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Beneficial Effects of Artocarpus Lakoocha Extracts in Rat Models Suffering from Atherosclerosis

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[1,3]Conception, planning of research and writing of manuscript, Discussion
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ABSTRACT

Objective: To evaluate the anti-inflammatory effects of A. lakoocha extracts by utilizing the rat models with induced hyperlipidemia.

Methodology: Twenty-four well-fed rats (Wistar breed), were selected for the current study, weighing average 150-250 grams each and divided into three groups; GROUP I served as healthy control group, fed with normal diet composed of carbohydrates, proteins and fats. Group II were hyperlipidemic rats, with atherosclerosis, and considered as disease group. They were fed with high fat diet. Group III rats were fed with extracts of A. lakoocha, extending from day 20 up to day 60 of this experiment.

Results: In the disease group, significantly decreased ($P < 0.001$) expression levels of IL-5 were found in (14.76 ± 2.65) compared with control group (26.54 ± 3.98). IN the group treated with A. lakoocha extracts (22.01 ± 4.53) significantly enhanced ($P < 0.01$) the expression levels of IL-5 were observed. In addition, plasma IL-6 levels were significantly enhanced ($P < 0.05$) in the disease group (44.19 ± 10.90) compared with the control group (32.59 ± 4.51). Treatment of rats with A. lakoocha extract (20.22 ± 2.75) caused significant reduction ($P < 0.001$) in the expression levels of IL-6 as compared with the disease group. Significantly raised ($P < 0.01$) expression levels of TNF-α within the disease group (31.78 ± 4.71) compared with the control group (23.34 ± 2.25) group. Moreover, A. lakoocha extracts (24.60 ± 4.26) significantly reduced ($P < 0.05$) the expression of TNF-α levels, compared with the disease group.

Conclusion: Current study demonstrates that A. lakoocha extracts significant protective and anti-inflammatory properties in selected rat models, subsequently leading to atherosclerosis. The present study highlighted the down-regulation of inflammatory cytokines such as IL-6, TNF-α, IL-6 and NF-κB, while simultaneously up-regulating the plasma levels of IL-15, in rat models treated with extracts of A. lakoocha.

Keywords: Artocarpuslakoocha, Anti-inflammatory, Atherosclerosis.

Introduction

Artocarpuslakoocha (A. Lakoocha) medicinal extract exhibits significant anti-hyperlipidemic actions, mainly attributed to its anti-inflammatory and immune-modulatory properties. A. Lakoocha is a tropical tree found in South Asia, Southeast Asia and extensively distributed throughout the Indian Subcontinent. This medicinal plant is known to possess diverse and essential medicinal values. The fruits of A. Lakoocha are known to contain beneficial biochemical phenolic ingredients such as, steroids, alkaloids, flavonoids, phenolic derivatives, saponins, tannins and arylbenzofurons.[1] These bioactive chemical ingredients are known to possess powerful anti-inflammatory and oxidant properties. In addition, these flavonoids and phenolic derivatives exert anti-platelet aggregation properties, thus clinically significant in preventing thrombotic events in susceptible individuals.[2] Other constituents such as saponins and tannins are considered effective against the treatment of fever caused

by malarial parasites plasmodium falciparum and plasmodium vivax, and also known to exert beneficial effects in treating infective diarrhea, overt diabetes and helminthic Taenia Saginata (tapeworm) infection.[3] The mildly sweet, aromatic and edible innermost pulp of the A. Lakoocha fruit potentiates the metabolic activity of liver, thus acting as metabolic booster and tonic.[4] The ripe fruits are purgative, appealing and are traditionally used to avoid over-production of bile within the hepatocytes, thus preventing formation of gall stones. Traditionally, A. Lakoocha extracts have been used in variety of inflammatory disorders such as rheumatoid arthritis, tuberculosis, ulcerative colitis and endocarditis.[5] The seed extracts of this tree are considered beneficial in the treatment of infective diarrhea and gastritis. The tree leaves are beneficial in the management of helminthic infestations, respiratory diseases such as bronchial asthma and cholesterol gall stones. Leaves of this tree are known to accelerate wound healing by enhanced synthesis of granulation tissue in the wounds, and also facilitate the process of lactation in breast-feeding women and domestic animals by promoting milk ejection. Similarly, the roots of this tree are considered beneficial in the treatment of various dermatological disorders such as dermatitis, eczema and impetigo. The bark of an old tree of A. Lakoocha has been traditionally used for managing malaria caused by plasmodium vivax and plasmodium falciparum. It also exerts beneficial effects in discharge of placenta after delivery in cows. The latex obtained from the tree is usually mixed with vinegar, and this combination stimulates healing of pyogenic abscesses, fibrotic nodules and snake bites.[5] The leaves of A. Lakoocha are also utilized in treating hepatitis, due to induction of liver enzymes and increasing the liver metabolism. The leaves extracts alleviate the clinical effects of hypotension by mild elevation of systolic blood pressure during resting stage, thus not recommended for hypertensive patients.

