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L-Carvone ameliorates lipopolysaccharide-induced acute kidney injury in mice model

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Abstract

Background:

Acute kidney injury (AKI) is a common critical illness which is usually associated with sepsis-related endotoxemia and rapid decline in kidney function over short period. Carvone is chiral monoterpenoid ketone present in the essential oils of dill, caraway, and spearmint. This monoterpene exhibits some interesting biological activities such as antimicrobial, anti-fungal, anticonvulsants and anti-inflammatory properties. However, there is no evidence to show the protective effects of L-carvone on lipopolysaccharide (LPS)-induced acute kidney injury (AKI). Therefore, we investigated the protective effects of L-carvone on LPS-induced AKI in mice and the underlying mechanism. Forty-eight BALB/c male (20–30g) were divided into six groups: control group (mice received normal saline, Group I), model group (received LPS 10 mg/kg as a single dose Group II), Vehicle group (mice received corn oil for 5 days then LPS 10 mg/kg as a single dose on day5, Group III). Treatment groups (received either [(25mg/kg/day), Group IV] or [(50mg/kg/day), Group V] or [(100mg /kg) (Group VI] of oral L-carvone for 5 consecutive days before LPS injection then LPS 10mg/kg as a single I.P dose administrated at day 5. Twenty-four hours after LPS injection, blood samples and kidneys were collected for evaluation.

Results:

The results revealed that pretreatment with 25 mg/kg or 50 mg/kg or 100mg /kg L-carvone significantly *(P<0.05)* ameliorated LPS-induced AKI as evidenced by decreased serum urea and creatinine level, kidney injury molecule -1 KIM-1 mRNA leve and renal pathological manifestations. Meanwhile low, medium and high dose of Carvone also strikingly suppressed the inflammatory response as evidenced by significant decreased in the gene expression of Toll-like receptor 4 (TLR-4), transcription factors [(nuclear factor-kappa (NF-κB), activator protein-1 (AP-1) and Interferon Regulatory Factor 3 (IRF3)] in renal tissue, resulting in attenuation of proinfammtory cytokines (IL-1 β and TNF-α), iNOS. Increasing the dose of L-Carvone to 100 mg/kg showed further suppression in serum urea and creatinine, KIM-1, TLR-4, NF-κB and proinfammtory cytokines level indicating that the effect of L-carvone is dose dependent against inflammatory markers in our study.

Conclusion

Our findings indicate that L-carvone has protective effects against LPS-induced AKI by inhibiting TLR4-MYD88 dependent and independent signaling pathway and improving the renal function and structural kidney damage. Hence L-carvone providing a potential therapeutic effects for the LPS-induced SA-AKI.

Introduction

Acute kidney injury (AKI) is a multi-etiological clinical condition that affects 18–39 persons per thousand people[(1)]. Sepsis is a leading cause of death and morbidity in critically ill people around the world [(2)]. The core of sepsis is systemic inflammatory reactions in which the kidney is the most vulnerable organ [(3)]. The underlying pathophysiology of AKI in sepsis involves endothelial dysfunction ,intrarenal hemodynamic alterations, infiltration of inflammatory cells in the renal parenchyma, and impairment of tubules with necrotic cells and debris [(4)]. Many evidences suggests there is an extensive stimulation of the innate immune response, which accomplishes a defensive response that involve both cellular and humoral components [(5)]. This results in the release of several cytokines, most notably IL-1, TNF-a, and IL-6, which leads to cytokine storm, hemodynamic instability, and ultimately organ dysfunction and septic shock. [(6)].The lipopolysaccharide (LPS) is still the secondary cause of systemic inflammatory response syndrome[(7)]. It is a glycolipid part of the cell wall of gram negative bacteria such as Escherichia and Salmonella species[(8)]. LPS is considered as a pathogen-associated molecular patterns PAMPs by which bacteria can be recognized by certain host receptors known as pattern recognition receptors (PRRs) such as Toll-like receptor (TLR) [(9)]. LPS functions as a toxin by excessively activating the TLR4 signaling pathway, thereby triggering a sequence of intracellular processes. TRL4 participates in the induction of innate immune and inflammatory responses via two major signaling pathways (Myeloid differentiation MyD88-dependant and TIR-domain-containing adaptor-inducing interferon- (TRIF)-dependent pathway) leading to nuclear translocation of nuclear transcription factor kappa-B (NF-B), a ubiquitous rapid response factor implicated in immune and inflammatory reactions that stimulates the release of IL-1β and TNF-α[(10)]. NF-B also causes ROS-mediated peroxidation of lipids, damage to nucleic acids, and oxidation of proteins, which subsequently leads to cell death and tissue damage[(11)]. Despite a greater understanding of pathophysiologic processes of sepsis, the therapy of septic AKI remains beyond satisfaction. Thus, medicines that decrease inflammation and oxidative stress may be beneficial in the treatment of AKI caused by sepsis.

Carvone is a (5-isopropenyl-2-methyl-2-cyclohexenone), a monocyclic monoterpene ketone, with ($C_{10}H_{14}O$) chemical formula which has an asymmetric carbon. Carvone is contained in the essential oils of several aromatic and medicinal plants species of the Lamiaceae and Asteraceae families including Mentha spp.[(12)], Origanum spp., Rosmarinus spp., Thymus spp., and many others [(13)] . This monoterpene shows some remarkable biological actions. It shows, for example, antibacterial [(14)] ,antitumor [(15)] anti- fungal [(16)] and anti-inflammatory properties [(17)]

We hypothesized that L-carvone would be effective against septic AKI by controlling inflammation, based on the traditional medical use of carvone and its pharmacological properties. The purpose of the present work is to evaluate the potential renoprotective effect of

carvone against LPS-induced AKI and to investigate its underlying molecular mechanisms in rodent model.

Materials and Methods

Chemicals and reagents

L- Carvone was purchased from (Sigma, USA). The purity of the compound was certified to be 98% by high-performance liquid chromatography (HPLC). Lipopolysaccharide (LPS) from Escherichia coli O55:B5 was purchased from Sigma Chem. Co. (St. Louis, MO, USA) Diethyl ether was obtained from ROMAN pure chemistry, UK. Formaldehyde was provided from Sinopharm chemical reagent Co., Ltd, China. ELISA kits (TNF-α) was purchased from Shanghai, China. Serum urea and Creatinine assay kit reagents were supplied by AGAPPE DIGNOSTICS SWITZERL AND GmbH (Germany). KIM-1, NF-κB, IRF3, IL-1B, AP, and iNOS, Primers were purchased from Macrogen / South Korea. RNA extraction kit was purchased from TransGen biotech/ China. EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix were purchased from TransGen, biotech / China. TransStart® Top Green qPCR Super Mix was purchased from TransGen, biotech. /China.

Animals

Forty-eight healthy adult male Albino, BALB/c weighing between 20–30 gm were brought from and maintained in the animal house of the College of Pharmacy, University of Baghdad. The mice were housed at constant room temperature with a 12:12 h light-dark cycle combined with ad libitum feeding with a standard rodent diet and tap water.

Experimental design

The present study was reviewed and approved by the scientific and ethical committee of the College of Pharmacy, University of Baghdad. The animals were randomly divided into six groups and each group contained 8 mice as follows:

- **Negative control group (Group I)**: mice received 0.1 ml normal saline then euthanized after 24 hours
- **LPS- model group (Group II)**: mice received a single dose of intraperitoneal LPS in dose (10mg/kg) then euthanized after 24 hours.
- **vehicle control group (GroupIII):** mice received corn oil (0.1ml) orally as vehicle through five consecutive days. On the fifth day mice received LPS (10mg/kg) then mice were euthanized after 24 hours
- **Treatment group (Group IV):** mice received L-Carvone (**25mg/kg/day)** orally through five consecutive days. On the fifth day mice received a single dose of intraperitoneal LPS (10mg/kg)
- **Treatment group (Group V)**: mice received L-carvone (**50 mg/kg/day)** orally once daily for five constitutive days. On the fifth day, mice received a single dose of intraperitoneal LPS (10mg/kg)

- **Treatment group (Group VI)**: mice received L-carvone (**100 mg/kg/day**) by oral gavage once daily, for constitutive five days. In the fifth day, mice received a single dose of intraperitoneal LPS

  Twenty-four hours after the LPS injection euthanization was done by diethyl ether followed by cervical dislocation. Blood is collected via retro-orbital sampling and then centrifuged at 3000 rpm for 20 minutes at 4℃. After that, the serum was stored at -20℃ for urea and creatinine measurement.

