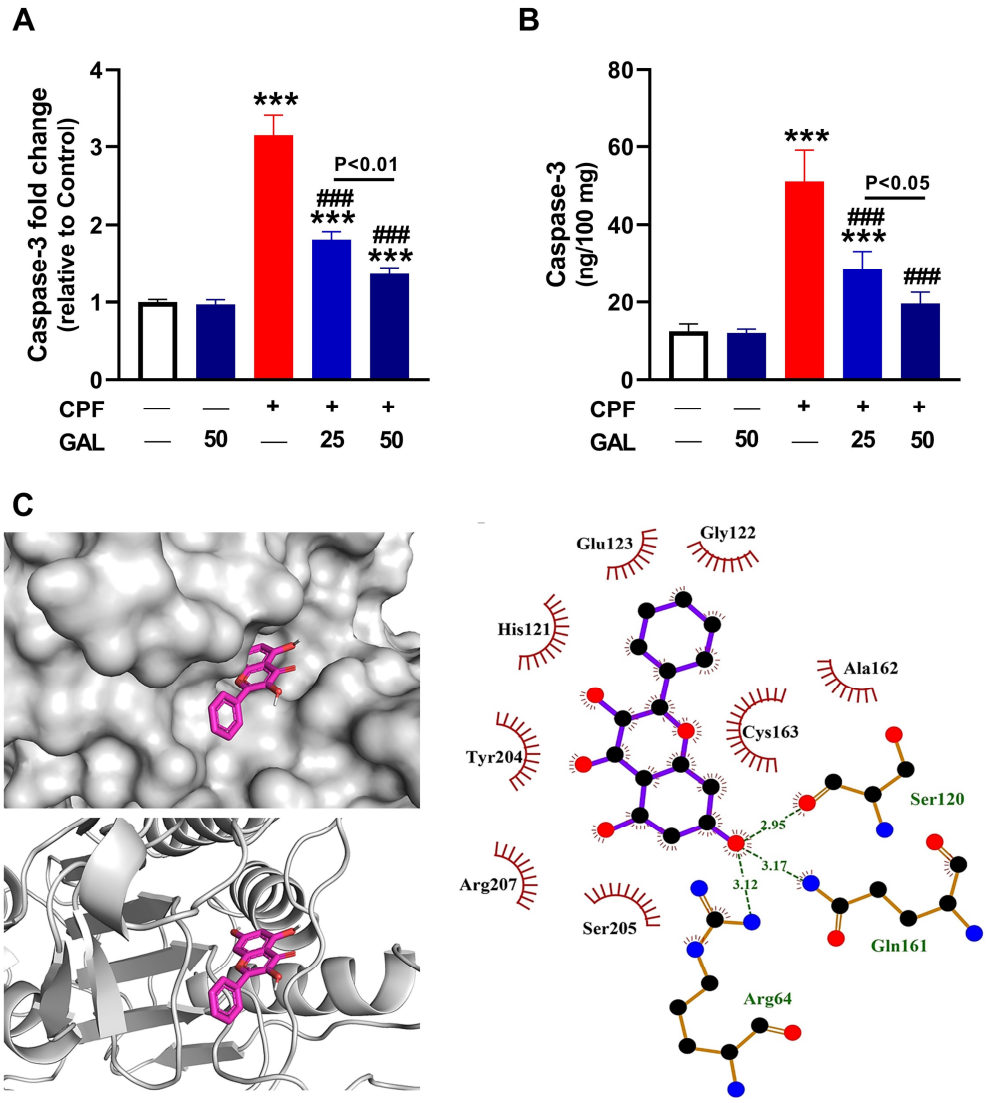

Fig. 5. GAL downregulated caspase-3 mRNA (A) and protein (B) in the kidney of CPF-administered rats. Data are mean ± SEM, (n = 6). ***P < 0.001 *vs* Control and ###P < 0.001 *vs* CPF. (C) Molecular docking of GAL with caspase-3 showing the crystal structure and amino acid residues involved in polar bonding and hydrophobic interactions.

