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Original Article

Anti-cytokine Storm Activity of Fraxin, Quercetin, and their Combination on Lipopolysaccharide-Induced Cytokine Storm in Mice: Implications in COVID-19

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Abstract

Background: Cytokine release syndrome (CRS) is the leading cause of mortality in advanced stages of coronavirus patients. This study examined the prophylactic effects of fraxin, quercetin, and a combination of fraxin+quercetin (FQ) on lipopolysaccharide-induced mice.

Methods: Sixty mice were divided into six groups (n=10) as follows: control, LPS only, fraxin (120 mg/Kg), quercetin (100 mg/Kg), dexamethasone (5 mg/Kg), and FQ. All treatments were administered intraperitoneally (IP) one hour before induction by LPS (5 mg/Kg) IP injection. Twenty-four hours later, the mice were euthanized. Interleukin one beta (IL1-β), interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α) were quantified using an enzyme-linked immunosorbent assay (ELISA), and lung and kidney tissues were examined for histopathological alterations. This study was conducted at Al-Nahrain University, Baghdad, Iraq, in 2022.

Results: FQ reduced IL-1β ($P<0.001$). All treatments significantly suppressed IL-6, fraxin, quercetin, dexamethasone, and FQ, all with $P<0.001$. The TNF-α level was reduced more with dexamethasone ($P<0.001$) and quercetin ($P<0.001$). Histopathological scores were significantly reduced mainly by quercetin and FQ in the lungs with scores of 12.30±0.20 ($P=0.093$), and 15.70±0.20 ($P=0.531$), respectively. The scores were 13±0.26 ($P=0.074$) and 15±0.26 ($P=0.222$) for quercetin and FQ in the kidneys, respectively.

Conclusion: All used treatments reduced proinflammatory cytokine levels and protected against LPS-induced tissue damage.

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Keywords ● Quercetin ● Cytokine release syndrome ● Lipopolysaccharides ● Fraxin ● COVID-19

What's Known

- A cytokine storm is a medically critical condition characterized by the accelerated release of proinflammatory cytokines, resulting in significant mortality and morbidity rates. Previous research has indicated that fraxin and quercetin have concurrent anti-inflammatory and antioxidant characteristics.

What's New

- This study is a new investigation into the impact of co-administered fraxin and quercetin on the cytokine storm induced by lipopolysaccharide in mice. Fraxin, quercetin, and their combination reduced proinflammatory cytokines levels and exhibited protective effects on both the lungs and kidneys against the deleterious effects of lipopolysaccharides.

Introduction

Cytokine storm syndrome (CSS) is a pathological condition characterized by systemic inflammation, elevated levels of circulating cytokines, and immune system hyperactivity.[1]

Various factors such as pathogens, cancers, autoimmune diseases, and autoinflammatory diseases, are the main causes of CSS.[1] Activation of the host immune response occurs in response to the recognition of pathogen-associated molecular patterns (PAMPs) by the toll-like receptors (TLR) located on the surface of

immune cells. Tumor necrosis factor-alpha (TNF-α), Interleukin 1 beta (IL-1β), and Interleukin 6 (IL-6) are among the produced proinflammatory cytokines. Lipopolysaccharides (LPS) are toxic compounds found in the outer membrane of Gram-negative bacteria, recognized as PAMP, by the TLR of immune cells, and are used to create an experimental model of a cytokine storm.[2]

It should be noted that viruses such as the Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) induce lethal hypercytokinemia in vulnerable hosts, which increases mortality.[1] The management of coronavirus disease 2019 (COVID-19) can be divided into two parts, which are restricting viral replication with antiviral medication and controlling systemic inflammation. The latter is described under "cytokine storm treatment", which can be managed by using glucocorticoids and new emerging anti-cytokine agents.[3]

Developing immunomodulating phytochemical drugs with improved bioavailability and minimal unwanted effects could offer a potential strategy to mitigate the CSS reported in COVID-19.[4]

Fraxin isolated from *Cortex fraxini* is 7, 8-dihydroxy-6-methoxy coumarin-8-beta-D-glucoside characterized by anti-inflammatory, antimicrobial, antiviral, antioxidant, immunomodulatory, and anti-hyperuricemia activities.[5] Based on its dual antioxidant and anti-inflammatory function, fraxin can play an important role in CSS attenuation.