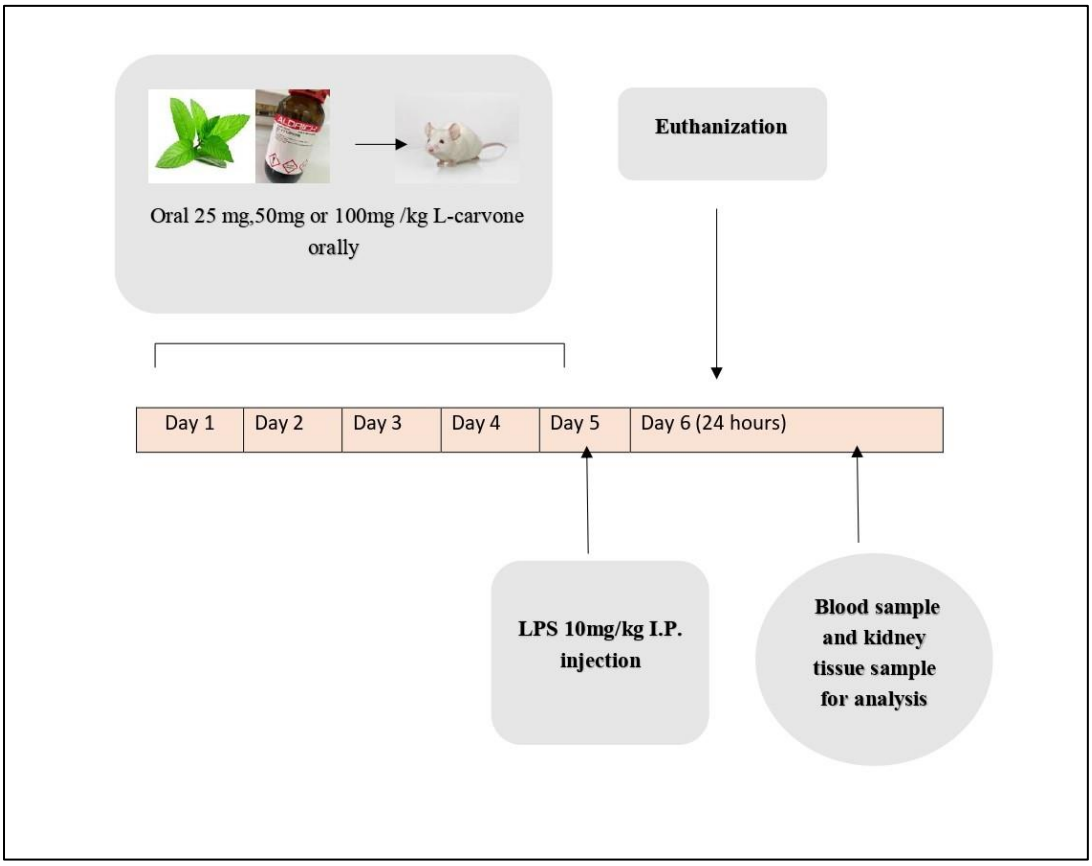


Figure (1): Schematic presentation of experiment protocol

Kidney tissue preparation:

Right kidneys were removed from all mice. Then, kidney tissue was divided into two parts for ELISA and PCR analysis.

Histopathological examination:

Twenty-four hours after LPS challenge, mice were sacrificed and their left kidneys were removed for histopathological examination. For morphological examination, each tissue was fixed in 10% formalin, embedded in paraffin, sectioned, and stained with Hematoxylin and Eosin.

The semi-quantitative score for kidney injury was considered for each animal observed in blinded manner. The percentage of tubules in renal cortex that showed epithelial necrosis, brush

border loss, and tubular dilation were scored as follows: 0, normal kidney; 1, <25%; 2, 25–50%; 3, 50–75%; 4, >75% [18] .

Biochemical measurements:

Renal function was assessed in mice of all groups at the end of experiment. Serum urea and creatinine, as important index of renal injury severity, were measured for the assessment of renal function. After 24 hours of treatment, serum was drawn from each animal and the concentrations of urea and creatinine were analyzed by semi-automated biochemical analyzer using commercial kit reagents following the manufacturer's instructions.

RNA Extraction and Real-Time RT-PCR

Real-Time PCR was used to determine the mRNA expression levels of NF–κB, IRF3, iNOS, IL-1b, AP and KIM-1. The kidney tissue had been isolated and washed to remove any blood then the tissue minced to small pieces. 50-100 mg from tissue was put in each tube containing 1 ml of TRIzol and frozen for later use and total RNA was isolated from the kidney tissue by using TransZol Up Plus RNA Kit (TransGen, biotech. ER501-01) according to the instructions of the manufacturer. Then DNA-free total RNA was reverse-transcribed and the complementary DNA was synthesized by using of EasyScript® one-step gDNA removal and cDNA synthesis supermix (**TransGen, biotech. AE311-02**). The mRNA expression levels of NF-kB, IRF3, iNOS, IL1-b, AP and KIM-1 were analyzed using qRT-PCR and the SYBR Green Supermix (**TransGen, biotech. AQ131-01**). For the purposes of qRT-PCR analysis, GAPDH was used as the reference for RNA quality and differences among samples. The primers were synthesized by, Macrogen / South Korea and the sequences are shown in table 1.

Table 1: Sequences of the primers for quantitative real-time PCR.

Primer	**Sequence (5′→3′ direction)**
F-GAPDH	CGGGTTCCTATAAATACGGACTG
R-GAPDH	CCAATACGGCCAAATCCGTTC
F-NF-κB	AAGACAAGGAGCAGGACATG
R- NF-κB	AGCAACATCTTCACATCCC
F-TLR4	TCCCTGCATAGAGGTAGTTCC
R-TLR4	TCAAGGGGTTGAAGCTCAGA
F- Kim-1 (Havcr1)	GGCTCTCTCCTAACTGGTCA
R- Kim-1 (Havcr1)	CCACCACCCCCTTTACTTCC
F-AP-1	AG GCTGCAGGATGATGCGAT
R-AP-1	TTCTAGCCAGGACGACTTGC
F-IRF3	CAATTCCTCCCCTGGCTAGA
R-IRF3	GGGATCCTGAACCTCGTTCG
F- iNOS	GGTGAAGGGACTGAGCTGTT
R-iNOS	ACGTTCTCCGTTCTCTTGCAG
F- IL-1β	TGCCACCTTTTGACAGTGATG
R- IL-1β	TGATGTGCTGCTGCGAGATT

Enzyme-Linked Immunosorbent Assay analysis of TNF-α

Various groups of kidney tissues were homogenized with PBS (pH7.4) on ice, and then separated by centrifugation for 20 minutes at 2000-3000 RPM. The supernatants were subsequently collected to determine the concentration of **TNF-α** accordance with the manufacturer 's instruction.

