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The protective effect of Iraqi Juniperus oxycedrus plant on acute kidney injury induced by lipopolysaccharide in mice model

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SUMMARY. **Inflammatory control is essential to diminish injury and make renal injury treatment simpler. Proposed therapeutics have primarily targeted pro-inflammatory variables. Juniperus oxycedrus was frequently used to treat a variety of infectious disorders, hyperglycemia, obesity, TB, bronchitis, inflammation, and pneumonia. Juniperus oxycedrus twigs and leaves were defatted with n-hexane using Soxhlet apparatus then the residue of plant material dried and re-extracted sequentially by two different solvents Ethylacetate and methanol. The pro-inflammatory markers IL-1 and iNOS, as well as the potential kidney biomarker KIM-1, TNF-α, and transcription factor NF-KB were measured using the Real-Time Quantitative qPCR method. The results showed that Juniperus oxycedrus Ethylacetate and methanol extracts inhibit IL-1β, iNOS, TNF-α, NF-KB, and KIM-1induced by LPS significantly ($p<0.05$) in 200 and 400mg/kg/day of both crude extract. In conclusion the data suggest that ethylacetate and methanol crude extracts have strong anti-inflammatory and reno-protective effects in acute kidney injury induced by LPS in a mouse model, which were demonstrated by the hampering effect on KIM-1, IL-1, NF-KB, TNF-α, and iNOS mRNA expression levels via NF-KB signaling pathways inhibition.**

INTRODUCTION

Juniperus L. comprises about 70 species and 36 varieties [(1)]. Juni¬perus genus is recognized as an exceptional source of antioxidants [(2)], and antimicrobial activity [(3)], also they are used as rheumatic, antiseptic, cardiac, diuretic, and stomachic drugs [(4,5)]. In addition to anti-cancer [(6)], analgesic, anti-inflammatory and antibacterial activities [(7)]. Furthermore, J. oxycedrus is used for bronchitis, parasitic disease, and urinary infections in folk medicine [(8-10)].

Juniperus oxycedrus consisted from flavonol, flavones, terpenes, volatile oil, sesquiterpenes, resin, tannin, and monoterpenes. Cade oil comprises (17-26)% phenols mainly guaiacol about 12%, cadinene (sesquiterpene), alcohol (cardinal) and carburs. The principle constituent in Juniperus oxycedrus Tar is cadinene, a sesquiterpene, but guaiacol and cresol were also recognized [(11-13)].

Inflammation is an innate response of immune system to injurious stimuli, such as damaged cells, pathogens, toxic, or irradiated compounds [(14)], and acts by eliminating harmful stimuli and introducing the

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healing process [15]. Prostaglandins (PGs), nitric oxide (NO), and inflammatory cytokines including interleukin-1 (IL-1) tumor necrosis factor (TNF) are among the inflammatory mediators that macrophages release after becoming activated by pathogens. Anti-inflammatory drugs focus heavily on inhibiting these mediators and cytokines because their excessive production and protracted secretion are linked to a number of acute and chronic inflammatory disorders [16].

The main element of Gram-negative bacteria, via downstream signaling cascades toll-like receptor (TLR)-4 activated that control immune responses in macrophages, including mitogen activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) / inhibitory κB (IκB) pathways [17-19]. The classical MAPKs are JNK, ERK, and p38 MAPK. Activation of MAPKs and NF-κB/IκB pass on the signal for inducing transcription activity of activator protein-1 (AP-1), and inflammation-related genes transcription is regulated by NF-κB, such as inflammatory cytokines, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS)[20,21].

The complex illness known as acute kidney injury (AKI) is recognized by a sudden drop in kidney function and the retention of metabolic waste products [22,23]. After an injury, cells quickly lose their cytoskeletal integrity and membrane polarity, and they perish through necrosis and apoptosis. Drug-induced AKI particularly targets cells in the proximal tubule of the kidney [24,25]. Dysfunction damaged the tubular epithelial cells, which may trigger the production of cytokines and the attraction of inflammatory cells [26]. Activated leukocytes and tubular epithelial cells both release a range of inflammatory mediators, such as IL-1, TNF-, IL-8, IL-6, TGF-, IL-18, MCP-1, MIP-2, and RANTES [27,28]. Interferon regulatory factor-1 (IRF-1) is a transcription factor that has been linked to pro-inflammatory signaling after acute kidney damage that is reportedly stimulated by reactive oxygen species [29].

KIM-1

Ichimura et al. discovered the type I membrane protein known as kidney injury molecule [30]. KIM-1 is a new biomarker that can be used to identify acute kidney damage. Ichimura et al. used representational variance analysis to examine variations in mRNA populations between normal kidneys and kidneys that were renewing following reperfusion or ischemia. They demonstrate that renewed proximal tubule epithelial cells expressed high quantities of protein and KIM-1 mRNA. In post-ischemia kidneys, the S3 section is extremely vulnerable to ischemic stress, KIM-1 mRNA was markedly up-regulated in S3 region of the proximal tubule [31].

Experimental work

Plant material collection and extraction

Juniperus oxycedrus twigs and leaves were collected from the north of Iraq in Akra city in July 2020. After plant collection, it identified and authenticated by the specialist Prof. Dr. Haees Sayel Jarjes Mohammed Al-Jowary at college of Agriculture and Forestry, University of Mosul, Iraq. The plant parts about 500 g were cleaned completely, then dried beneath shade, and ground using a mechanical grinder to a fine powder, weighted and subsequently subjected to defatting using a Soxhlet apparatus with nonpolar solvent as n-hexane; the residue of plant material was dried and re-extracted with semi polar solvent as Ethylacetate then filter, the residue of plant material was dried again and re-extracted with polar solvents as methanol sequentially then the crude fraction was filtered, concentrated under reduced pressure using a rotary evaporator to (26.6 g) of Ethylacetate and (92.11 g) of methanol extracts.