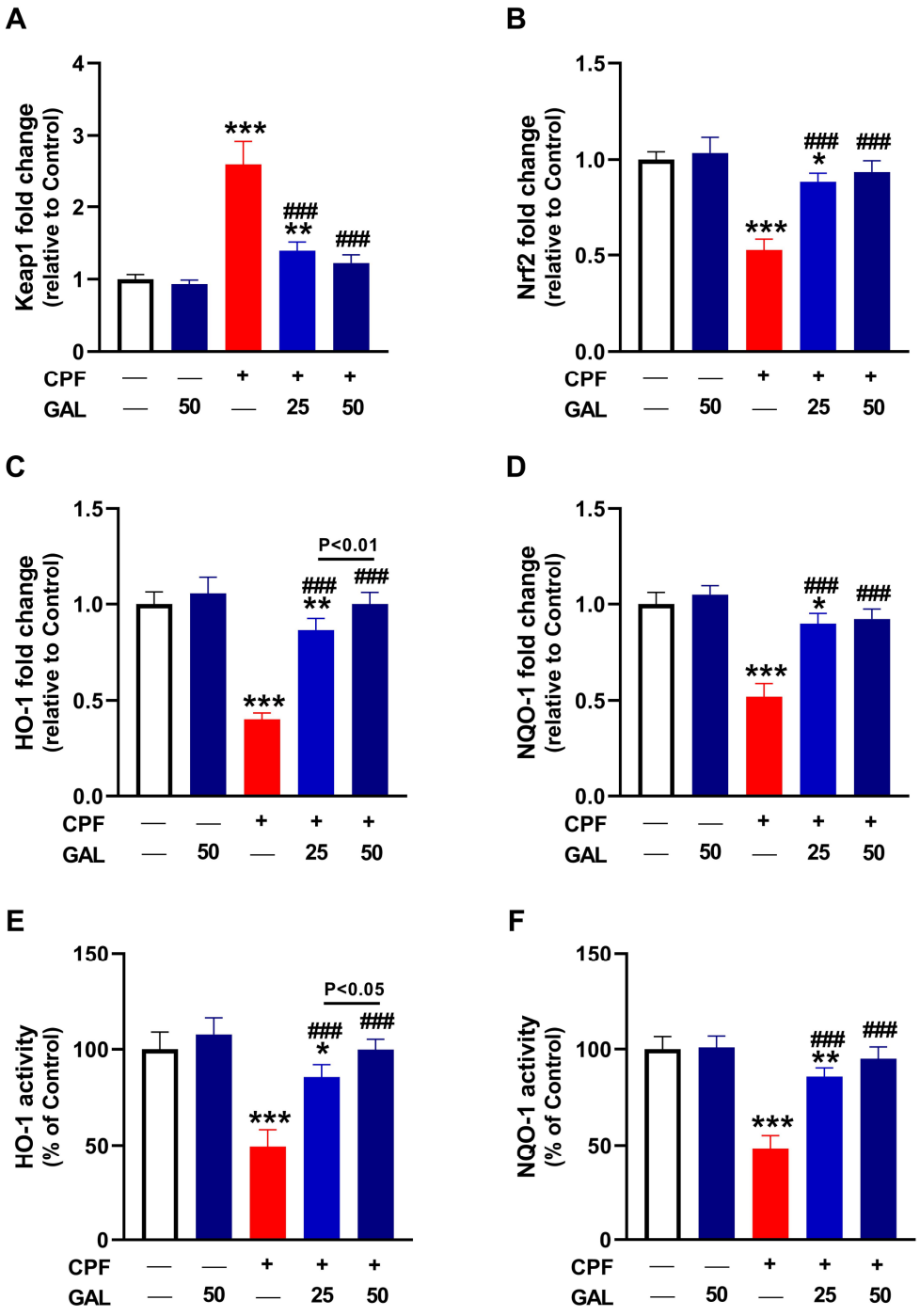

Fig. 6. GAL upregulated Nrf2/HO-1 signaling in the kidney of CPF-administered rats. GAL decreased Keap1 mRNA (A), Nrf2 (B), HO-1 (C) and NQO-1 mRNA (D), and HO-1 (E) and NQO-1 (F) activities in CPF-administered rats. Data are mean ± SEM, (n = 6). *P < 0.05, **P < 0.01 and ***P < 0.001 *vs* Control, and ###P < 0.001 *vs* CPF.