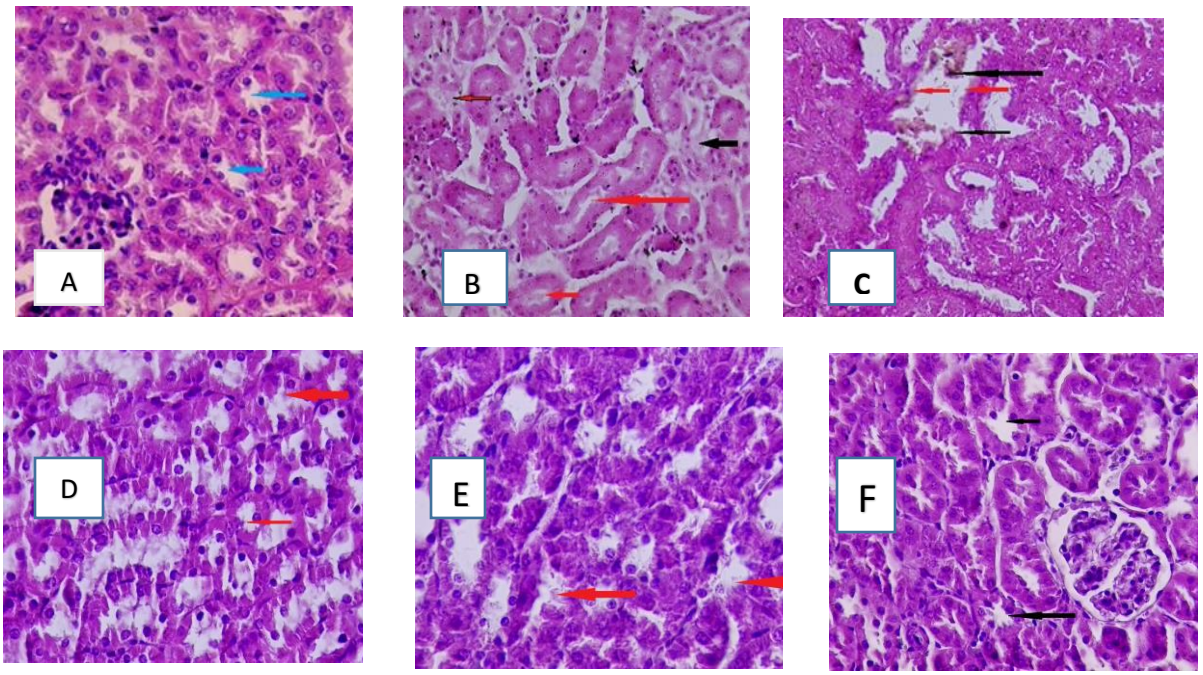
Statistical analysis

The data were presented as the mean ± standard error of mean (SEM). The analysis was performed using Statistical Package for the Social Sciences, version 25 (SPSS, version 25). One-way analysis of variance (ANOVA) and the Tukey test were utilized to assess the group differences. Independent t-test was used to compare the means among treatment groups. The differences between the groups were considered statistically significant when the *P* value was less than 0.05 *(P<0.05).*

Results:

Effects of Carvone on LPS-induced histopathological abnormality

Hematoxylin and Eosin (H&E) staining revealed that LPS injection induced histological alterations, such as tubular cells sloughing, loss of the brush border, and tubular dilation in the cortex (figure 2B) compared with the control group (figure 2A). The tubular score results revealed that these histopathological changes were significantly ($p < 0.05$) attenuated in treatment groups (group IV, group V & group VI) when compared with group II (LPS group) as shown in figure 3.



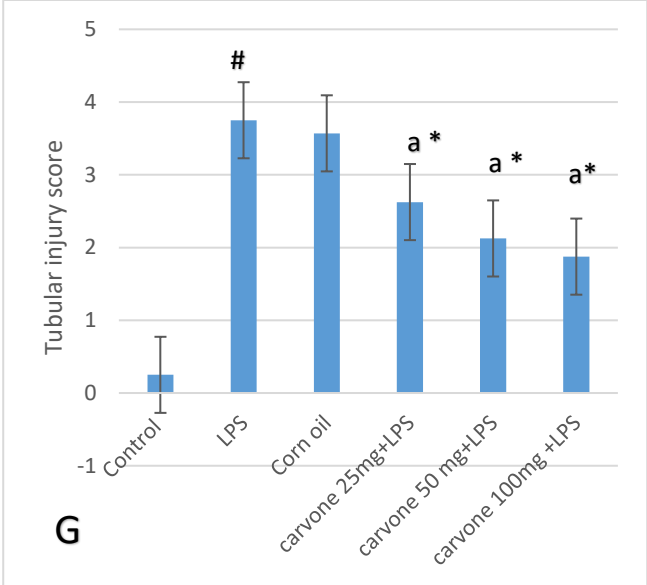


Figure (2): Effects of L- Carvone on LPS-induced pathological kidney changes. Representative histopathological images stained by H&E and formalin-fixed kidney tissues from each group (X40 magnification) Blue arrow: normal proximal tubules. Black arrow: tubular cells sloughing. Red arrow: Tubular dilation and loss of brush border.
(A) normal (B) LPS group (C) corn oil group (D) carvone 25 mg+LPS (E) carvone 50 mg+LPS (F) carvone 100mg +LPS (G) Tubular injury score.
Each value represents mean ± SEM
***denotes significant difference (*P*<0.05) vs LPS group.**
 # denotes significant difference (P<0.05) vs control group.
(a, b and c) non -identical small letters denotes significant difference (P<0.05) among treatment groups.

Effects of L-Carvone on kidney function in LPS-induced AKI model

As shown in (figure 4 A, B) after 24 hours of LPS injection the animals showed sharp increase in serum urea and creatinine level compared to control group (saline group). For creatinine level (LPS ,80.99 ±2.60 Vs control 23.34 ± 2.08) and for urea (116.35±17.35 µmol/L vs 34.59 ±3.55 µmol/L). Moreover, the doses of L-carvone in group IV, V & VII were significantly decreased the serum levels of creatinine when compared to LPS group [(31.3891±1.01 µmol/L, 31.38 ±1.19 µmol/L & 22.63± 0.99 µmol/L) Vs 80.99± 2.60 µmol/L)] respectively. Our current results showed, there were a significant difference in creatinine level in group IV (25 mg/kg carvone) &V (50mg/kg carvone) when compared with the high dose of L-carvone that mice received in group VI (100 mg/kg L-carvone) (31.38 ±1.01 & 31.3891±1.19 vs 22.63 ±0.99) respectively.

Regarding urea serum concentration, the results revealed that, L-carvone doses (25 mg/kg ,50mg/kg,100 mg/kg) reduced the serum urea level compared to urea level in LPS administered group [(57.41±5.12 mg/dl, 40.31±1.92 mg/dl & 40.48±2.14 mg/dl) Vs 116.35±17.35 mg/dl)]

respectively. Our findings regarding the effect of three doses of L-carvone on serum urea concentration, showed a significant difference *(P<0.05)* in urea concentration between GPIV (25 mg/kg) and GPV (L- carvone 50 mg /kg) (57.41±5.12 vs 40.31±1.92) and significant difference in urea concentration between group IV (25 mg/kg) and Group VI (100 mg/kg) & (57.41±5.12 vs 40.48 ±2.14).