Animal and experimental protocol

The present study done on thirty six albino mice weighing (20-35 g) that approved by the ethical, and scientific committee of Pharmacy College, University of Baghdad. Mice were allocated into groups, and kept in cages at standard conditions of temperature, humidity, and the light/dark cycle (12/12 hrs.) with free access to diet (commercial pellets), and water. Mice were randomly subdivided into six groups (n=6 per group). Group I received normal saline 0.1ml for 4 consecutive days then euthanize on day five; Group II received LPS 10mg/kg by intra-peritoneal injection, and euthanize after 24hr.; Group III and Group IV received Ethylacetate crude extract 200 and 400 mg/kg/day respectively dissolved in 0.5% carboxy methyl cellulose (CMC) using vortex to facilitate dissolving processes was done at 8:00 AM daily orally for four days and LPS 10 mg /kg on day 4 by intraperitoneal injection then euthanized on day five; Group V and Group VI received methanol crude extract 200 and 400 mg/kg/day respectively dissolved in 0.5% carboxy methyl cellulose (CMC) using vortex to facilitate dissolving processes was done at 8:00 AM daily orally for four days and LPS 10 mg /kg on day 4 by intra-peritoneal injection then euthanized on day five. Euthanization was done in the morning on day 5 (after 24 hrs. of LPS injection) using diethyl ether followed by cervical dislocation.

Kidney Tissue Homogenate Preparation

At the end of the experiment (on day 5), after euthanization the abdomen was dissected by a midline incision then the kidneys of six mice in each group were isolated and washed with cold phosphate buffer saline (PBS, pH 7.4) to remove excess blood and other debris, then the

tissue was dried with filter paper for PCR analysis and weighed by sensitive balance. For PCR analysis (50-100 mg) of kidney tissue was added to 1 ml of TRIzol solution then frozen to be used later.

Determination of mRNA expression of KIM-1, iNOS, IL-1β, and GAPDH in kidney tissue

The gene expression levels of iNOS, IL-1β, KIM-1, TNF-α, NF-kB, and GAPDH in kidney tissue were estimated using the real-time quantitative polymerase chain reaction RT-qPCR method, which involved kidney tissue isolation, total RNA extraction and purification, cDNA preparation and real-time quantitative polymerase chain reaction (RT-qPCR) steps, and then data analysis. GAPDH is a housekeeping gene used as a reference gene.

RNA Extraction:

RNA was extracted from a tissue sample according to the protocol of the RNA extraction kit. After mice euthanization, (50-100 mg) of kidney tissue was added immediately to one ml solution of TRIzol, freeze for later use; RNA was separated total from kidney tissue via using TransZol Up Plus RNA Kit (TransGen, biotech. ER501-01) as stated by the manufacturer instructions. DNA-free total RNA is a reverse-transcribed and by using of cDNA synthesis supermix (TransGen, biotech. AE311-02) and EasyScript® one-step gDNA removal complementary DNA was synthesized. The levels of mRNA expression of IL-1β, KIM-1, TNF-α, NF-kB, and iNOS were analyzed by using the SYBR Green Supermix (TransGen, biotech. AQ131-01) and qRT-PCR. For qRT-PCR purposes analysis, the housekeeping for RNA differences and quality between samples was GAPDH. Macrogen/South Korea synthesized the primers, and their sequences are presented in table 1.

Primer	Sequence (5'→3' direction)
GAPDH	
Forward	CGGGTTCCTATAAATACGGACTG
Reverse	CCAATACGGCCAAATCCGTTC
KIM-1	
Forward	GGCTCTCTCCTAACTGGTCA
Reverse	CCACCACCCCCTTTACTTCC
iNOS	
Forward	GGTGAAGGGACTGAGCTGTT
Reverse	ACGTTCTCCGTTCTCTTGCAG
IL-1β	
Forward	TGCCACCTTTTGACAGTGATG
Reverse	TGATGTGCTGCTGCGAGATT
TNF-α	
Forward	TAGCCCACGTCGTAGCAAAC
Reverse	ACAAGGTACAACCCATCGGC
NF-KB	
Forward	AAGACAAGGAGCAGGACATG
Reverse	AGCAACATCTTCACATCCC

Table (1): The primer sequence of genes used in this research

Statistical analysis

The information was displayed as the mean and standard error of the mean (SEM). Version 25 of the Statistical Package for the Social Sciences was used to conduct the analysis (SPSS, version 25). The Tukey test and one-way analysis of variance (ANOVA) were employed to assess group differences. The means of the various treatment groups were compared using an independent t-test. When ($P<0.05$) P value was less than 0.05, it was believed statistically the significant differences between groups.

RESULTS AND DISCUSSION

Acute kidney injury brought on by sepsis, is considered a global health concern due to increased incidence rates, significant morbidity, and unacceptable in-hospital mortality [32]. LPS is thought to be the main contributor to systemic inflammatory response syndrome [33]. During sepsis, LPS is released into the bloodstream, where it binds to TLR4 and activates a number of signaling pathways to cause significant inflammatory and immune reactions [34]. The inflammatory response is thought to be a key aspect of sepsis as a direct cause of AKI by a significant body of evidence. According to the results of qRT-PCR, the mRNA levels of KIM-1, IL-1β, TNF-α, NF-KB, and iNOS are up regulated significantly by LPS in kidney tissues of mice model groups when compared with the normal control group.

As a result, one of the most widely used animal models for understanding the mechanisms behind sepsis-induced AKI is LPS-induced AKI. Current therapies for septic AKI include early antibiotic delivery, sufficient vasopressors, and fluid resuscitation. Unfortunately, there is no effective way to prevent this terrible disease, and the most current treatments are ineffective and nonspecific [35]. Therefore, it appears to be of great therapeutic significance to find novel, efficient treatments or medications for septic-AKI.

