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The Effect of *Albizia lebbeck* on Impairment of Interlukin-2 (IL-2) Gene Expression

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Abstract: The determine of incorporated impacts of an ethanolic *Albizia lebbeck* leaf extract and Killed Whole Cell Sonicated Ag-*Klebsiella pneumoniae* (KWCSAg-KP) on diminish of IL-2 gene expression. Five groups of eight rabbits each were used in this experiment. Subcutaneous injection of 1000 µg/ml KWCSAg-KP was given to the first Group, while the second received subcutaneous KWCSAg-KP vaccination (1000 µg/ml) and oral *Albizia lebbeck* extract (300 mg/kg). Oral *Albizia lebbeck* extract at a dosage of 150 mg/kg and 1000 µg/ml KWCSAg-KP were given to the third group. For the fourth group, oral dosage of *Albizia lebbeck* extract was only 300 mg/kg. The fifth group was given PBS to serve as a control. Assessments of interleukin-2 (IL-2) gene expression by Real time-quantitative polymerase chain reaction were used to evaluate the outcomes. The first, second and third groups revealed an increase in IL-2 gene expression after vaccination compared to the control group; in contrast the fourth group exhibited a decrease in IL-2 gene expression compared to the other groups throughout the study and the changes were statistically significant ($P< 0.05$) among groups. It was concluded that the *Albizia lebbeck* extraction has anti-inflammatory properties by impairs IL-2 gene expression.

Keywords: Interlukine-2(IL-2), Gene expression, Real time PCR, *Albizia lebbeck*, *Klebsiella pneumoniae*

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Introduction

Inflammation is an immunological response provoked as a defense mechanism against harmful stimuli such as, microbes, infections cell, tissue injury, or toxinic compounds. the series of events that take place at the site of tissue injury or infection are called inflammatory reactions, it involves the activation of immune cells, release of chemical signals (such as cytokines and chemokines), and changes in blood flow to the affected area (1). A major cytokine with pleiotropic impacts on the immune system is interleukin-2 (IL-2). The production of IL-2 is limited to antigen-activated T cells, and the expression of IL-2 receptors is exclusive to lymphocytes. Maturated lymphocytes, particularly T cells, benefit from IL-2 in three ways: survival, differentiation, and proliferation Therefore, IL-2 is required for a normal acquired cellular immune response that targets specific antigens, and it can also enhance the host's innate defenses by stimulating the activity of natural killer cells. The amount, rate, and duration of the immunological

response mediated by T cells are controlled in vivo by IL-2. The amount of protective immunity is also heavily influenced by the availability of IL-2, since its quantity determines the size of the memory cell pool (2). Interleukins have the potential to influence the transcription of target genes that are important in cellular survival and proliferation by stimulating multiple signaling pathways (3). Furthermore, they mediate the immune system (4).

Autonomously regulation and management of inflammatory reactions at physiological levels are essential for cellular role and growth, while chronic and fatal concentrations, resulting in severe deterioration of vital organs such as pancreas, brain, liver, heart, lungs, kidneys as well as reproductive systems. An unregulated inflammatory response or persistent activation of immune cells is responsible for the development of various diseases such as rheumatoid arthritis, diabetes, hypertension, or obesity (5). Despite the fact that inflammation can be successfully decreased with corticosteroids as well as nonsteroid anti-inflammatory medications, they also have the potential to cause significant adverse effects when used for an extended period of time (6). Hence, it is imperative to explore safer pharmaceuticals, specifically derived from natural sources, for the treatment of infectious and inflammatory illnesses (7,8).

Plants and their phytochemical constituents have historically been used in traditional medicine as potential alternative sources of natural anti-inflammatory, antimicrobial and antioxidant materials for the treatment of several disorders (9-13)

.*Albizia lebbeck* is distribution worldwide, rapidly growing, deciduous tree. All parts of this plant including (leaves, bark, seeds, and roots) are utilized in traditional Indian and Rumanian medicine (14).Paw edoema irritation caused by Freund's adjuvant, dextran, carrageenan and cotton pellet can be inhibited several extracts of *A. lebbeck* stem barks (15), and the most active components are alkaloids, flavonoids, tannin, and saponins found in its leaves (16). Multiple studies have shown that dietary flavonoids have anti-inflammatory and antioxidant properties (17-19). Sun *et al*. (2015) also revealed that saponin has anti-inflammatory properties. Adequate consumption of *A. lebbeck* leaves may offer health benefits due to their high presence of these bioactive substances. The abundant presence of bioactive chemicals in *A. lebbeck* leaves makes them a promising source of health benefits when consumed in sufficient quantities. The main objective of this research is to evaluation of anti-inflammatory effect of *Albizia lebbeck* extract using cytokine expression and impair it's effect as pro-inflammatory mediator.