The flavonoid quercetin is prominently present in many fruits and vegetables. Research suggests that quercetin has several pharmacological activities with the potential to exhibit antimicrobial, anti-inflammatory, and antioxidant properties making it suitable for evaluation as an anti-cytokine storm agent.[6] Herbal medicine combinations are highly effective with fewer negative effects than isolated herbal agents, and the assumption is that the various mechanisms of their different components may be the reason behind the improved efficacy.[7]

The objective of this investigation is to evaluate the prophylactic efficacy of fraxin, quercetin, and their combination (fraxin+quercetin) against LPS-induced cytokine storms in murine models through the quantification of proinflammatory cytokine levels in murine serum, as well as histopathological examination of any protective effects of these treatments against LPS-induced pulmonary and renal tissue damage.

Materials and Methods

This study was conducted in 2022 at Al-Nahrain University, College of Medicine, Research Laboratories in Baghdad, Iraq.

Chemicals and Reagents

Quercetin hydrated or 2-(3,4-dihydroxyphenyl) -3,5,7-trihydroxychromen-4-one, dihydrate (purity≥96%), fraxin (7,8-dihydroxy-6-methoxy coumarin-8-beta-D-glucoside) (purity≥98%), dexamethasone (purity≥98%), and LPS (from *Escherichia coli* 055:B5) were all purchased from Hangzhou-Hyper Chem. Limited, China. Dimethyl sulfoxide (DMSO) was bought from Thomas Baker, India. Mouse enzyme-linked immunosorbent assay (ELISA) kits of IL-1β, IL-6, and TNF-α were purchased from Shanghai YL Biont, China. Formalin 10% (containing formaldehyde) was bought from Panreac. AppliChem., Spain. Diethyl ether was obtained from Thomas Baker, India. Normal saline 0.9% (NS) was acquired from PSI, Saudi Arabia.

Mice Used

For the anti-cytokine storm study, 90 Swiss albino BALB/c mice (20-25 g, 7 to 8 weeks old) were used. A total of 90 mice were divided into two study areas. First, the acute toxicity study included 30 male and female mice equally divided into five groups, n=6. In each group, there were three males and three females. Second, the cytokine quantification study included 60 mice (all males) divided into six groups, n=10. The mice were purchased from the Animal House Facility, National Center for Drug Quality Control and Research in Iraq. The mice were randomly housed as five in each cage, with a 12-hour light/dark cycle. On arrival, the mice were acclimated for a week and kept in a pathogen-free place with a temperature of 23±2 °C and a humid atmosphere between 30%-60%. Regular rodent food and water, *ad libitum*, were available to all the mice. The Animal Ethics Committee of Al-Nahrain University, accepted the experimental protocol under the issue number Nah. Co. Pha.12. Animal randomization method block was used for each study described in this research.

The First Stage: Acute Toxicity Study on 30 Animals (15 Males and 15 Females)

Animals were divided into five groups. The control group received 0.9% NS, and the other four groups (n=6, three males and three females) received different doses of FQ (2.5 g/Kg, 1.25 g/Kg, 0.625 g/Kg, and 0.312 g/Kg), intraperitoneally (IP). Three mice for each gender formed one block. Possible balanced combinations with M (male) and F (female) subjects were calculated as 10 blocks, five for M (Block AM, Block BM,

Block CM, Block DM, Block EM), and five for F (Block AF, Block BF, Block CF, Block DF, Block EF). Only five of the following combination blocks were randomly chosen to determine the assignment of the participants in the groups.
AMAF, AMBF, AMCF, AMDF, AMEF, BMAF, BMBF, BMCF, BMDF, BMEF, CMAF, CMBF, CMCF, CMDF, CMEF, DMAF, DMBF, DMCF, DMDF, DMEF, EMAF, EMBF, EMCF, EMDF, EMEF

Blocks were randomly chosen to determine the assignment of all thirty animals; *e.g.*, one random sequence was AMEF/BMDF/CMCF/DMBF/EMAF.

The Second Stage: Cytokine Quantification Study in 60 Male Animals

Animals were divided into six groups, and the control group received NS. The LPS group received 5 mg/Kg LPS IP, while treatment groups included the fraxin group with a dose of 120 mg/Kg, IP, the quercetin group with a dose of 100 mg/Kg, IP, the dexamethasone group with a dose of 5 mg/Kg, IP, and the combination FQ group, who were given quercetin (50 mg/Kg) IP, and fraxin (60 mg/Kg) IP.

All treatments were administered as a single dose, IP, one hour before the IP injection of LPS (5 mg/Kg). Each group contained ten animals (all were males). Every five mice formed a block and there were twelve blocks: Block A, Block B, Block C, Block D, Block E, Block F, Block G, Block H, Block I, Block J, Block K, and Block L. For each of the six groups, two blocks were randomly chosen to determine the assignment of the mice in the groups. One random sequence as an example was AL, BK, CJ, DI, EH, FG.