Natural products have received a lot of attention as a possible source of useful bioactive compounds for drug discovery [36]. Juniperus oxycedrus L. twigs and leaves Ethylacetate and methanol extracts are two of the natural products, have previously been investigated for their potential to reduce inflammation [37, 38].

Effect of Ethylacetate, and methanol extracts doses on the gene expression of KIM-1 in renal tissue of LPS-induced AKI

KIM-1 was measured to evaluate renal dysfunction. Numerous studies have established that KIM-1

is a specific and sensitive marker of kidney damage and a predictor of prognosis, particularly in acute renal injury [39]. For additional examining the influence of Ethylacetate fraction on tubular injury, KIM-1 mRNA expression was measured. As presented in table (2) and figure (1), the statistical analysis of data revealed that KIM-1 expression was significantly raised (P<0.05) in LPS group II when compared to negative control group I (11.07±2.80 Vs. 2.65±1.18 folds). Besides, Pretreated mice with F2 200 mg /kg, and 400 mg/kg for four days before LPS, exhibited a significant decline in tubular injury marker, KIM-1 level when compared with the LPS injected mice in group III and IV [(1.38±0.42, and 0.56±0.06 folds) Vs. (LPS, 11.07±2.80 folds)]. On other hand pretreated mice with F3 200 mg /kg, and 400 mg/kg for four days before LPS, also exhibited a significant decline in tubular injury marker, KIM-1 level when compared with the LPS injected mice in group V and VI [(2.18±0.50, and 0.48±0.10 folds) Vs. (LPS, 11.07±2.80 folds)]. This study give a novel result related to the Ethylacetate, and methanol fraction doses.

Interestingly increasing the dose of Ethylacetate and methanol extracts from 200 mg to 400 mg/kg/day resulted in significant attenuation (P<0.05) (0.56±0.06, and 0.48±0.10 folds) respectively in KIM-1 gene expression when compared to its expression in renal tissue of mice treated with 200mg/kg of F2 (1.38±0.42 folds), and (2.18±0.50 folds) of methanol fraction in the other hand, there is a significant variance (P>0.05) in KIM-1 level was revealed in mice treated with 200, and 400 mg/kg/day of Ethylacetate and methanol extracts according to t-test, as in table(2) and figure (2). Indeed, KIM-1 possesses an ideal marker of proximal tubule epithelium injury because it is expressed in small amounts in a normal kidney. However, when the kidney is damaged by ischemia or toxins, KIM-1synthesis is aggravated in damaged tubular cells and its expression is increased on the apical cell membrane [40].

Interestingly the co-administration of Ethylacetate and methanol extracts (200 and 400 mg/kg/day) with LPS markedly reduced the gene expression of KIM-1. This could be attributed to the Ethylacetate and methanol extracts effect in attenuation of inflammatory cytokines in this study and the oxidative insult in renal tubules as revealed by many studies [41,42]. Together with the enhancement of kidney function as well as the improvement of the pathological features of proximal tubules, this study can conclude that Ethylacetate and methanol extracts challenge has a protective effect on kidney function and against tubular damage.

Experimental groups	Gene expression of KIM-1 (fold)
Negative control group I	2.65±1.18
LPS model group II	11.07±2.80*
Ethylacetate fraction 200mg/kg	1.38±0.42#
Ethylacetate fraction 400mg/kg	0.56±0.06#^a
methanol fraction 200mg/kg	2.18±0.50#
methanol fraction 400mg/kg	0.48±0.10#^b

Table (2): Effect of Ethylacetate and methanol extracts doses on KIM-1 gene expression
Values presented as mean±SEM, (N=6) for each group.
(*) Denotes a significant difference of LPS model/Group II as compared to the negative control/Group I (P<0.05)
(#) Denotes significant difference of treatment Groups (III, IV, V, and VI) as compared to LPS model/Group II (P<0.05).
(^a) Denote significant differences between treatment Groups III and IV (P<0.05).
(^b) Denote significant differences between the treatment Groups V and VI (P<0.05).

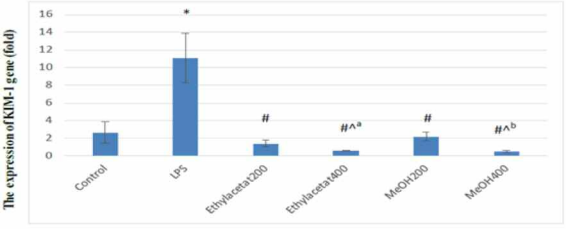

Figure (1): Effect of Ethylacetate and methanol extracts doses on KIM-1 gene expression level in renal tissue of LPS-induced AKI
Values presented as mean±SEM, (N=6) for each group.
(*) Denotes a significant difference of LPS model/Group II as compared to the negative control/Group I (P<0.05)
(#) Denotes significant difference of treatment Groups (III, IV, V, and VI) as compared to LPS model/Group II (P<0.05).
(^a) Denote significant differences between the treatment Groups III and IV (P<0.05).
(^b) Denote significant differences between the treatment Groups V and VI (P<0.05).