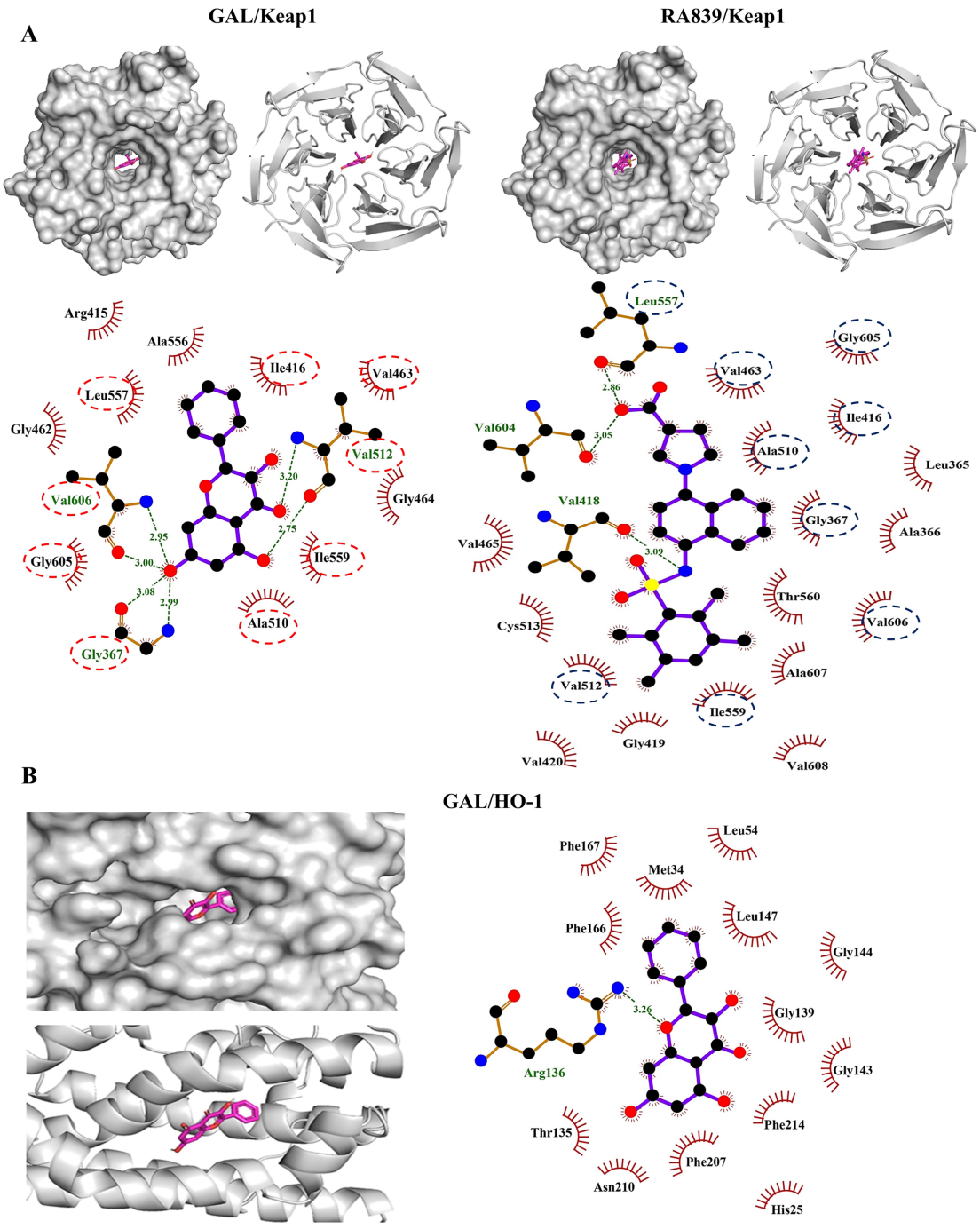

Fig. 7. Molecular docking of GAL and RA839 with Keap1 (A) and GAL with HO-1 (B) showing the crystal structure and amino acid residues involved in polar bonding and hydrophobic interactions. Circled amino acid residues in GAL/Keap1 and RA839/Keap1 complexes are the same.