Investigating Agent Preparation

Fraxin, quercetin hydrated, and dexamethasone were freshly prepared on the same day of use, and dissolved in 1% DMSO and NS, according to the manufacturer's instructions.

Induction of Cytokine Storm Model

LPS (from *E. coli*, serotype 055:B5) was injected IP into each mouse once with a dose of 5 mg/Kg. The preparation of LPS stock solution was done by dissolving LPS crystalline powder in NS to prepare a concentration of 0.5 mg/mL according to the manufacturer's instructions.[8]

Acute Toxicity Study

According to protocol 425, established by the Organization for Economic Cooperation and Development (OECD), thirty Swiss albino mice (8-10 weeks old, non-pregnant females and males) were used for the acute toxicity study of the FQ combination.[9] After five days of adaptation, the animals were divided into five groups, each group consisting of three males and three females. One of the groups served as the control and was only administered with NS, while the other five groups received five doses of a combination of FQ (2.5 g/Kg, 1.25 g/Kg, 0.625 g/Kg, and 0.312 g/Kg). All doses were administered IP as a single dose, starting from the lowest dose. If all animals survived, the next highest amount was administered, and the animals were monitored for any signs of mortality and morbidity 4 hours after administration of each dose, and then monitored every 24 hours, for the following 48 hours. The results were recorded, and the LD50 for the combination was obtained according to a straight-line equation calculated from the dose-response curve.[8, 10]

Animal Grouping and Study Design

A total of sixty male mice were divided into six separate groups, with each group consisting of ten mice. The mice received LPS in a dose of 5 mg/Kg IP once. The control group received only NS 300 µL IP, treatment groups included fraxin (120 mg/Kg) IP, quercetin (100 mg/Kg) IP, dexamethasone (5 mg/Kg) IP, and the FQ combination, where quercetin was administered with a dose of 50 mg/Kg and fraxin 60 mg/Kg. All treatments were administered IP one hour before the IP injection of LPS (5 mg/Kg). The doses for the anti-cytokine storm study were determined based on the acute toxicity study and previous research on fraxin and quercetin.

Serum Collection

After 24 hours from the LPS injection, the mice were euthanized under the effects of a high dose of inhalational diethyl ether (Thomas Baker, India) and cervical dislocation. Blood was obtained from the jugular veins of all animals included in the study 24 hours after the LPS stimulation. A series of samples were obtained and transferred into gel tubes, followed by applying the centrifugal force of 5,000 rotations per minute (rpm) for 10 min, to separate the serum from the remaining components. Subsequently, the serum was transferred to Eppendorf tubes (Eppendorf, Germany) and subjected to freezing at a temperature of -20 °C to facilitate subsequent thawing and the quantitative measurement of cytokines. The ELISA technique, employing commercial assays, was utilized for the measurements, and each sample was tested in triplicate to maintain consistency and accuracy.

Quantification of the Cytokine Level

The proinflammatory cytokines IL-1β, IL-6, and TNF-α in mouse serum were quantified using ELISA kits as directed by the manufacturer (Shanghai YL Biont, China).

ELISA Test Principle

Based on the biotin double antibody sandwich technology, a kit measured mouse interleukins (IL-1β, IL-6, and TNFα). Interleukin was added to pre-coated wells with monoclonal antibodies and then incubated. Then, biotin-labeled anti-IL- antibodies were added to combine with streptavidin-horseradish peroxidase (HRP) to generate an immune complex. After incubation, free enzymes were removed and washed. Substrates A and B were added. The blue solution, influenced by the acid effect, soon changed to a yellowish color. The shades of the solution were positively correlated with the concentration of Interleukin at 450 nm. The optical density (OD) was determined. The linear regression equation for the standard curve was computed by utilizing standardized concentrations and OD values. Cytokine levels detected within the serum samples were quantified in pg/mL and subsequently expressed as such, according to the manufacturer, Shanghai YL Biont (China).

Histopathological Analysis

After 24 hours passed from the LPS injection, the mice were euthanized by a high dose of inhalational diethyl ether and cervical dislocation. Dissection and organ collection were performed on all animals. Then, the collected organs were fixed with 10% formalin and embedded in paraffin, and sent for slide preparation. The tissues were dehydrated with 50%, 70%, 80%, and 95% alcohol for 10 min each, and 100% ethanol for 30 min each. Moreover, they were immersed in paraffin wax at a low melting temperature. Next, they were sectioned to 4 mm thick slices, deparaffinized in xylene, and rehydrated with 100%, 95%, 70%, and 50% alcohol for 3 min. Hematoxylin and eosin stain (H&E) were used after being sliced into four µm sections using a manual rotatory microtome (LEICA RM2245-Germany), and samples were analyzed by an expert pathologist blinded to different treatment groups. Note that each organ, from each animal, in all groups, was given a distinct code to identify it.