Ethical Approval

Both the experimental design and the procedures that were utilized in this study were examined and authorized by the Scientific Committee of the Department of Microbiology, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq under Animal Utilization Protocol Certificate P.G. 2317.

Material and methods

Plant material

Albizia lebbeck leaves were gathered from veterinary medicine collage gardens at the University of Baghdad located in Baghdad, Iraq. The Department of Biological Sciences of Sciences Collage, University of Baghdad, verified the authenticity of the plant samples. The leaves were shade dried at room temperature. Using 96%

of ethanol (1:10) as extracting solvent in soxhelt apparatus,the powdered plant material was extracted for 16 hours. The crude extracts obtained were made free from the solvents by placing them in an incubator at 60ºC for 12 hours and stored in a refrigerator (21).

Gas chromatography–mass spectrometry (GC–MS) analysis

Gas chromatography–mass spectrometry (GC–MS) analysis was conducted on *Albizia lebbeck* crude extract using aAgelint(7820A) USA GC–MS equipped with an Agelint HP-5 MS fused silica capillary column. The volume of the sample (*Albizia lebbeck* extract) injected in to GC is 1µL . The injection type is specified as splitless, which means the entire sample is injected into the GC without splitting.The injector temperature was set at 250 ◦C. the oven program specifies the temperature profile during the analysis:

-Ramp 1: The temperature is initially set at 60 ˚C and held for 3 minutes.

-Ramp 2: The temperature is then ramped from 60 ˚C to 180 ˚C at a rate of 7 ˚C per minute

-Ramp 3: The temperature is further ramped from 180 ˚C to 280 ˚C at a rate of 8 ˚C per minute.

-Ramp 4: The temperature is held at 280 ˚C for 3 minutes.

The relative percentage of volatile constituents was expressed as percentages through peak area normalization. The identification of components within the crude extract relied on GC retention time on the HP-5 capillary column and the computer matching of mass spectra with those of standard.

Acute toxicity test

The acute toxicity test for the ethanolic *Albizia lebbeck* extract was conducted using the up-and-down method, according to (22). Overnight-fasted albino rats, organized into groups of six animals each, were employed for the study. The extract was administered to all animals in a group at an initial single dose of 2000 mg/kg. The animals were closely observed for a 2-hour period, followed by intermittent observations over the next 4 hours to assess the severity of any toxic signs and mortality. In the absence of observed mortality, the same dose was administered to an additional animal. If no mortality occurred at this dose, the same procedure was iterated for dose levels of 3000, and 4000 mg/kg B.W.of the extract in separate and subsequent groups.

***Klebsiella pneumoniae* and Antigen preparation**

The isolated *Klebsiella pneumoniae* was identified and characterized using standard and molecular methods (23) at Microbiology department, Veterinary Medicine Collage, University of Baghdad, on ordinary and HiChrom media to prepare antigen. The Killed Whole-Cell Sonicated Antigen of *Klebsiella pneumoniae* (KWCSA-KP) has been produced in accordance with the procedure described in the reference (24-26).

Experimental Lab Animals

Forty Albino rabbits (male and female) weighing (1000-1500)g and aged (6-12) months were collected from the animal house of College of Veterinary Medicine at the University of Baghdad. The animals were housed under regular circumstances for controlled measurements.

Experimental design

Each of the five groups that the animals were divided into had eight rabbits in it. The first group received an immunization with 1000 µg/ml KWCSAg-KP, the second group received 300 mg/kg *Albizia lebbeck* extract alternated with immunization

with 1000 µg/ml KWCSAg-KP, starting a week before day 1 of the trial and continuing on alternate day, group three followed a similar protocol, but with a dose of 150 mg/kg *Albizia lebbeck* extract, the fourth group received *Albizia lebbeck* extract at a dose of 300 mg/kg, while the fifth group, which served as a control, received PBS. The first, second, and third groups received booster doses of 1000 µg/ml KWCSAg-KP on the 14th day. Furthermore, the second and third groups received an additional dose of *Albizia lebbeck* extract. Blood was collected for serum assays on the 28th, 42nd, and 56th days for all groups.