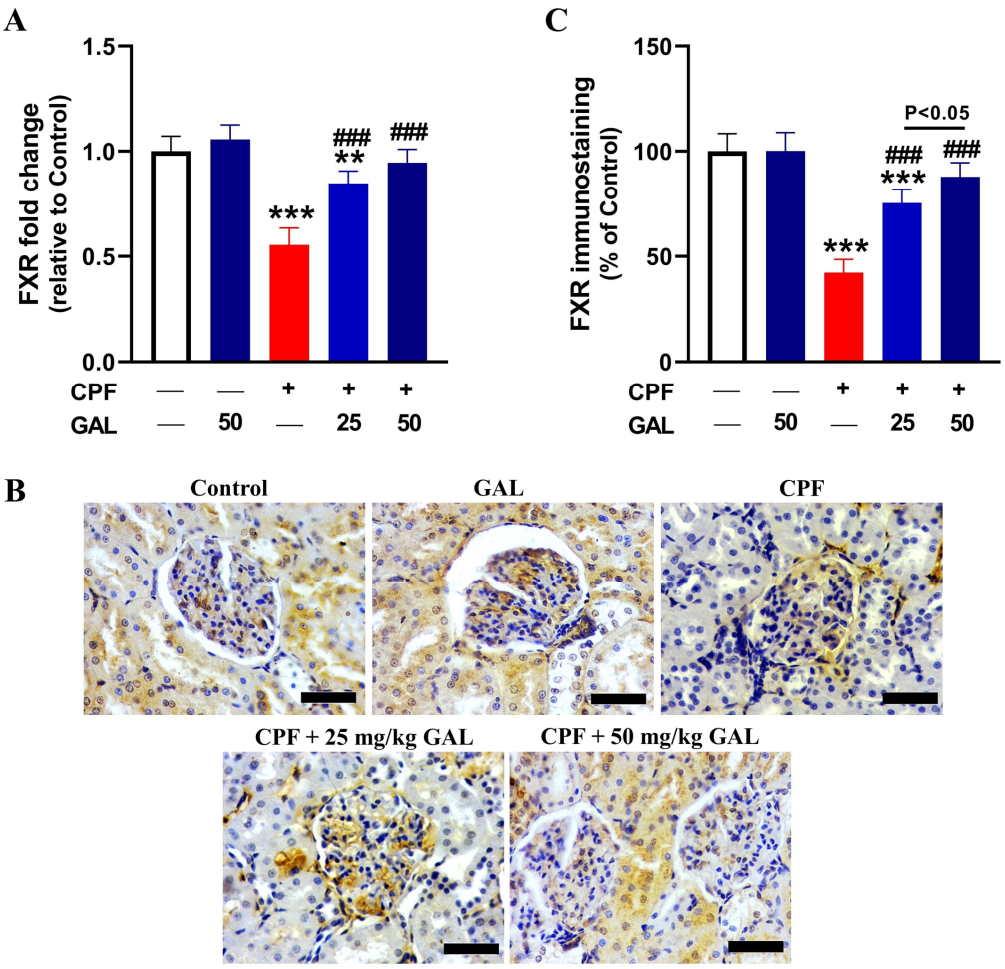

Fig. 8. GAL upregulated FXR in the kidney of CPF-administered rats. GAL increased FXR mRNA (A) and protein (B-C) in CPF-administered rats. Scale bar = 50 µm. Data are mean ± SEM, (n = 6). $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* Control, and $^{###}P < 0.001$ *vs* CPF.