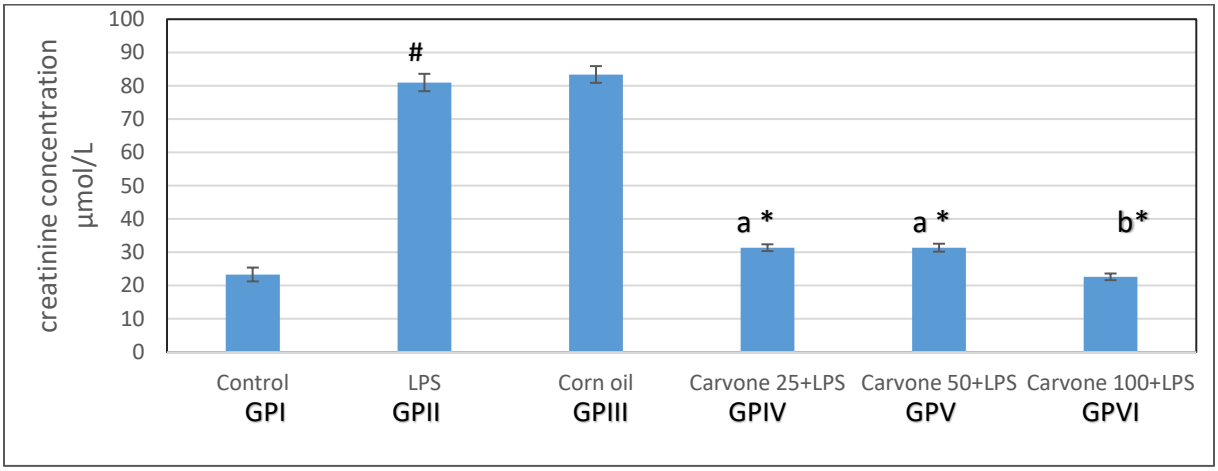


Figure (3A): Effect of L-carvone on serum creatinine concentration.
Values are indicated as mean ± SEM. (n=8) for each group
*** denotes significant difference (P<0.05) vs LPS model group (GPII).**
 # denotes significant difference (P<0.05) vs control group (GPI).
(a, b and c) non -identical small letters denotes significant difference (P<0.05) among treatment groups

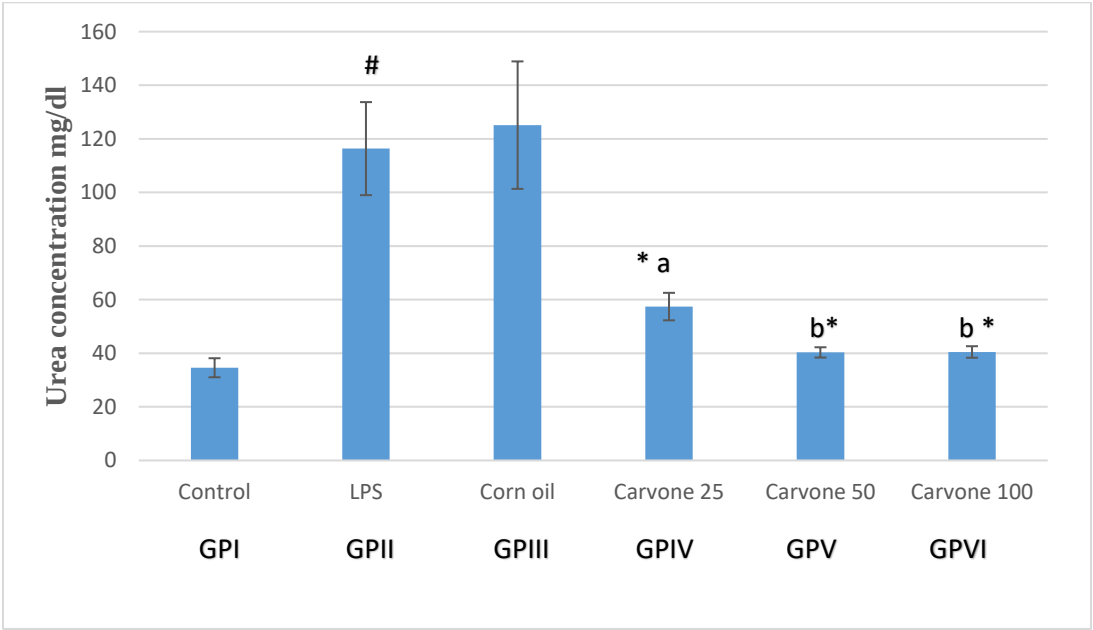


Figure (3B): Effect of L-carvone on serum urea concentration.
Values are indicated as mean ± SEM. (n=8) for each group

*** denotes significant difference (P<0.05) vs LPS model group (GPII).**
 # denotes significant difference (P<0.05) vs control group (GPI).
(a, b and c) non -identical small letters denotes significant difference (P<0.05) among treatment groups

Effect of L-Carvone on Kidney injury molecule-1 (KIM-1) in LPS induced AKI model

The results revealed that KIM-1 gene expression significantly elevated (*P*<0.05) in LPS group when compared to control group (54.95±7.38 vs 3.38±1.00) as shown in figure (5). Pretreatment with L-Carvone (25 mg /kg ,50 mg/kg and 100 mg/kg) exhibited a significant decline in KIM-1 level when compared with the LPS model group [(31.97±2.00 , 23.23±4.97 & 20.88± 4.29) vs (LPS, 54.95 ±7.38)]. On the other hand, the results showed that, there was a significant difference in KIM-1 expression in mice group treated with lowest dose of L-carvone GPIV (25mg /kg) compared to high dose in GPVI (100mg/kg) , while there was no significant difference *(P>0.05)* in KIM-1 level in GPV compared to GPIV &GPVI.

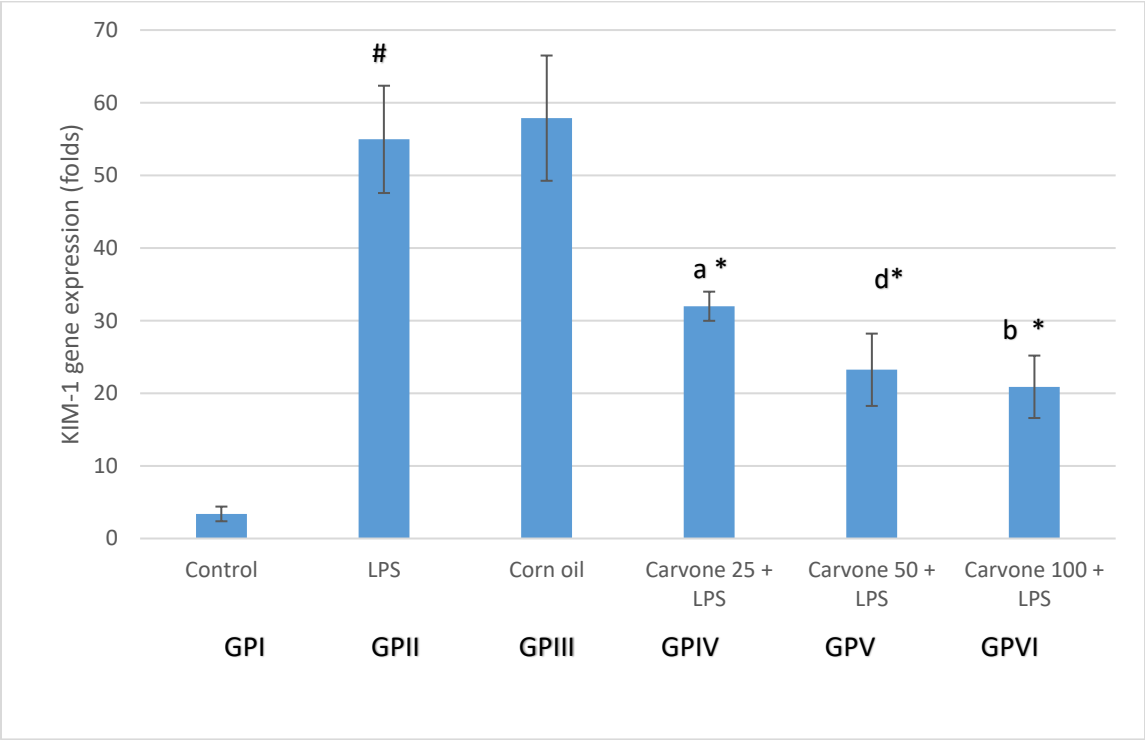


Figure (4): Effects of carvone on KIM-1 gene expression level in renal tissue in LPS induced AKI.
Values are indicated as mean ± ± SEM. (n=8) for each group
*** denotes significant difference (*P<0.05*) vs LPS group.**
 # denotes significant difference (*P<0.05*) vs control group (GPI).
(a, b and c) non -identical small letters denotes significant difference (*P<0.05*) among treatment groups
(d) small letter denotes no significant difference (*P>0.05*) compared to GPIV& GPVI treatment groups

Effect of L-Carvone on Toll-like receptor TLR4 expression level in LPS induced -AKI model

As shown in figure (6), LPS injection in GPII significantly up-regulated the expression of TLR4 compared to control group (15.74 ±2.92 vs 1.98± 0.72). The results revealed that, mice received L-carvone 25mg/Kg showed TLR4 mRNA downregulation significantly compared to LPS group (0.94±0.43 vs. 15.74 ±2.92). Furthermore, administration of carvone 50 mg/Kg also significantly attenuated the mRNA level of TLR4 compared to non-treated mice (LPS group) (1.23±0.35 vs. 15.74 ±2.92) respectively. However, the increment in L-carvone dose to 100mg/kg/day showed significant reduction in TLR4 level (1.33± 0.15) compared to LPS group (15.74 ±2.92). L-Carvone seems to have dose-independent effect on TLR4 renal expression.