Effect of Ethylacetate, and methanol extracts doses on TNF-α level of kidney tissue in LPS – induced AKI model

TNF-α is a crucial participant in innate and adaptive immunity; it's pro-inflammatory activities are well defined which lead mostly to the activation and recruitment of inflammatory cells, cause local activations of vascular endothelium, nitric oxide release, and hence local vasodilation, and increase vascular permeability [43]. An uncontrollably prolonged and vigorous inflammatory reaction is the chief characteristic feature

of serious sepsis. Accordingly, the inflammatory mediator TNF-α level was measured and the results are presented in table (3), figure (2). The level of TNF-α in renal tissue was increased significantly (P<0.05) after 24 hours of LPS injection in group II compared to the normal saline received group I (P<0.05) (19.39±3.28 Vs. 6.99±4.21 fold). Data analysis also reveal a significant difference (P<0.05) in TNF-α level between the Ethylacetate fraction 200mg/kg/day received mice and LPS-injected mice (4.98± 1.48 Vs. 19.39±3.28 fold); increase Ethylacetate fraction dose to 400mg/kg/day in group IV also give a significant difference (P<0.05) in TNF-α level compared to LPS group II (0.30±0.11 Vs. 19.39±3.28 fold)

Furthermore, raising the dose of Ethylacetate fraction to 400 mg/kg/day showed a significant reduction (P<0.05) in TNF-α renal tissue level in comparison to the lowest dose (200 mg/kg/day) (4.98±1.48 Vs. 0.30±0.11). on the other hand methanol fraction at dose 200 mg/kg/day group V revealed a significant difference in TNF-α level compared to LPS group II (3.77±1.11 Vs. 19.39±3.28 fold); beside that increase methanol fraction dose to 400mg/kg/day group VI also revealed a significant difference in TNF-α level compared to LPS group II (1.65±0.19 Vs. 19.39±3.28 fold). Simultaneously, no significant difference (P>0.05) in tissue TNF-α level was demonstrated between the dose of 200 and 400 mg/kg/day F3 treated mice according to t-test. This study exhibited a novel result of Ethylacetate and methanol extracts on TNF-α level compared to LPS treated mice.

Experimental groups	Gene expression of TNF-α (fold)
Negative control group I	6.99±4.21
LPS model group II	19.39±3.28*
Ethylacetate fraction 200mg/kg	4.98±1.48 #
Ethylacetate fraction 400mg/kg	0.30±0.11 #^a
Methanol fraction 200mg/kg	3.77±1.11 #
Methanol fraction 400mg/kg	1.65±0.19 #^b

Table (3): Effect of Ethylacetate and methanol extracts doses on TNF-α gene expression
Values presented as mean±SEM, (N=6) for each group.
(*) Denotes a significant difference of LPS model/Group II as compared to the negative control/Group I (P<0.05)
(#) Denotes significant difference of treatment Groups (III, IV, V, and VI) as compared to LPS model/Group II (P<0.05).
(^a) Denote significant differences between the treatment Groups III and IV (P<0.05).
(^b) Denote no significant differences between the treatment Groups V and VI (P>0.05).

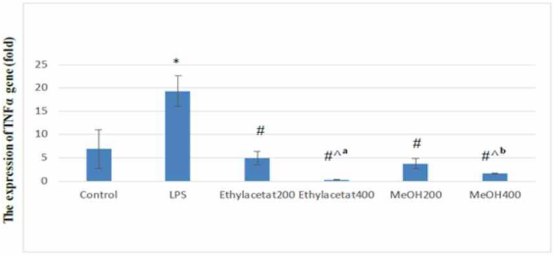

Figure (2): Effect of Ethylacetate and methanol extracts doses on TNF-α level in renal tissue in LPS-induced AKI
Values presented as mean±SEM, (N=6) for each group.
(*) Denotes a significant difference of LPS model/Group II as compared to the negative control/Group I (P<0.05)
(#) Denotes significant difference of treatment Groups (III, IV, V, and VI) as compared to LPS model/Group II (P<0.05).
(^a) Denote significant differences between the treatment Groups III and IV (P<0.05).
(^b) Denote no significant differences between the treatment Groups V and VI (P>0.05).

Effect of Ethylacetate and methanol extracts on IL-1β gene expression in renal tissue in the LPS-induced AKI

LPS induces tubular epithelial and endothelial cells to produce inflammatory mediators such as cytokines and chemokines, which contribute to the recruitment of leukocytes into the kidneys [44]. With IL-1β, these cytokines increase the adhesion molecules on endothelial cells such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-Selectin, the generation of other cytokines and chemokines, and the expression of "class I and class II major histocompatibility complex molecules" [45].

To further assess the Ethylacetate fraction effect on the production of cytokines in LPS-induced AKI, the expression of pro-inflammatory IL-1β cytokine was measured. The real-time PCR data analysis revealed that gene expression of IL-1β in renal tissue was significantly elevated in the LPS-injected mice in comparison to the normal saline-injected mice (9.73±2.39 Vs. 1.79±0.35 fold) as shown in table (4), figure (3).

Moreover there was a significant difference in IL-1β expression level between Ethylacetate fraction at 200mg/kg/day group III when compared to group II LPS-injected mice (1.68±0.39 Vs. 9.73±2.39 fold), increase the dose to 400mg/kg/day group IV also give significant differences in IL-1β expression level compared with group II LPS-injected mice (0.76±0.22 Vs. 9.73±2.39 fold). On the contrary, there was no significant difference (P>0.05) between group III and group IV according to t-test.

Notably, the treatment with methanol fraction

(200 and 400mg/kg/day dose) down-regulated the renal IL-1β mRNA expression and the reduction were "statistically" significant (P<0.05) (1.15±0.26, 0.44±0.21) respectively when compared to IL-1β mRNA expression in renal tissue of mice in LPS model group II (9.73 ± 2.39). Moreover, the findings of the current study demonstrated there was no significant variation (P>0.05) in IL-1β expression among group V and VI of methanol fraction according to t-test. This study is a novel one that express a significant effect of Ethylacetate and methanol extracts on IL-1β expression compared to LPS group.