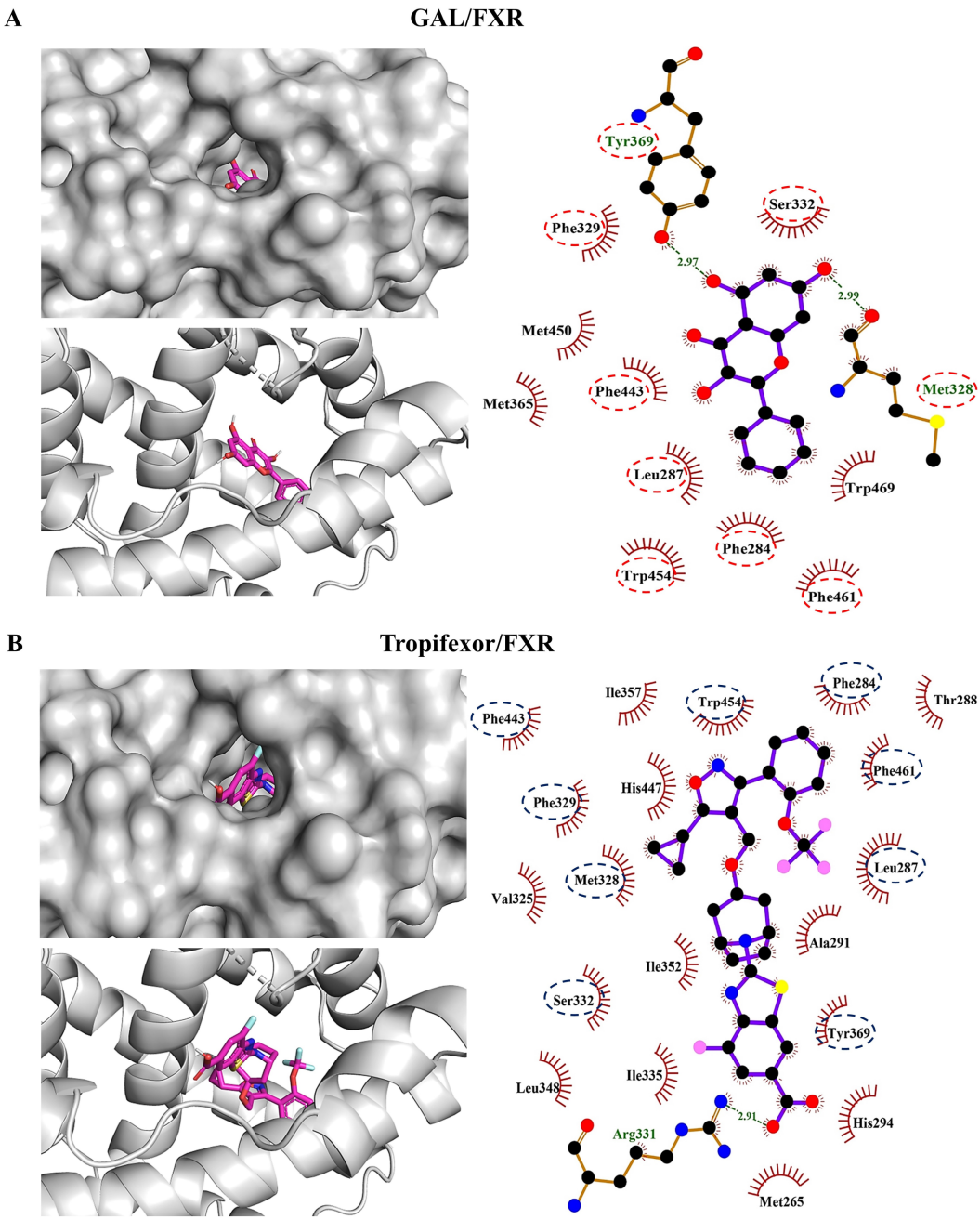

Fig. 9. Molecular docking of GAL (A) and tropifexor (B) with FXR showing the crystal structure and amino acid residues involved in polar bonding and hydrophobic interactions. Circled amino acid residues in GAL/FXR and tropifexor/FXR complexes are the same.