Histological scoring parameters in lung tissues included signs of edema, intra-alveolar cell infiltration, congestion, and alveolar hemorrhage. The score was recorded as one of the following four grades for each animal used in this study: normal (0), mild (1), moderate (2), and severe (3).[11] At ×400 magnification, the following parameters were used to assess the degree of kidney damage: score 1 (0-25% damage), score 2 (up to 50%), score 3 (from 50% to 75%), and score 4 (up to 100%).[12] A Zeiss Imager M2 microscope (Carl Zeiss Microimaging, Germany) equipped with an Axio Cam MRc CCD camera (Zeiss, Germany) was used to photograph the slides.

Statistical Analysis

The study used the practice of three replicated (triplicate) results as follows. Results were presented as a mean±SEM and analyzed using one-way analysis of variance ANOVA with a subsequent Tukey *post-hoc* test. SPSS version28 software was utilized for the analysis. Significance was assessed at a P value of <0.001. Diagrams were rendered using Microsoft 365 Excel Spreadsheet Software. The histological differences between groups were examined using the Dunn-Bonferroni *post-hoc* test, with statistical significance set at $P<0.05$.

Results

Acute Toxicity Study

During the 14-day observation period, no deaths were recorded after treatment with FQ. The animals behaved normally and showed no signs of toxicity compared to the control group. FQ appears to have an LD50 value greater than 2.5 g/Kg/day in mice.

Measurement of Cytokine Levels

Figures 1 show IL-1β, IL-6, and TNF-α levels, respectively, measured in all groups. In comparison to the LPS-only control, treatments with fraxin, quercetin, dexamethasone, and FQ significantly reduced IL-1β, IL-6, and TNF-α concentrations in mice serum ($P<0.001$), as shown in table 1.

Histopathology Analysis

The histological findings of all treatment groups are shown in figures 2 and 3, from A to F. The control group, which received only NS, is shown in figures 2A and 3A. All tissue sections of the control group showed a normal histological structure. The lungs section shows normal respiratory bronchioles surrounded by the alveolar septa and the alveolar duct. The kidney section shows the normal structure of glomeruli, both proximal (PCT) and distal convoluted tubules (DCT), and the histoscore was zero for all tissue sections, which indicates no damage at all in this group. In contrast,

in the LPS-induced group in figures 2B and 3B, serious pulmonary edema with the destruction of alveoli in the lung sections was recorded in this group, showing inflammatory degenerative changes in the renal tubules epithelial cells. Tissue damage was reduced in treatment groups compared to the LPS-induced group in all tissue sections. Figures 4 and 5 and table 2 show histopathological scores for both lung and kidney in all groups.

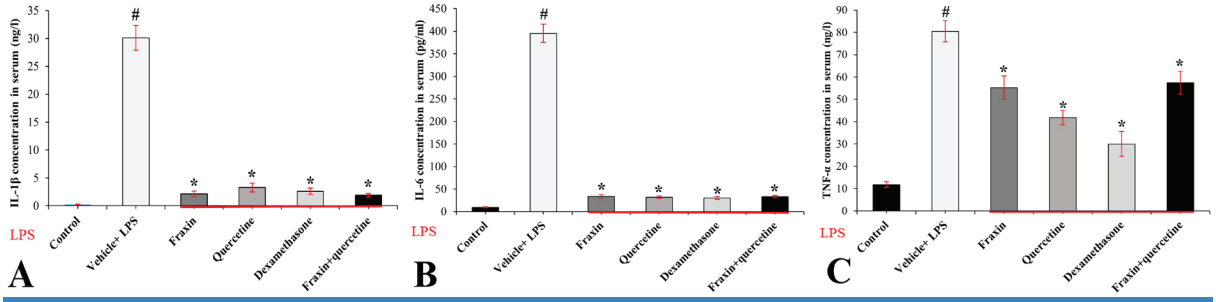

Figure 1: Level of IL-1β in LPS-induced mice serum (A); level of IL-6 in LPS-induced mice serum (B); and the level of TNF-α in serum from LPS-induced mice (C). The data is presented as mean±SEM, analyzed using one-way ANOVA, followed by a Tukey *post-hoc* test for multiple comparisons. *Denotes highly significant P<0.001 when compared to the LPS-only control; #Shows highly significant P<0.001 when compared to the control; the red line indicates groups induced with LPS.