Experimental groups	Gene expression of IL-1β (fold)
Negative control group I	1.79±0.35
LPS model group II	9.73±2.39*
Ethylacetate fraction 200mg/kg	1.68±0.39 #
Ethylacetate fraction 400mg/kg	0.76±0.22 #^a
Methanol fraction 200mg/kg	1.15 ± 0.26#
Methanol fraction 400mg/kg	0.44 ±0.21#^b

Table (4): Effect of Ethylacetate and methanol extracts doses on IL-1β gene expression
Values presented as mean±SEM, (N=6) for each group.
(*) Denotes a significant difference of LPS model/Group II as compared to the negative control/Group I (P<0.05)
(#) Denotes significant difference of treatment Groups (III, IV, V, and VI) as in contrast to LPS model/Group II (P<0.05).
(^a) Denote no significant differences between the treatment Groups III and IV (P>0.05).
(^b) Denote no significant differences between the treatment Groups V and VI (P>0.05).

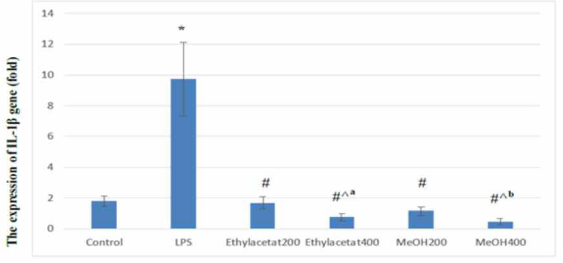

Figure (3): Effect of Ethylacetate and methanol extracts on IL-1β level in renal tissue in LPS-induced AKI.
Values presented as mean±SEM, (N=6) for each group.
(*) Denotes a significant difference of LPS model/Group II as compared to the negative control/Group I (P<0.05)
(#) Denotes significant difference of treatment Groups (III, IV, V, and VI) in contrast to LPS model/Group II (P<0.05).
(^a) Denote no significant differences between the treatment Groups III and IV (P>0.05).
(^b) Denote no significant differences between the treatment Groups V and VI (P>0.05).

Effect of Ethylacetate and methanol extracts on iNOS gene expression in renal tissue in LPS-induced AKI

LPS-associated AKI is accompanied by changes in blood flow that are strongly influenced by the increased production of NO caused by the cytokine-mediated up regulation of iNOS. This leads to high levels of NO, which change renal hemodynamics and cause peroxynitrite-related tubular injury by causing RNSs to form locally [46]. NO produced by iNOS during sepsis modulates glomerular function and causes systemic vasodilatation. For these causes, selective inhibition of iNOS, leaving physiologically present eNOS intact, might be a superior approach to treat sepsis-induced AKI [47]. Extending the spectrum of the inflammatory markers measured in this study, important renal inflammatory player iNOS gene expression level was evaluated. Data presented in tables (5), and figure (4) revealed a significant up regulation (P<0.05) in iNOS gene expression in renal tissue after 24 hours of LPS administration in contrast with control group (12.83±4.09 Vs. 3.22±0.86 fold).

In contrast, pre-treatment with Ethylacetate extract at a dose of (200 mg/kg/day) exposed a significant down regulation in iNOS gene expression compared with the LPS model group (4.69±0.82 Vs. 12.83±4.09 fold). Additionally, doubling the dose of Ethylacetate fraction to 400mg/kg/day exhibited a significant reduction (P<0.05) in iNOS levels in comparison to the LPS model group (0.52±0.11Vs. 12.83±4.09 fold). Regarding the impact of Ethylacetate fraction treatment among the two doses on the renal expression of iNOS, the results exhibited a significant change (P<0.05) in iNOS mRNA level between group III and IV in t-test. There was also significant differences (P<0.05) in iNOS mRNA level in methanol fraction doses (200 and 400mg/kg/day) in group V and VI compared to group II LPS received mice (3.69±1.26, and 0.45±0.06 respectively Vs. 12.83±4.09 fold). T-test also revealed significant differences (P<0.05) between group V and VI.

Experimental groups	Gene expression of iNOS (fold)
Negative control group I	3.22±0.86
LPS model group II	12.83±4.09*
Ethylacetate fraction 200mg/kg	4.69±0.82 #
Ethylacetate fraction 400mg/kg	0.52 ± 0.11#^a
Methanol fraction 200mg/kg	3.69±1.26 #
Methanol fraction 400mg/kg	0.45±0.06 #^b

Table (5): Effect of Ethylacetate and methanol extracts doses on iNOS gene expression

Values presented as mean±SEM, (N=6) for each group.
(*) Denotes a significant difference of LPS model/Group II as compared to the negative control/Group I (P<0.05)
(#) Denotes significant difference of treatment Groups (III, IV, V, and VI) as compared to LPS model/Group II (P<0.05).
(^a) Denote significant differences between the treatment Groups III and IV (P<0.05).
(^b) Denote significant differences between the treatment Groups V and VI (P<0.05).

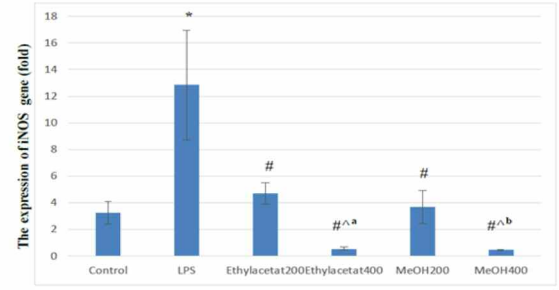

Figure (4): Effect of Ethylacetate and methanol extracts on iNOS level in renal tissue in LPS-induced AKI
Values presented as mean±SEM, (N=6) for each group.
(*) Denotes a significant difference of LPS model/Group II as compared to the negative control/Group I (P<0.05)
(#) Denotes significant difference of treatment Groups (III, IV, V, and VI) as compared to LPS model/Group II (P<0.05).
(^a) Denote significant differences between the treatment Groups III and IV (P<0.05).
(^b) Denote significant differences between the treatment Groups V and VI (P<0.05).