Reverse Transcription_ quantitative Polymerase Chain Reaction (RT_qPCR)

RT_qPCR analysis was done by SYBR Green technique for quantifying gene expression. The total RNAs were extracted from the serum samples using the FavorPrep Total RNA Mini Kit (Korea), following the manufacturer's instructions. Subsequently, the whole RNA samples (1µl) were transformed into complementary DNA (cDNA) using random primers in a Reverse transcription II system (Promega Corporation, Madison, WI, USA) (Table:2), after reading the instructions sheet on the packaging. Real-Time PCR Amplification was performed using designated primer according to NCBI program (Table:1), combining with the cDNA template, the PCR master mix, and nuclease-free water in PCR tubes or plates then the reaction mixtures are placed in a real-time PCR instrument, after that the PCR cycling program was performed in the real-time PCR instrument. This typically involves an initial denaturation step to separate the DNA strands, followed by a series of cycles consisting of denaturation, primer annealing, and melting curve (Table:3). The instrument monitors the fluorescence intensity at each cycle, allowing real-time detection of the accumulating PCR product (Table:3). Analyze the real-time PCR data using appropriate software provided by the instrument manufacturer. The software calculates the threshold cycle (Ct) value, which represents the cycle number at which the fluorescence signal crosses a defined threshold. The Ct value is inversely proportional to the initial amount of target mRNA in the sample. the relative expression levels of the target gene was calculated by comparing the Ct values of the target gene with a reference gene or control sample. The comparative Ct method (ΔΔCt method) was used for relative quantification.

Finally, statistical analysis of variance (ANOVA) was used on the obtained data to determine the significance of any differences in gene expression between samples.

Table (1): The sequence of primers that used this study.

Primer	Sequence	Primer sequence 5'- - 3'	Tm (°C)	GC %	
IL-2	F	TCCAAACTTTCCAGGATGCTCA	61.6	45	Primer design
	R	TCCCCCATGAGAGTTTTTGCC	63.8	52	
B ACTIN Reference gene	F	CAACTGGGACGACATGGAGAA	62.8	52	
	R	CGTCTCGAACATGATCTGGGT	61.9	52	

Conversion of RNA to cDNA Protocol

1. A new sterile 0.2 tube have prepared for each sample and

the following components were added

Table (2): Reverse transcription II system for conversion of RNA to cDNA

Component	Volume
Random Primer(N9)	1 µl
2×ES Reaction Mix	10 µl
EasvScript®RT/RI Enzyme Mix	1 µl
RNase-free Water	Up to 20 µl
Eluted RNA	5 µl

2. Incubation

Incubate at 25 °C for 11 minutes ,42 °C for 35 minutes and 85 °C for 5 min (for qPCR).

Table (3): Real-Time PCR Cycling program.

qPCR Steps	Temp.	Time/ Cycle(s)
Initial denaturation	95℃	1min/ 1 15 sec/ 45 30 sec/45 15 sec/100
Denaturation	95℃	
Annealing	60℃	
Melting curve	61-90C	

Statistical Study:

Statistical software package for social sciences (SPSS) version 26 and Microsoft Office Excel 2010. The numeric data were reported as the mean and standard deviate on after doing a Kolmogorov-Smirnov normality test and determining whether the variables were normally or abnormally distributed. The One-Way ANOVA test was employed to examine the disparity in means among many groups, assuming that the variable follows a normal distribution. The level of significance was set at a P-value of less than 0.05, with a highly significant level at 0.01 or below (27).

3.Results:

Gas chromatography–mass spectrometry (GC-MS) analysis of Albizia lebbeck

The crude extract of Albizia lebbeck analyzed using GC-MS,GC-MS had the following major composition:

Heptanedioic acid, dimethyl ester (1.21%), Hexadecanoic acid, methyl ester (11.99%), Pentadecanoic acid(5.39%), 9,12-Octadecadienoic acid (Z,Z)-methyl ester(7.62%),9-Octadecenoic acid, methyl ester(21.20%),11-Octadecenoic acid, methyl ester(3.58%),Methyl stearate(6.05%), 2-Methyl-Z,Z-3,13 octadecadienol (21.20%),Heptane, 1-(ethenylthio)(2.58%), N-Trifluoroacetylimidazole(1.13%).