Table 1: Proinflammatory cytokine levels among all groups in LPS-induced mice serum

Groups \ Cytokines	IL-1β (ng/L)		IL-6 (pg/mL)		TNF alpha (ng/L)	
	Mean±SEM	P value	Mean±SEM	P value	Mean±SEM	P value
Control	0.25±0.10	<0.001[a]	9.65±4.15	<0.001[a]	11.71±4.23	<0.001[a]
LPS	30.10±7.02	<0.001[b]	395.90±63.81	<0.001[b]	80.50±14.87	<0.001[b]
Fraxin	2.16±1.50	<0.001[c]	33.62±11.69	<0.001[c]	55.18±16.63	0.002[c]
Quercetin	3.28±2.52	<0.001[c]	31.87±9.00	<0.001[c]	41.75±10.25	<0.001[c]
Dexamethasone	2.60±1.79	<0.001[c]	30.27±11.13	<0.001[c]	29.99±17.53	<0.001[c]
Fraxin+quercetin	1.87±0.99	<0.001[c]	33.09±8.30	<0.001[c]	57.45±16.28	0.007[c]

Values presented as mean±SD were analyzed using one-way ANOVA, followed by the Tukey *post-hoc* test for multiple comparisons. The P<0.001, multiple comparisons were done as follows: [a]Means the control group compared with the LPS group. [b]Means the LPS group compared with the control group. [c]Means all other treatment groups compared with the LPS group.

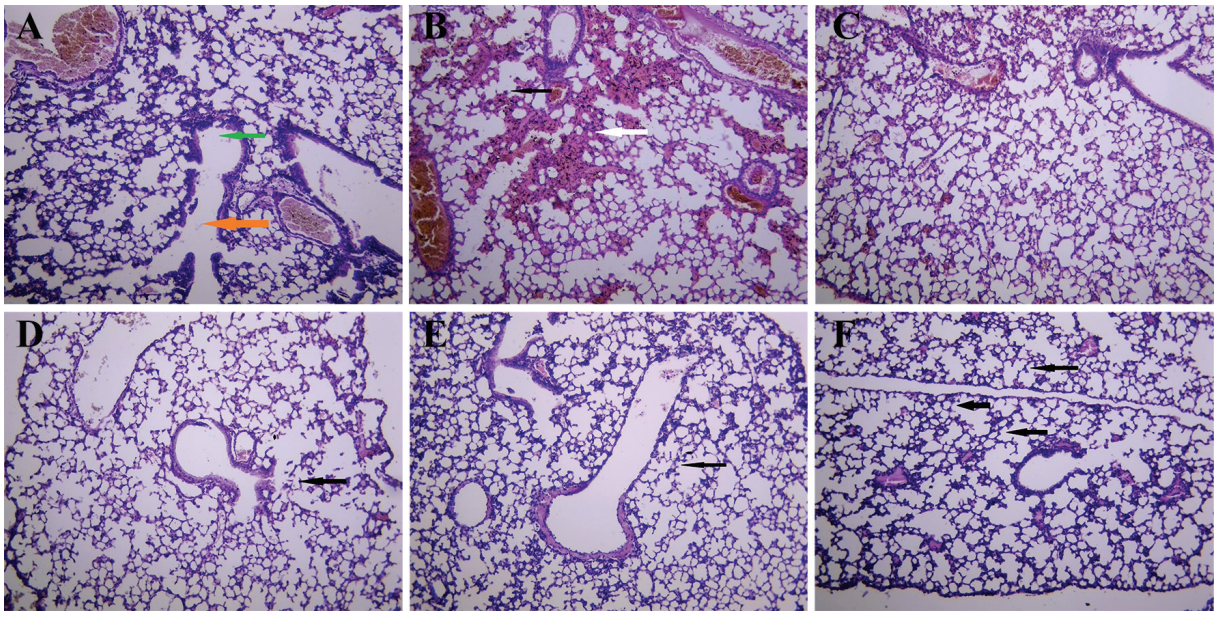

Figure 2: Histopathology of the lung sections. The control group has a normal appearance of respiratory bronchioles (green arrow), and alveolar duct (orange arrow) (A). The LPS-only group has signs of serious pulmonary edema (white arrow) with destruction of the alveoli (black arrow) (B). The fraxin+LPS group has slight congestion with mild destruction to the alveolar septa (C). The quercetin+LPS group has a mild destruction of the alveolar septa (black arrow) (D). The dexamethasone+LPS group has a near-normal appearance with a mild destruction of the alveolar septa (black arrow) (E). The FQ+LPS sample has mild destruction of alveolar septa (black arrow), H&E stain, lung ×10 (F).