Effect of Ethylacetate and methanol extracts on gene expression of NF-ϰB in LPS-induced AKI

Transcription factors (TFs) are the principal modulators of basic physiologic processes due to their ability to control multiple gene expressions. In AKI, many TFs are upregulated strongly at different times, and at different levels [48]. It functions via immunological and epithelial cells to link the coordination of inflammation and cell death [49]. Besides, NF-ϰB activation involves two key signaling routes, the canonical and alternative non-canonical pathways, both are required for regulating immunological and inflammatory responses despite their distinct signaling mechanisms [50]. After measuring the inflammatory markers, the next step is to explore the upstream players in the inflammatory event. After measuring the inflammatory markers, the next step is to explore the upstream players in the inflammatory event. A common and important signaling event of pattern recognition receptors is the stimulation of the canonical NF-ϰB pathway, which is accountable for transcriptional initiation of pro-inflammatory cytokines production.

Accordingly, the gene expression of transcription factor NF-ϰB was measured in mice renal tissue to evaluate the anti-inflammatory effect of Ethylacetate and methanol fraction and to explore the site of its action. As shown in table (6) and figure (5) the expression of NF-ϰB mRNA after 24 hours of LPS administration (group II) was significantly spiked (P<0.05) compared to the normal saline-injected mice (group I) (8.55±1.20 Vs. 2.35±0.47 fold). A significant (P<0.05) difference was revealed in NF-ϰB mRNA in F2 200mg/kg/day administered group III when compared to the LPS model group (4.26±1.00 Vs. 8.55±1.20 folds).

Interestingly the pre-administration of Ethylacetate fraction at a dose of 400mg/kg/day group IV significantly down regulated the NF-ϰB expression when compared to the LPS model group II (0.46±0.17 Vs. 8.55±1.20 folds). This result revealed that the inducing action of NF-ϰB on the synthesis of the pro-inflammatory cytokines was attenuated by Ethylacetate fraction. Furthermore, there was a significant differences (P<0.05) between group III, and IV in NF-ϰB expression level according to t-test.

On the other hand 200mg/kg/day as in group V of methanol fraction give significant difference (P<0.05) in the mRNA level of NF-ϰB when in contrast to LPS model group II (1.41±0.61 Vs. 8.55±1.20 folds). Furthermore, raising the dose of methanol fraction to 400mg/kg/day in group VI revealed a significant (P<0.05) attenuation in NF-ϰB expression when compared to LPS model group II (0.12±0.04 Vs. 8.55±1.20 folds). In addition to a significant differences (P<0.05) between group V, and VI in NF-ϰB expression level according to t-test.

Experimental groups	Gene expression of NF-ϰB (fold)
Negative control group I	2.35±0.47
LPS model group II	8.55±1.20*
Ethylacetate fraction 200mg/kg	4.26 ±1.00 #
Ethylacetate fraction 400mg/kg	0.46 ±0.17 #^a
Methanol fraction 200mg/kg	1.41±0.61 #
Methanol fraction 400mg/kg	0.12±0.04 #^b

Table (6): Effect of Ethylacetate and methanol extracts doses on NF-ϰB gene expression
Values presented as mean±SEM, (N=6) for each group.
(*) Denotes a significant difference of LPS model/Group II as compared to the negative control/Group I (P<0.05)
(#) Denotes significant difference of treatment Groups (III, IV, V, and VI) as compared to LPS model/Group II (P<0.05).
(^a) Denote significant differences between the treatment Groups III and IV (P<0.05).

(^b) Denote significant differences between the treatment Groups V and VI (P<0.05).

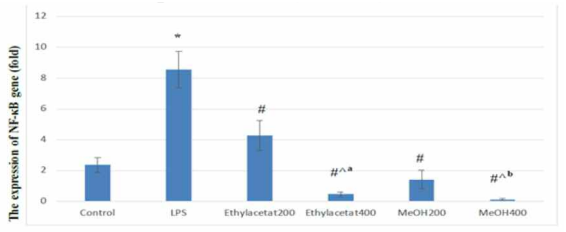

Figure (5): Effect of Ethylacetate and methanol extracts on NF-ϰB level in renal tissue in LPS-induced AKI
Values presented as mean±SEM, (N=6) for each group.
(*) Denotes a significant difference of LPS model/Group II as compared to the negative control/Group I (P<0.05)
(#) Denotes significant difference of treatment Groups (III, IV, V, and VI) as compared to LPS model/Group II (P<0.05).
(^a) Denote significant differences between the treatment Groups III and IV (P<0.05).
(^b) Denote significant differences between the treatment Groups V and VI (P<0.05).

The previous results give an indication that Juniperus oxycedrus L. twigs and leaves have anti-inflammatory effects according to the gene expression of KIM-1, NF-kB, TNF-α, iNOS, and IL-1β in comparison with LPS induce inflammation, this study noted that the presence of secondary metabolites such as alkaloids, flavonoids, terpene, phenolic acid, anthraquinone glycosides, essential oils, and saponins may give synergistic effect on kidney inflammation in mice.

CONCLUSIONS

According to the results obtained from this study, it's appear that Ethylacetate and methanol extracts from Juniperus oxycedrus plant exhibited anti-inflammatory effects against acute kidney injury induced by LPS in mice models via its strong anti-inflammatory and reno-protective effects manifested by the hampering effect on KIM-1, IL-1β, and iNOS mRNA expression levels through the inhibition of inflammatory signaling pathways. These outcomes provide a novel insight into a talented candidate to treat kidney damage caused by sepsis.