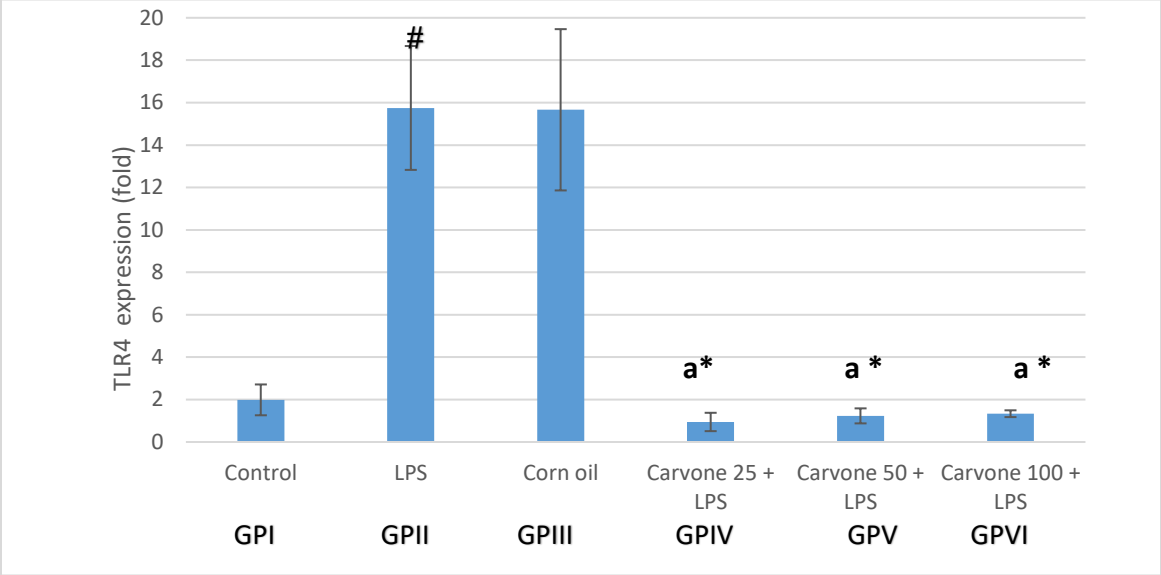


Figure (5): Effects of carvone on TLR4 gene expression level in kidney tissue in LPS induced AKI. Values are indicated as mean ± ± SEM. (n=8) for each group
*** denotes significant difference (P<0.05) vs LPS group.**
 # denotes significant difference (P<0.05) vs control group (GPI). Identical small letters denote non-significant difference (*P>0.05*) among treatment groups.

Effect of L-Carvone on gene expression of NF-κB in LPS-induced AKI model

Data presented in figure (7) showed significant elevation in the expression of NF-κB (*P<0.05*) after 24 hours from LPS administration (group II) compared to the saline control group (21.70 ± 2.50 versus 1.37± 0.39) respectively.

The pre-administration with L-carvone at dose of 25 mg/kg/day (group IV) significantly decreased the NF-κB expression (3.41± 0.46) when compared to LPS group (group II) (21.70 ± 2.50). Furthermore, increasing the dose of L-carvone to 50 mg/kg/day and 100mg/kg/day revealed a significant reduction in NF-κB expression (3.05±0.60 and 1.13±0.37) respectively. Furthermore, the statistical analysis indicated a significant variation between GPIV (the lowest dose of L-carvone) and GPVI (highest dose) (3.41±0.46 VS 1.13±0.37) respectively. A

significant difference also revealed when we compared (50mg/kg) dose of carvone with the highest dose (100mg/kg in GPVI) (3.05± 0.60 Vs 1.13± 0.37) respectively, while no significant difference revealed in (50mg/kg) treated mice GPV compared to (25mg/kg) treated mice in GPIV.

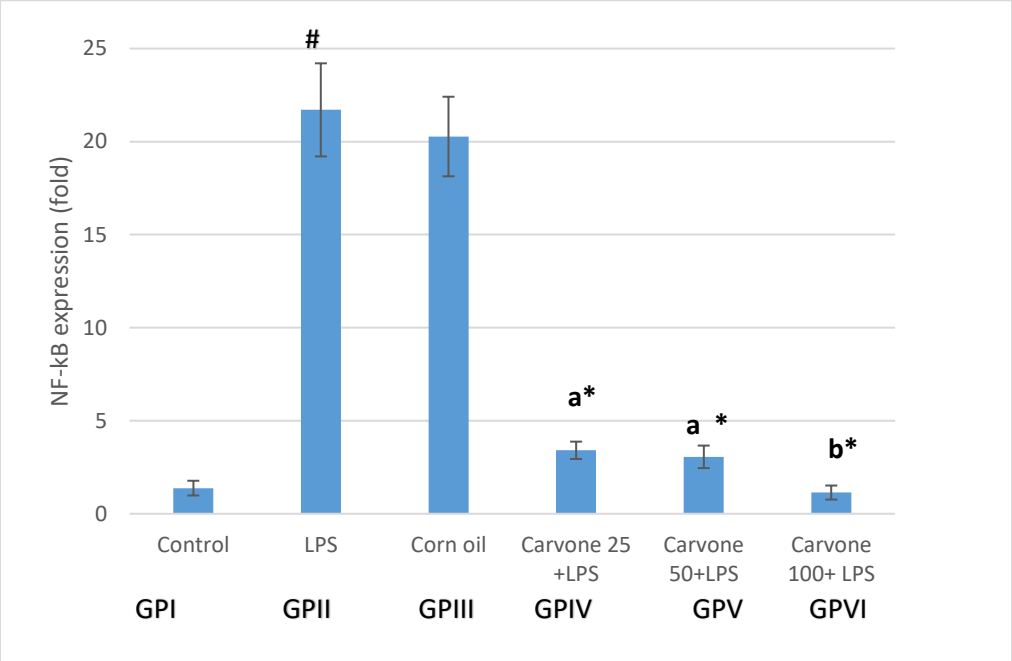


Figure (6): Effects of carvone on NF-κB gene expression level in kidney tissue.
Values are indicated as mean ± SEM. (n=8) for each group
*** denotes significant difference (P<0.05) vs LPS group.**
 # denotes significant difference (P<0.05) vs control group (GPI).
(a, b and c) non -identical small letters denotes significant difference (P<0.05) among treatment groups

Effect of L-Carvone on activator protein-1 (AP-1) in LPS induced AKI model

The results of this study exhibited that administration of LPS in group II resulted in a significant increase of AP-1 gene expression compared to the negative control group (group I) (18.16 ±1.98 vs 1.41±0.47) respectively. The administration of L-carvone (25 mg ,50mg and 100 mg/kg (group IV, V & VI) revealed a significant attenuation in AP-1 mRNA expression when compared to LPS group [(4.48±0.96, 2.49± 0.50 & 1.16± 0.35) vs (18.16 ±1.98)] respectively. Additionally, significant difference *(p<0.05)* was demonstrated between GPIV (L-carvone 25mg/kg) and GPVI (L-carvone 100mg/kg) in mRNA level of AP-1 (4.48± 0.96 Vs 1.16±0.35) respectively. While our findings showed that there was no significant difference (*P>0.05)* in AP-1 renal tissue level in GPV compared to GPIV and GPVI.