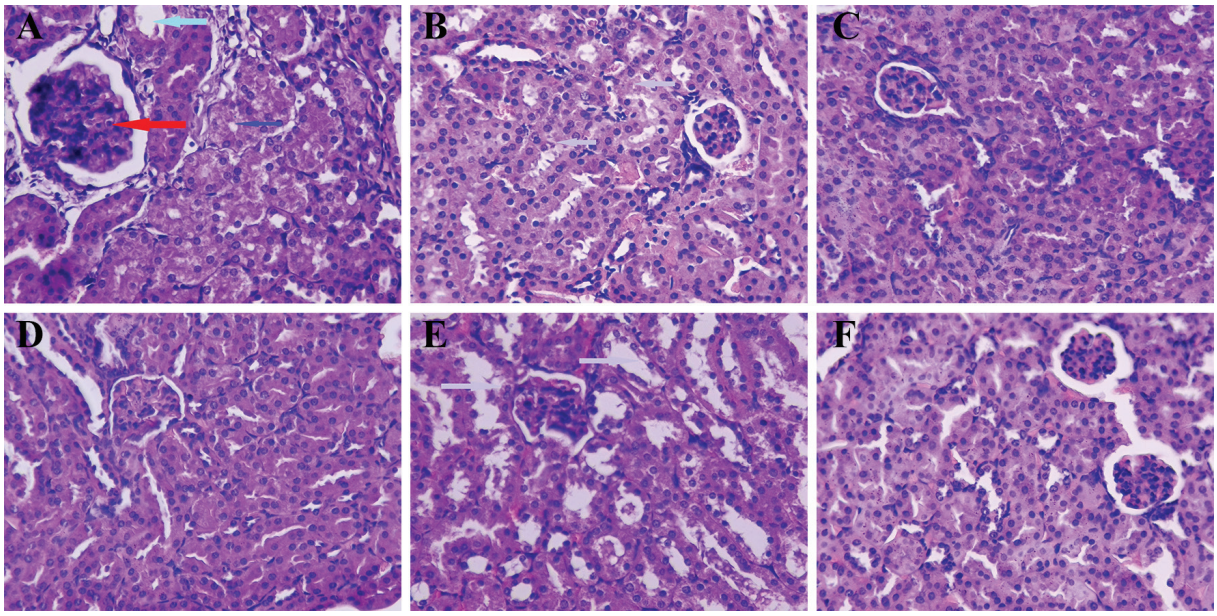

Figure 3: The histopathology of kidney sections. The control group has a normal histological structure consisting of glomeruli (red arrow), a proximal convoluted tubule (blue arrow), and a distal convoluted tubule (light blue arrow) (A). The LPS-only group shows inflammatory degenerative changes in renal epithelial cells of renal tubules (gray arrow) (B). The fraxin+LPS group has a near-normal appearance (C). The quercetin+LPS group has near normal appearance (D). (F) The dexamethasone+LPS group has degenerative changes in renal tubules (E). The FQ+LPS group has a near-normal appearance, H&E stain, kidney ×40.

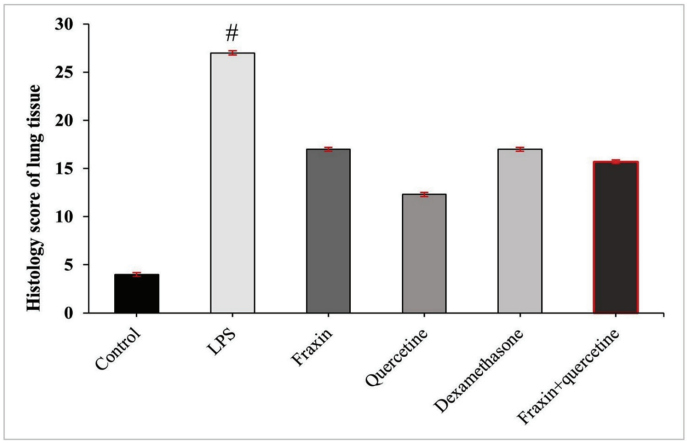

Figure 4: Histopathological scoring in tissues of the lungs for all groups, with data presented as a mean±SEM, using the Kruskal–Wallis, Dunn-Bonferroni *post-hoc* test. LPS: Lipopolysaccharide; #Means significant at P<0.05 for LPS compared with the control group. The red outlined box in the graph indicates the FQ combination.