REFERENCES

1. Mrid R Ben, Bouchmaa N, Bouargalne Y, Ramdan B, Karrouchi K, Kabach I, et al. Phytochemical Characterization, Antioxidant and In Vitro Cytotoxic Activity Evaluation of Juniperus oxycedrus Subsp. Oxycedrus Needles and Berries. Molecules. 2019;24(3):1-18.
2. Höferl M., Stoilova I., Schmidt E., Wanner J., Jirovetz L., Trifonova D., Krastev L., Krastanov A. Chemical composition and antioxidant properties of Juniper Berry (J. communis L.) Essential oil. Action of the essential oil on the antioxidant protection of Saccharomyces cerevisiae model organism. Antioxidants.2014;3: 81–98.
3. Peruč D., Gobin I., Abram M., BroznićD., Svalina T., Štifter S., Stavar M.M., Tićac B. Antimycobacterial poten¬tial of the juniper berry essential oil in tap water. Arhives of Industrial Hygiene and Toxicology. 2018; 69: 46–54.
4. Stoilova I.S., Wanner J., Jirovetz L., Trifonova D., Krastev L., Stoyanova A., Krastanov A. Chemical com¬position and antioxidant properties of juniper berry (Juniperus communis L.) essential oil. Bulgarian Journal of Agricultural Science. 2014;20: 227–237.
5. Charles D.J.Antioxidant properties of spices, herbs and other sources. In: Charles D.J.: Juniper. (1st Ed.). New York, Springer. 2013; 357–362.
6. De Marino S., Festa C., Zollo F., Rusolo F., Capone F., Guer¬riero E.,Costantini S., de Felice V., Iorizzi M. Phy¬tochemical profile of Juniperus oxycedrus ssp. oxycedrus berries: A new monoterpene glucoside and evaluation of the effects on cancer cell lines. Phytochemistry Letters. 2014; 10: 152–159.
7. Eryilmaz M., Tosun A., Tumen I. Antimicrobial activity of some species from pinaceae and cupressaceae. Turkish Journal of Pharmaceutical Sciences. 2016;13: 35–40.
8. Živić N, Milošević S, Dekić V, Dekić B, Ristić N, Ristić M, et al. Phytochemical and antioxidant screening of some extracts of Juniperus communis L.and Juniperus oxycedrus L. Czech J Food Sci. 2019;37(5):351–8.
9. Faraj HAY. Morphological and Some Wood Properties of Juniperus Oxycedrus Subsp . Macrocarpa in Derna Region , East Libya. Libyan Journal of Ecological & Environmental Sciences and Technology.2020;2(1):18-26.
10. Lesjak MM, Beara IN, Orčić DZ, Anačkov GT, Balog KJ, Francišković MM, et al. Juniperus sibirica Burgsdorf. As a novel source of antioxidant and anti-inflammatory agents. Food Chem. 2011;124(3):850–6.
11. Barrero AF, Oltra JE, Altarejos J, Barragan A, Lara A and Laurent R. Minor components in the essential oil of Juniperus oxycedrus L. wood. Flavour and Fragrance Journal 1993; 8: 185-189.
12. Barrero AF, Sanchez JF, Oltra JE, Altarejos J, Ferrol N and Barragan A. Oxygenated sesquiterpenes from the wood of Juniperus oxycedrus. Phytochemistry 1991; 30: 1551-1554.