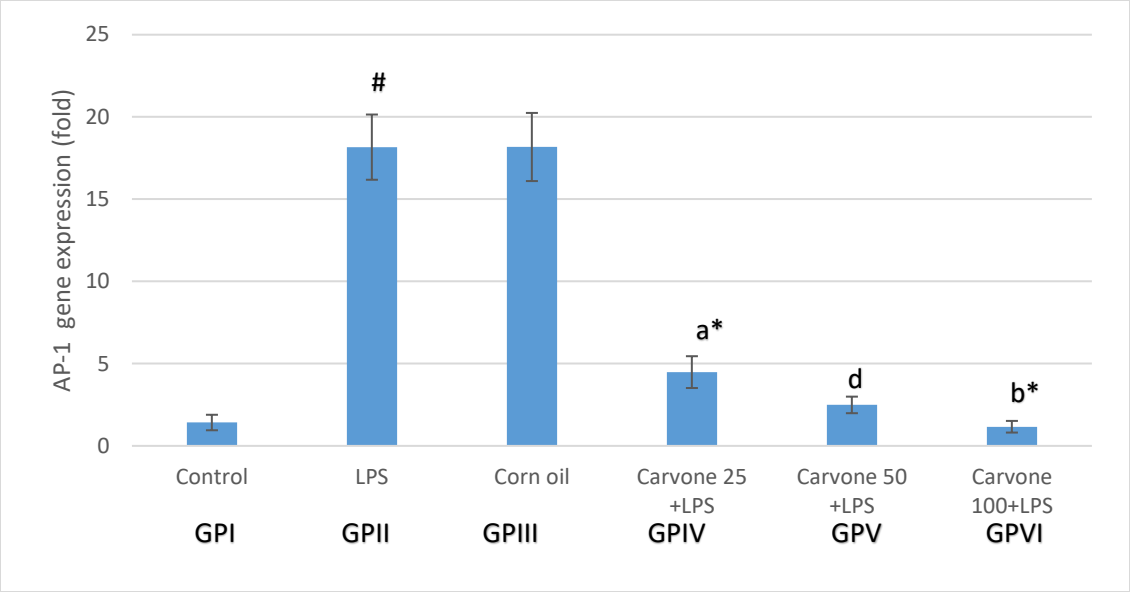


Figure (7): Effect of Carvone on gene expression levels of AP-1 in kidney tissue in LPS induced AKI. Values are indicated as means ± SEM. (n=8) for each group.
*** denotes significant difference (*P<0.05*) vs LPS group.**
 # denotes significant difference (*P<0.05*) vs control group (GPI).
(a, b and c) non -identical small letters denotes significant difference (P<0.05) among treatment groups.
(d) small letter denotes no significant difference (*P>0.05*) in GPV compared to GPIV& GPVI treatment groups

Effects of L-Carvone on TNF-α level of kidney tissue in LPS –induced AKI model

Renal tissue level of TNF-α was increased after 24 hours of LPS injection in GPI compared to control group (*p<0.05*) (655.97±19.13 Vs 191.29 ±13.08). Moreover, the results of our study showed that TNF-α levels in tissue homogenate for mice in all treated groups (GPIV (25 mg/kg L-carvone, GPV 50 mg/kg L-carvone & GPVI 100mg/kg) were significantly reduced (*P<0.05*) (285.88±17.59, 235.23±20.21 & 228.93±18.57 ng/L) compared to TNF-α levels in tissue homogenate for mice of LPS group (655.97±19.13 ng/L) as showed figure (9). Furthermore, increment the dose of L-carvone to 100mg/kg showed significant reduction (*P<0.05*) in TNF-α renal level compared to lowest dose 25mg/kg (228.93± 18.57 Vs 285.88 ±17.59) respectively. While the difference between (GPIV &GPV) as well as between (GPV&GPVI) in TNF-α level was non-significant (*P>0.05).*

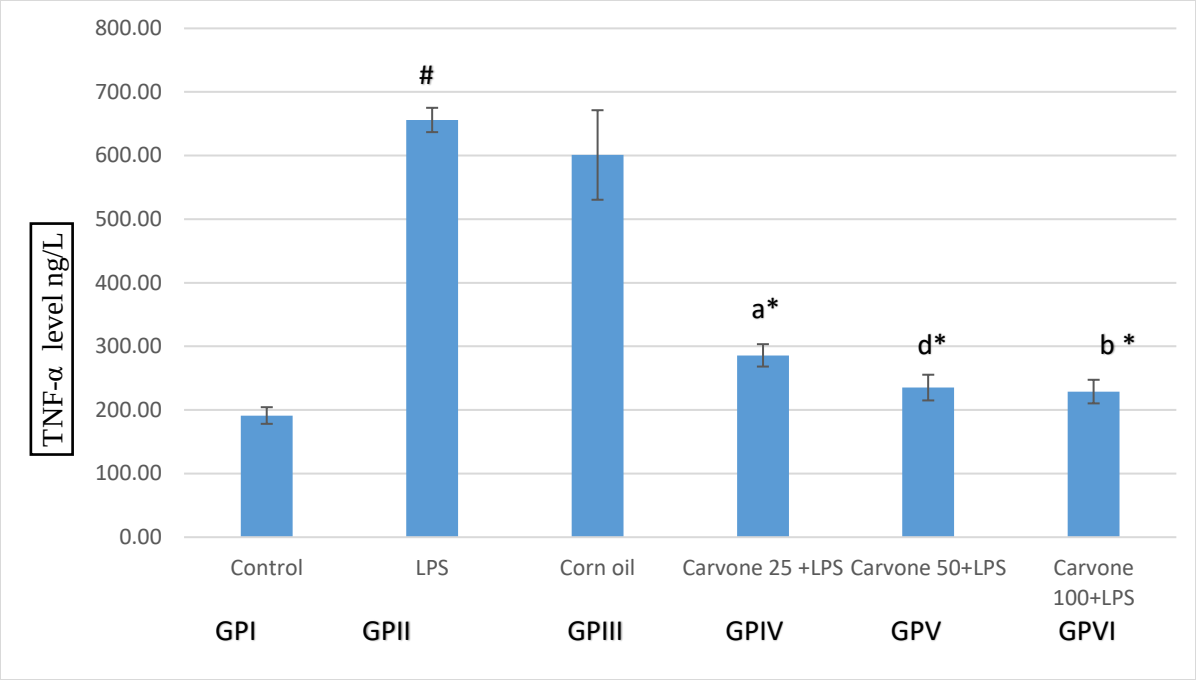


Figure (8): Effect of Carvone Effects of L-carvone on TNF-α level in kidney tissue in AKI mice model. Values are indicated as mean ± SEM. (n=8) for each group
*** denotes significant difference (*P<0.05*) vs LPS group.**
 # denotes significant difference (*P<0.05*) vs control group (GPI).
(a, b and c) non -identical small letters denotes significant difference (*P<0.05*) among treatment groups
(d) small letter denotes no significant difference (*P>0.05*) compared to GPIV& GPVI treatment groups

Effects of L-carvone on Interleukin 1 β (IL-1β) gene expressions in LPS induced AKI model

The real-time PCR data exhibited that the gene expression levels of IL-1β in renal tissue significantly increased in LPS group in comparison to control group *(P<0.05*) (49.08±5.23 vs 3.58±1.98) as shown in figure (10). While IL-1β levels in tissue homogenate in pre-treated mice with (25 mg/kg, 50 mg/kg & 100 mg/kg carvone) showed a significant reduction (*P<0.05)* (22.24±2.67, 12.65± 2.68 and 10.28±1.53) respectively when compared to IL-1β mRNA level in tissue homogenate of mice in model group (49.08±5.23). Statistically L-Carvone seems to have dose-independent effect on IL-1B.