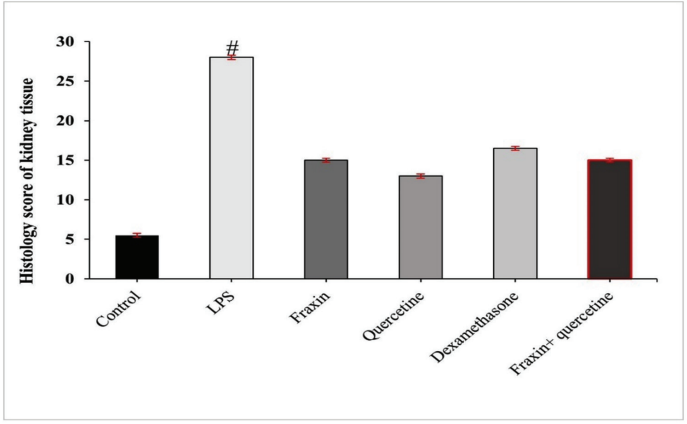

Figure 5: Histopathological scoring for all kidney tissue in all groups. The data is presented as mean±SEM, using the Kruskal-Wallis, Dunn-Bonferroni *post-hoc* test. LPS:Lipopolysaccharides; #Means significant at P<0.05 for LPS compared to the control group. The red outlined box in the graph indicates the FQ combination.

Table 2: Histopathological scores for kidney and lung tissues among all groups

Histological score / Groups	Kidney		Lung	
	Mean±SEM	P value	Mean±SEM	P value
Control	5.50±0.26	<0.001[a]	4±0.20	<0.001[a]
LPS	28±0.26	<0.001[b]	27±0.20	<0.001[b]
Fraxin	15±0.26	0.222[c]	17±0.20	0.939[c]
Quercetin	13±0.26	0.074[c]	12.30±0.20	0.093[c]
Dexamethasone	16.50±0.26	0.466[c]	17±0.20	0.939[c]
Fraxin+quercetin	15±0.26	0.222[c]	15.70±0.20	0.531[c]

Values are presented as mean rank. Analysis was conducted using the Kruskal-Wallis, Dunn-Bonferroni *post-hoc* test. Multiple comparisons were made as follows: [a]The control group compared with the LPS group. [b]The LPS group compared with the control group. [c]All other treatment groups compared with the LPS group.

Discussion

The results are that fraxin, quercetin, and their combination (FQ) reduced proinflammatory cytokine levels in the LPS-induced cytokine storm model, exerting a prophylactic effect against LPS-induced damage to lung and kidney tissue. Concurrent use of fraxin and quercetin did not result in discernible superiority of either agent individually, as evidenced by equivalent quantification of proinflammatory cytokines and comparable histopathological scores in both the lungs and the kidneys. The fraxin and quercetin combination had an LD50 greater than 2500 mg/Kg.

Fraxin and quercetin exhibited diverse pharmacological activity, including anti-inflammatory, antioxidant, and immune-modulatory activities.[13] Previous research has shown that the mechanisms by which both fraxin and quercetin antagonize cytokine release induced by LPS can be summarized as follows. Possible mechanisms of fraxin may involve the downregulation of the inflammatory cell signaling pathways nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 (NLRP3), as evidenced in previous studies.[14] Furthermore, fraxin may inhibit the synthesis of inflammatory factors, including IL-6, TNF-α, and IL-1β, suppressing the activation of mitogen-activated protein kinase (MAPK) signaling pathways.[15] The anti-inflammatory effects of quercetin after LPS-induced systemic inflammation were mainly gained by increasing IL-10 which is an anti-inflammatory cytokine. Pretreatment with dihydroquercetin (DHQ) reduced mortality in LPS-stimulated mice by altering both TNF-α and IL-6 production in mice serum.[16] Recent research suggests that quercetin inhibits the TLR-NF-κB and TLR-mediated MAPK signaling pathways and dramatically reduces the mRNA expression of TLRs.[17]

In terms of the histopathological aspects, the TLR/NF-κB pathway and other inflammatory pathways are believed to play a significant role in the pathophysiology that occurs after LPS injection. As a result, the synthesis of numerous proinflammatory cytokines is accelerated, leading to the activation and sequestration of neutrophils. The generation of proinflammatory substances such as oxidants, proteases, and leukotrienes can cause harm to epithelial tissue.[18, 19]

The results indicate that fraxin protects against ischemic/reperfusion-induced kidney damage due to its antioxidants and anti-inflammatory activities.[14] Fraxin has a favorable impact on kidney and lung tissue by raising superoxide dismutase (SOD) and total antioxidant status and concomitantly reducing the total oxidant status, myeloperoxidase (MPO), and malondialdehyde (MDA) levels in an experimental model of CLP-induced sepsis.