The retention time of the above compounds was:17.158, 20.205, 20.785, 22.404, 22.499, 22.585, 22.819, 23.053, 23,321,28,402, respectively.

3.1 ReverseTranscription-quantitative Polymerase Chain Reaction (RT_qPCR)

Reverse transcription-quantitative polymerase chain reaction test was performed to finding out the interlukin-2 (IL-2) gene expression in rabbits serum. The serum was obtained at days 28, 42, 56 post immunization. Finding

detected that the mean of IL-2 gene expression was higher in first, second and third groups in comparison with other groups (Figure:1,2), while lower in fourth group than of other groups in all period and differences among groups were significant (P<0.05) for the three periods (Figure:3). The differences among the groups were statistically significant (P < 0.05) (Table 4). The study's results demonstrate that the combination of KWCSA-KP and *Albizia* significantly enhances IL-2 gene expression in rabbits post-immunization. The KWCSA-KP + *Albizia* 300 mg/kg group, in particular, showed the highest IL-2 fold change at 28 days, suggesting a synergistic effect of the combined treatment on IL-2 expression.The elevated IL-2 levels in the KWCSA-KP and KWCSA-KP + *Albizia* groups indicate a robust immune response, which could be beneficial for vaccine efficacy or immunotherapy. The significant differences observed among the groups (P < 0.05) underscore the potential of these treatments in modulating immune responses.the study provides valuable insights into the effects of KWCSA-KP and *Albizia* on IL-2 gene expression, highlighting their potential as immunomodulatory agents. Further research is warranted to explore the underlying mechanisms and long-term effects of these treatments.

Table (4): Gene expression of IL-2 transcripts means fold changes in immunized and control groups.

Periods	Control	Group1	Group2	Group3	Group4	P value
28	1.93 ± 0.54	10.94 ± 3.32	15.29 ±5.19	10.35 ± 1.94	6.87± 2.06	0.001**
42	1.68± 0.74	5.46 ± 2.84	7.49 ±3.12	8.67±2.689	4.29±2.46	0.039*
56	1.26±0.64	3.31±1.71	3.84±0.96	7.59±2.33	1.37±0.07	0.027*
P value	0.301	0.68	0.013	0.780	0.141	

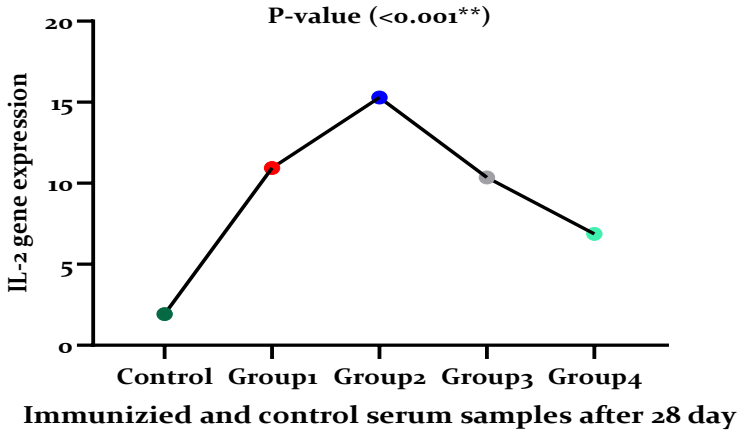

Figure (1): Gene expression of IL-2 transcripts means fold changes in immunized and control groups at 28 days. Here, mean ± standard error of groups 1, 2, 3, 4, and the negative control :10.94 ± 3.23, 15.29 ± 5.19, 10.35 ± 1.94, 6.87 ± 2.06 and 1.93 ±0.54, respectively, P value=0.001.