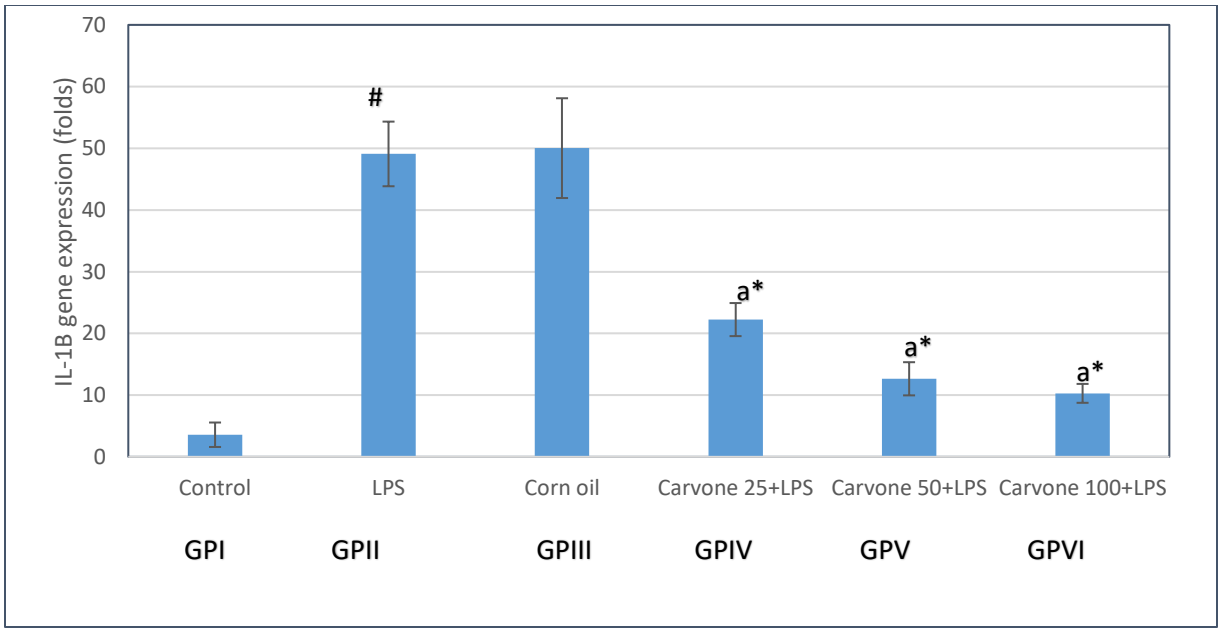


Figure (9): Effect of Carvone on gene expression levels of IL-1β in kidney tissue in LPS induced AKI. Values are indicated as mean ± SEM. (n=8) for each group
*** denotes significant difference (P<0.05) vs LPS group.**
 # denotes significant difference (P<0.05) vs control group (GPI).
(a, b and c) non -identical small letters denotes significant difference (*P<0.05*) among treatment groups

Effect of L-Carvone on inducible nitric oxide synthase (iNOS) in LPS induced AKI model

The Data presented in figure (11) showed a significant elevation (*P<0.05*) in iNOS gene expression in renal tissue after 24 hours from LPS administration as compared with the control group (40.87±5.59 vs 2.23±1.00). Interestingly the pretreatment with L-carvone at a dose of (25mg/kg) produced a significant reduction (*P<0.05*) in iNOS gene expression compared with the LPS model group (2.72±0.82 vs 40.87±5.59). Furthermore, multiplication the dose of L-carvone to 50mg/kg and 100 mg/kg exhibited significant reduction (*P<0.05*) in iNOS levels as compared to the LPS model group (2.26±0.89 and 1.66±0.73)respectively vs (40.87 ± 5.59). Regarding the effect among the three doses of L-carvone on iNOS expression, the results exhibited that, there was no significant variation *(P>0.05)* in iNOS mRNA level, hence the effect of L-carvone was dose -independent.

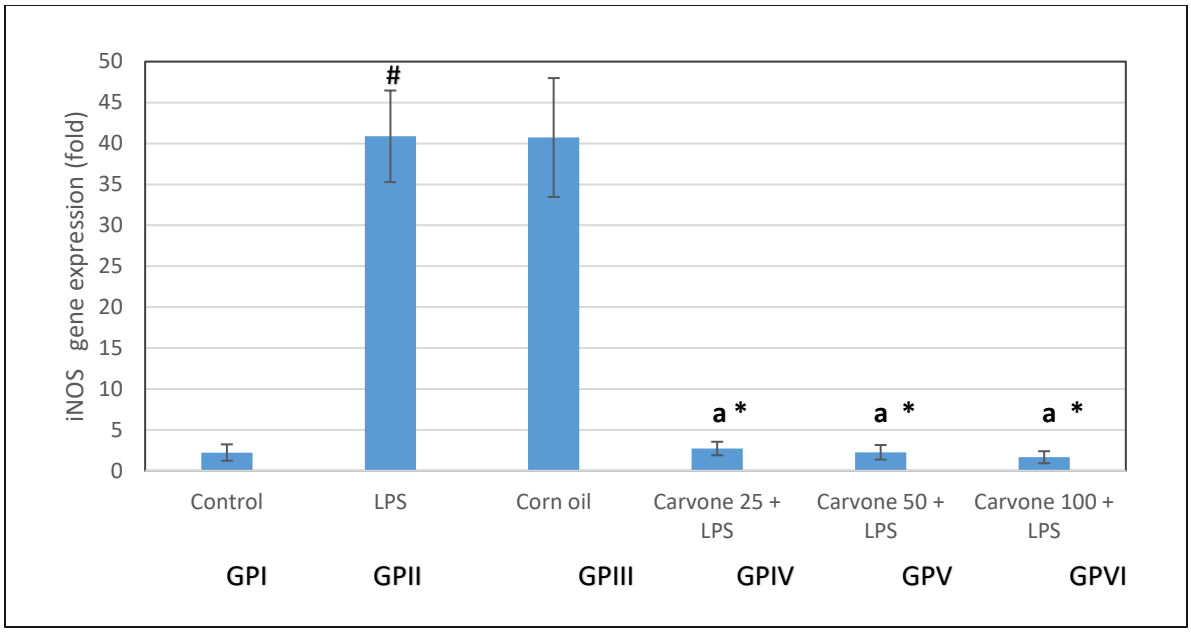


Figure (10): Effect of Carvone on gene expression levels of iNOS in kidney tissue in LPS induced AKI. Values are indicated as means ± SEM. (n=8) for each group.
*** denotes significant difference (P<0.05) vs LPS group.**
 # denotes significant difference (P<0.05) vs control group.
(a, b and c) non -identical small letters denotes significant difference (*P<0.05*) among treatment groups

Effect of L-carvone on Interferon Regulatory Factor 3 (IRF3) in kidney tissue of LPS induced AKI model

Our results revealed that administration of LPS in group II resulted in a significant increase in renal IRF3 levels as compared to the negative control group (*P<0.05*) (37.14 ±2.99 vs 2.10 ± 0.84) respectively. Besides, the pretreatment with L-carvone with different doses in group IV, V& VI exhibited significant attenuation in IRF3 level in comparing with LPS group ((2.94±0.95, 3.46± 0.64 & 3.05 ±1.17) vs (37.14 ±2.99)) respectively. At the same time, there were no significant differences *(P>0.05)* in the levels of IRF3 among the treatment groups IV, V& VI. Hence the effect of L-carvone on IRF3 renal expression was dose-independent.