13. Salido S, Altarejos J, Nogueras M, Sanchez A, Pannecouque C, Witvrouw M and De Clercq E. Chemical studies of essential oils of Juniperus oxycedrus ssp. badia. Journal of Ethnopharmacology 2002; 81: 129-134.
14. Medzhitov R. Inflammation 2010: new adventures of an old flame. Cell. 2010; 140:771–776.
15. Ferrero-Miliani L, Nielsen O, Andersen P, Girardin S. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1β generation. Clin Exp Immunol. 2007; 147:227–235.
16. Srisook K, Mankhong S, Chiranthanut N, Kongsamak K, Kitwiwat N thanit, Tongjurai P, et al. Anti-inflammatory effect of trans-4-methoxycinnamaldehyde from Etlingera pavieana in LPS-stimulated macrophages mediated through inactivation of NF-κB and JNK/c-Jun signaling pathways and in rat models of acute inflammation. Toxicol Appl Pharmacol. 2019;371(January):3–11.
17. Achek, A., Yesudhas, D., Choi, S., 2016. Toll-like receptors: promising therapeutic targets for inflammatory diseases. Arch. Pharm. Res. 39, 1032 1049.
18. Lu, Y.C., Yeh, W.C., Ohashi, S.P., 2008. LPS/TLR4 signal transduction pathway. Cytokine 42, 145–151.
19. Udomphong, S., Mankhong, S., Jaratjaroonphong, J., Srisook, K., 2017. Involvement of p38 MAPK and ATF-2 signaling pathway in anti-inflammatory effect of a novel compound bis [(5-methyl)2-furyl] (4- nitrophenyl) methane on lipopolysaccharide stimulated macrophages. Int. Immuno pharmacol. 50, 6–13.
20. Prekumar, V., Dey, M., Dorn, R., Raskin, I., 2010. MyD88-dependent and independent pathways of toll-like receptors are engaged in biological activity of Triptolide in ligand stimulated macrophages. BMC Chem. Biol. 10, 1–13.
21. Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. J Biol Chem. 1999;274(16):10689–92.
22. Shareef SM, Kathem SH. Gentiopicroside ameliorates lipopolysaccharide-induced acute kidney injury by inhibiting TLR4/NF-κB signaling in mice model. J Pharm Negat Results. 2022;13(4):135–45.
23. K. Abdel-Kader, P.M. Palevsky, Acute kidney injury in the elderly, Clin. Geriatr. Med. 2009;25(3):331–358.
24. D.P. Basile, M.D. Anderson, T.A. Sutton, Pathophysiology of acute kidney injury, Comp. Physiol. 2012;2(2):1303–1353.
25. J.V. Bonventre, L. Yang, Cellular pathophysiology of ischemic acute kidney injury, J. Clin. Invest. 2011;121 (11):4210–4221.
26. J.V. Bonventre, Pathophysiology of AKI: injury and normal and abnormal repair, Contrib. Nephrol. 2010;165:9–17.
27. M. Haq, J. Norman, S.R. Saba, G. Ramirez, H. Rabb, Role of IL-1 in renal ischemic reperfusion injury, J. Am. Soc. Nephrol. 1998;9(4):614–619. Apr.
28. A. Akcay, Q. Nguyen, C.L. Edelstein, Mediators of inflammation in acute kidney injury, Mediat. Inflamm. 2009;137072.
29. Y. Wang, R. John, J. Chen, J.A. Richardson, J.M. Shelton, M. Bennett, X.J. Zhou, G.T. Nagami, Y. Zhang, Q.Q. Wu, C.Y. Lu, IRF-1 promotes inflammation early after ischemic acute kidney injury, J. Am. Soc. Nephrol. 2009;20 (7):1544–1555.
30. Ichimura T, Bonventre JV, Bailly V, et al. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. J Biol Chem. 1998;273:4135–4142.
31. Yin C, Wang N. Kidney injury molecule-1 in kidney disease. Ren Fail. 2016;38(10):1567–73.
32. Gómez H, Kellum JA. Sepsis-induced acute kidney injury. Critical care nephrology. 2019 Jan 1:524-33.
33. Stasi A, Intini A, Divella C, Franzin R, Montemurno E, Grandaliano G, et al. Emerging role of Lipopolysaccharide binding protein in sepsis-induced acute kidney injury. Nephrol Dial Transplant. 2017;32(1):24–31.
34. Peerapornratana S, Manrique-Caballero CL, Gómez H, Kellum JA. Acute kidney injury from sepsis: current concepts, epidemiology, pathophysiology, prevention and treatment. Kidney Int. 2019;96(5):1083–99.
35. Nguyen HB, Rivers EP, Abrahamian FM, Moran GJ, Abraham E, Trzeciak S, et al. Severe Sepsis and Septic Shock: Review of the Literature and Emergency Department Management Guidelines. Ann Emerg Med. 2006;48(1):54.e1.
36. Kang HG, Lee HK, Cho KB, Park S Il. A review of natural products for prevention of acute kidney injury. Med. 2021;57(11).
37. Sousa C, Leitão AJ, Neves BM, Judas F, Cavaleiro C, Mendes AF. Standardised comparison of limonene-derived monoterpenes identifies structural determinants of anti-inflammatory activity. Sci Rep. 2020;10(1):1–14.
38. Chen L, Liu J cheng, Zhang X nan, Guo Y yan, Xu Z hui, Cao W, et al. Down-regulation of NR2B receptors partially contributes to analgesic effects of Gentiopicroside in persistent inflammatory pain. Neuropharmacology. 2008;54(8):1175–81.
39. Vaidya VS, Ozer JS, Dieterle F, Collings FB,

Ramirez V, Troth S, et al. Kidney injury molecule-1 outperforms traditional biomarkers of kidney injury in preclinical biomarker qualification studies. Nat Biotechnol. 2010;28(5):478–85.
40. Karmakova TA, Sergeeva NS, Kanukoev KY, Alekseev BY, Kaprin AD. Kidney injury molecule 1 (KIM-1): A multifunctional glycoprotein and biological marker (review). Sovrem Tehnol v Med. 2021;13(3):64–80.
41. Asle-Rousta M, Amini R, Aghazadeh S. Carvone suppresses oxidative stress and inflammation in the liver of immobilised rats. Arch Physiol Biochem . 2020;0(0):1–6.
42. Elmastaş M, Dermirtas I, Isildak O, Aboul-Enein HY. Antioxidant activity of S-carvone isolated from spearmint (Mentha spicata L. Fam Lamiaceae). J Liq Chromatogr Relat Technol. 2006;29(10):1465–75.
43. Jang DI, Lee AH, Shin HY, Song HR, Park JH, Kang TB, Lee SR, Yang SH. The role of tumor necrosis factor alpha (TNF-α) in autoimmune disease and current TNF-α inhibitors in therapeutics. International journal of molecular sciences. 2021 Mar 8;22(5):2719.
44. Edelstein CL, Akcay A, Nguyen Q. Mediators of inflammation in acute kidney injury. Mediators Inflamm. 2009;2009.
45. Dolmatova E V., Wang K, Mandavilli R, Griendling KK. The effects of sepsis on endothelium and clinical implications. Cardiovasc Res. 2021;117(1):60–73.
46. Oliveira FR, Assreuy J, Sordi R. The role of nitric oxide in sepsis-associated kidney injury. Bioscience Reports. 2022 Jul;42(7):BSR20220093.
47. Heemskerk S, Masereeuw R, Russel FG, Pickkers P. Selective iNOS inhibition for the treatment of sepsis-induced acute kidney injury. Nature Reviews Nephrology. 2009 Nov;5(11):629-40.
48. Piret SE, Mallipattu SK. Proximal Tubular Transcription Factors in Acute Kidney Injury: Recent Advances. Nephron. 2020;144(12):613-5.
49. Kezić A, Stajic N, Thaiss F. Innate immune response in kidney ischemia/reperfusion injury: potential target for therapy. Journal of immunology research. 2017 Oct;2017.
50. Song N, Thaiss F, Guo L. NFκB and kidney injury. Frontiers in immunology. 2019 Apr 16;10:815.