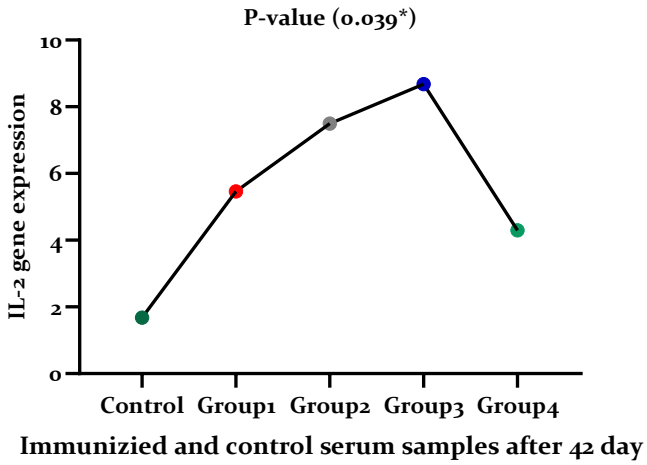

Figure (2): Gene expression of IL-2 transcripts means fold changes in immunized and control groups at 42 days. Here, mean ± standard error of groups 1, 2, 3, 4, and the negative control : 5.46 ± 2.84, 7.49 ± 3.12, 8.67 ± 2.68, 4.29 ± 2.46 and 1.68 ± 0.74, respectively, P value=0.039.

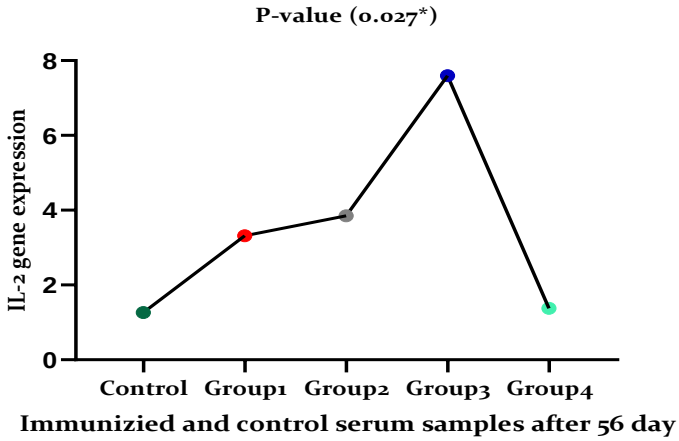

Figure (3): Gene expression of IL-2 transcripts mean fold changes in immunized and control groups at 56 days. Here, mean ± standard error of groups 1, 2, 3, 4, and the negative control : 3.31 ± 1.73, 3.84 ± 0.96, 7.59 ± 2.33 and 1.37 ± 0.073, 1.26 ±0.64, respectively, P value=0.027.

Discussion

The main objective of this research is to determine the immunological response of rabbits to a sonicated antigen of *Klebsiella pneumoniae* with different concentrations of an adjuvant made of extract from *Albizia lebbeck* leaves. Because eliciting a modulatory immune response from an adjuvant is crucial for the purpose of "studying the effects of herbal immunisation on the cellular immune system," numerous studies have been performed.

An earlier investigation by Praengam *et al.* (28) found that the aqueous *Albizia lebbeck* fraction suppressed the levels of COX-2, IL-8, IL-6, TNF-α and MCP-1. This was due to the presence of carotenoids, flavonoids and other active compounds in the water-soluble *Albizia lebbeck* leaves fraction modulating

immune responses by certain bioactive compounds which present in *Albizia lebbeck* extract could potentially help regulate the production and activity of various immune cells and signaling molecules (28). Findings from this study demonstrate that IL-2 expression was a strong regulator of immunological and inflammatory responses, which in turn affects cell-mediated immunity. *Albizia lebbeck* extract prior treatment decreased IL-2 expression in *Albizia* immunization groups. This finding was in line with a study by Sulekha *et al*. (2018) (29) that confirmed the "immunomodulatory effects of extract, which have revealed significantly reduced levels of Interlukine-6 (IL-6), Interlukine-4(IL-4), tumor necrosis factor-α (TNF- α), neutrophils, eosinophils as well as OVA sIgE, while increasing levels of IFN-γ in both blood and BAL fluid in rats".

A paradigm shift in molecular immunology occurred with the identification of interleukin-2 (IL-2). Because of IL-2's pleiotropic effects, understanding the molecular mechanisms of immune regulation requires elucidating the signaling pathways by which this cytokine regulates the development and maintenance of T cells that are both pro- and anti-inflammatory (30).

Conclusion

It was conclude that the *Albizia lebbeck* leaves extract have anti-inflamlammatory activity represent in impair IL-2 gene expression .

Confliction of interest

The authors declare that they have conflicts of interest.

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