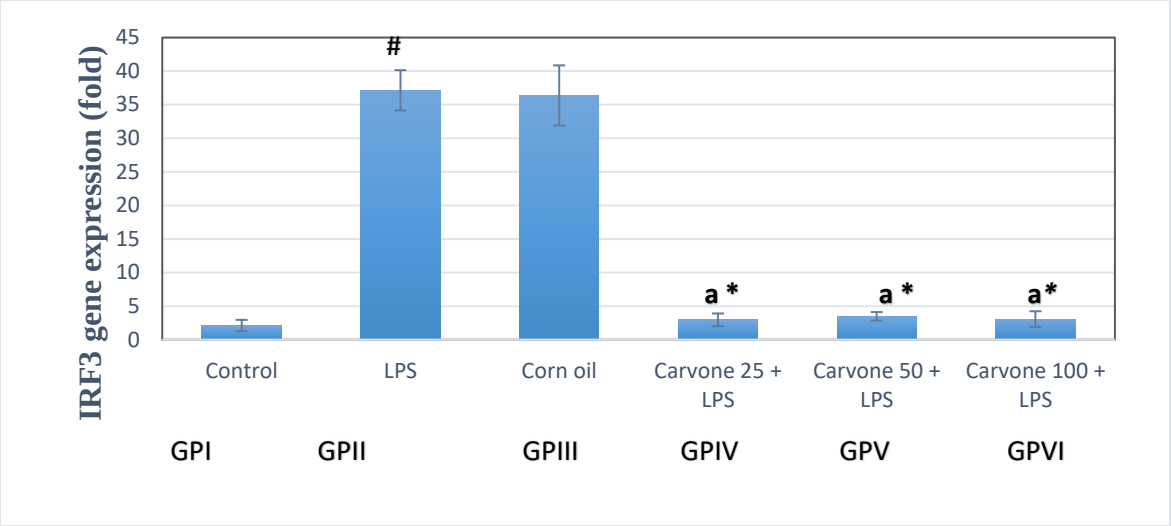


Figure (11): Effect of Carvone on gene expression levels of IRF3 gene expression in kidney tissue in LPS induced AKI. Values are indicated as means ± SEM. (n=8) for each group.
*** denotes significant difference (P<0.05) vs LPS group.**
 # denotes significant difference (P<0.05) vs control group (GPI).
(a, b and c) non -identical small letters denotes significant difference (*P<0.05*) among treatment groups

Discussion

AKI is a prevalent and difficult worldwide problem[19]. It has been established that sepsis-induced AKI demonstrates considerable damage mediated by the inflammatory response Therefore, there is an urgent necessity to explore therapeutic medicines that can halt the development of AKI caused by LPS. Interestingly the effects and exact mechanisms of Carvone in the pathophysiology of LPS-induced SA-AKI have not been studied previously.

In this study, an animal model of AKI was established by LPS I.P injection in mice. It was found that, the injection of 10 mg /kg LPS induced renal dysfunction by increasing serum urea and creatinine and induced renal pathological changes, observed as a tubular cells sloughing , loss of the brush border, and tubular dilation in the cortex which was consistent with previous studies [20] [21]. The current results showed that increasing the dose of L- carvone to 100mg/kg further attenuated the creatinine and urea concentration. These outcomes in part could be attributed to the effect of LPS on the up-regulation of inflammatory cytokines, such as TNF-α and IL-1β. Studies showed that TNF-α was closely related to the extensive tubular damage and IL-1β also plays an important role in the pathogenesis of kidney inflammation and tissue damage[22][23]. Inhibition of these inflammatory cytokines could protect against LPS-induced AKI. Recent studies of AKI have focused on identifying biomarkers that are characterized as. KIM-1 is one of the most promising biomarker that refers to the structural damage of kidney. It is early, noninvasive, and sensitive indicators of AKI [24]. Overexpression in KIM-1 level after 24 hours of I.P. LPS injection was shown in current study as with other study[25]. In deed our results revealed that, pretreatment with all doses of L-carvone decreases KIM-1 level in renal tissue. Furthermore the release of KIM-1 from damaged renal tissue also leads to the

downstream activation of transcription factors that regulate the expression of inflammatory cytokines and chemokines [24].

TLR4 is extremely expressed in immune cells, glomeruli in both, podocytes and mesangial cells and mainly in tubular epithelium.TLR-4 expression appears dynamic with a low constitutive level that increases in response to LPS [26]. Our findings regarding TLR4 receptor showed that the expression was significantly increased in the LPS model group after 24 hours compared to the control saline group which was consistent with previous studies [20] At the same time, L-Carvone in dose of 25,50 and 100 mg/kg can cause significant reduction in its level which may prove the anti-inflammatory activity of L-carvone against AKI. In our study the downstream signaling of TLR4 was studied to prove the anti-inflammatory effect of L-Carvone. TLR4 signaling upon binding to LPS can be divided into MyD88-dependent and -independent pathways [27]. The MyD88-dependent pathway is responsible for NF-κB and pro-inflammatory cytokines expression [28]. NF-κB is a transcription factor that plays a central role in the onset of inflammation [29].The activation of NF-κB induces the expression of various inflammatory genes, including iNOS and tumor necrosis factor-α (TNF-α). The gene expression of these inflammatory mediators is also regulated by AP-1, another transcription factor that is crucial for inflammation[30]. The results of current study that NF-κB signalling pathway was activated in the in renal tissue of mice injected with LPS and the pre-treatment with L-carvone was significantly attenuated the NF-κB , AP-1 expression compared to LPS model group. Interestingly further attenuation in the level of these transcription factors were observed with 100 mg /kg.Our results revealed that all doses of carvone exhibited a significant attenuation in proinfammtory cytokines level (TNF-α & IL-1β) compared with LPS group ,these results in agreement with other study in which L-carvone 50mg/kg & 100mg/kg produced a significant reduction in TNF-α level in irinotecan-induce intestinal mucositis in mice[31]

The pathogenesis of sepsis-induced AKI involves systemic vasodilation leading to decrease in systemic vascular resistance by iNOS activation[32]. Nitric oxide NO plays a crucial role in tubular endothelial cells function[33]. Additionally, excessive induction of iNOS in the kidney, predominantly in the proximal tubules the medulla may be the basis of peroxynitrite-related tubular injury as a consequence of local production of reactive oxygen and nitrogen species (ROS and RNS) during systemic inflammation[34].In the present study, L-Carvone significantly inhibited the mRNA expression of iNOS in renal tissue along with restoring tubular function.

After early TLR4 activation (MyD88 dependent), the receptor is forwarded towards the endosome and stimulates a late phase of NFκB stimulation and IFN production [26]. The activation of the MyD88-independent pathway includes phosphorylation and nuclear localization of transcription factor IRF3, triggering the type I IFN response and also prompting late-phase NF-κB activation with the subsequent inflammatory cytokines gene expression [35]. Our findings showed that LPS induced significant expression in IRF3 tissue level compared to normal control group. These results are consistent with other studies in which LPS injection showed significant expression of IRF3induced [36].The results of current study demonstrated

that, low, medium & high dose of L-carvone revealed a significant attenuation in IRF3 mRNA compared to LPS group. Hence this finding provides an evidence on the effect of L-carvone in attenuation of late-phase NF-κB expression and in inhibiting of type I IFN[37-39] .

Conclusion

This is the first study demonstrating that L-carvone had a promising pharmacological intervention in the inhibition of LPS-induced kidney injury with potent anti-inflammatory. Pretreatment with different doses of L- carvone in this study improved renal pathological changes, inflammatory reactions, these curative effects of might be achieved by downregulating the TLR4 and NF-kB pathways as well as consequently suppressing the production of inflammatory cytokines. These findings offer novel insights into this promising candidate for treating sepsis-associated kidney damage.

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