Fraxin's preventive role against LPS lung tissue-damaging effect is supported by earlier research; fraxin was shown to lessen the harm that LPS causes to the lung in a dose-dependent manner.[20-22] Furthermore, fraxin reduced damage to the alveolar networks, alveolar septa, and the infiltration of inflammatory cells induced by LPS.[15, 23] Sahib and his colleagues found only a few degenerative changes in epithelial renal tubules in the quercetin complex-treated group.[8] Quercetin demonstrated reno-protective properties, alleviated kidney dysfunction, reduced histopathological damage blood urea nitrogen (BUN) and serum creatinine levels, and inflammatory cell accumulation in acute kidney injury induced by LPS.[24]

Quercetin has been found to provide a greater improvement in injured lung lesions in septic rats than in the control group.[25] The antifibrogenic and anti-inflammatory properties of quercetin were observed in pulmonary fibrosis, which was partially attributed to its ability to modulate nuclear factor erythroid 2-related factor 2 (Nrf2). In septic rats, quercetin demonstrated a significant preventive and therapeutic impact on lung histology and notably reduced damage to lung parenchymal tissue.[26]

Consistent with our results, previous studies suggested that fraxin and quercetin each had an LD50 greater than 2500 mg/Kg.[18, 27, 28] Another study using quercetin in combination with rutin and curcumin found that the LD50 increased beyond 2000 mg/Kg/day.[7] As discussed above, both fraxin and quercetin exhibited remarkable anti-inflammatory activities. However, few studies focused on the possible synergy between them and any other bioflavonoids. Surprisingly, the synergy with the fraxin and quercetin combination was not as expected. This can be rationalized since the synergy of phytochemical combinations can arise due to many factors, including improving the bioavailability of each other, increasing antioxidant capacity, interacting with the gut microbiome, targeting different cells and inflammatory markers or pathways or the same inflammatory signaling pathways.[29] In the case of the FQ combination, based on previous studies, it can be concluded that both may share the same inflammatory pathways targeting inducible nitric oxide synthase (iNOS), Cyclooxygenase-2 (COX-2), Nrf2/hemoxygenase 1 (HO-1), NF-κB, NLRP3, TLR-4, and upregulation of peroxisome proliferator–activated receptor γ (PPAR-γ), suppression of proinflammatory releases as a result.[15-17, 20, 19, 30-33] Although each phytochemical in the combination has a specific way of interacting with inflammatory pathways, it can be hypothesized that the targets shared by the two phytochemicals in a combination given at a lower concentration can reach the maximum threshold for activating the specific pathway equally even when each of the combination agents is administered at a higher or double dose.

In contrast with our results, Li and colleagues found that when combined with catechin, quercetin exerts a synergistic effect through inhibition of activation of the TLR4-Myeloid differentiation primary response 88 (MyD88)-mediated NF-κB pathway.[34] Additionally, combining phytochemicals from the same source or raw extract exerts synergistic anti-inflammatory effects; for example, combining quercetin with resveratrol attenuates TNF-α, IL-6, and monocyte chemoattractant protein-1 (MCP-1) in rats.[35]

This study had some limitations as follows. A single concentration was tested for each treatment, and there was a small sample size of animal groups. Furthermore, the site of blood withdrawal can affect the level of cytokines, and the time of sampling can be influential as the cytokine level can fluctuate over time. Finally, the accuracy and range of the kits used may affect the results.

Conclusion

The findings derived from this investigation demonstrated that fraxin, quercetin, and the FQ combination exhibit significant anti-cytokine storm effects *in vivo* on LPS-challenged mice by affecting various signaling pathways. Histopathological analysis revealed that all treatments used in this study decreased the devastating damage of LPS on the kidneys and lungs. Histopathological scores were reduced compared to the LPS-only treatment group. Further investigations are needed to study the exact mechanism of action, potential synergy for using an FQ combination, and whether these agents can be considered in cytokine storm treatments in clinics in the future.

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Authors' Contribution

N.Sh and H.S: Study concept, study design, drafting, and critical reviewing; N.T: Study concept, study design, data acquisition, interpretation, and reviewing the manuscript; All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

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