Apart from the anti-inflammatory beneficial effects, the extracts are commonly employed over the skin as anti-aging agents. The anhydrous extract of the heartwood of this tree has been utilized as a traditional anti-helminthic agent especially in children and infants.[6]

Hyperlipidemia is a metabolic disorder marked by high plasma levels of lipid fractions, predominantly, triacylglycerols (TAG), cholesterol and low-density lipoprotein (LDL). Hyperlipidemia is considered a major risk factor for the development of atherosclerosis and coronary artery diseases (CAD), namely, angina pectoris and myocardial infarction. Characteristic inflammation is a hallmark in the progression and advancement of atherosclerotic plaque in coronary arteries associated with lipid accumulation in the arterioles.[7] Most atherogenic are LDL particles which are oxidized and subsequently engulfed by macrophages within the atherosclerotic plaque. These macrophages containing oxidized LDL are called Foam cells.[8]

Presence of these foam cells in vascular walls accelerates endothelial dysfunction, which further contributes towards progression of atherosclerosis.[9] Atherosclerotic insult to the vessel wall activates smooth muscles cells and vascular endothelial cells resulting in the excessive production of a variety of pro-inflammatory chemokines, cytokines, growth factors and cell adhesion molecules.[10]Interleukin-5 (IL-5) cytokine is secreted by T-helper cells (Th2) and mast cells, and exhibit an athero-protective role altogether. IL-5 promotes humoral immunity by stimulating B lymphocytes cells to synthesize and secrete immunoglobulins by rapidly differentiating into plasma cells.[11] Few studies in the past have demonstrated the progression of atherosclerosis in cases of Il-5 deficiency, and it was related to Il-5 ability to stimulate humoral responses against oxidation of LDL particles in the vascular walls.[12]IL-5 induces the differentiation of B lymphocytes, thus accentuating the humoral responses in an individual. In addition, IL-5 enhances maturation and cellular differentiation of eosinophils, which is the reason that its role in allergic conditions like urticaria and asthma has been studied in the past.[13] Earlier, it was documented that asthmatic patients receiving A. Lakoocha therapy, had decreased incidence of atherosclerosis and subsequent coronary artery diseases.[14]

Interleukin-6 is another pro-inflammatory cytokine, and is produced in excess in conditions such as atherosclerosis, GIT carcinomas and neuro-degenerative diseases mainly Alzheimer disease and rheumatoid arthritis.[15] In addition, IL-6 significantly enhances the plasma levels of fibrinogen along with reduced plasma levels of albumin. Overall, these effects augmented the clotting of blood and enhanced platelet reactivity; thus contributing further towards progression of atherosclerosis in humans.[16]

Tumor Necrosis factor-α (TNF-α) potentially activates the endothelial cells, leading to exaggerated inflammatory response.[17] TNF-α exerts excitatory and neuro-inflammatory properties, mainly mediated by its release from the microglia cells in the central nervous system. In turn, TNF-αpromotes the synthesis and release of a variety of neuro-transmitters in the central nervous system, thereby altering the delicate balance between excitatory and inhibitory neuro-transmitters. This imbalance of neuro-transmitters is postulated to produce cytotoxicity mediated by glutamate.[18]

NF-κB is a pro-inflammatory pathway, highly activated at inflammatory sites, and mediates the transcription of leukocyte adhesion molecules in vascular walls, enhanced secretion of pro-inflammatory mediators and a variety of chemokines. NF-κB is kept sequestered in the cytoplasm in a dormant form by associating with inhibitory molecules called inhibitors of κB(IκB) .[19] Once activated, NF-κ Btranslocates in the nucleus andcauses enhanced transcription of IL-1 and IL-6 by activated leukocytes and synoviocytes (Zhang 2013).[20]

The current study was intended to evaluate the beneficial effects of A. lakoocha extracts on the expression of atherogenic cytokines IL-5, IL-6, TNF-α and NF-κB, in hyperlipidemia associated atherosclerosis, using rat models.

Methodology

The research work was conducted on hyperlipidemic rat models with atherosclerosis, and estimation of anti-inflammatory effects of A. lakoocha extracts on IL-5, IL-6, TNF-α and NF-κB were studied in the biochemistry laboratory at The University of Health Sciences, Lahore.

234g of Tris base was obtained and dissolved in 500ml of water by a stirrer and 58.4 ml of glacial acetic acid was added with little quantity of water, then 150 ml of 0.5M EDTA was added in the mixture, and the volume was made up to one liter, and then kept at room temperature.

Twenty four well-fed rats (Wistar breed), were selected, weighing average 150-250 g each, and were kept in the research laboratory of The University of Health Sciences, Lahore. All these experimental rats were retained at fixed room temperature (22-24 ºC) with humidity maintained at (45-65%). The twenty four selected rats were allocated 3 groups, each group comprised of eight rats.

Group I (Healthy control group): These rats were fed with normal, composed of carbohydrates, proteins and fats.

Group II (Hyperlipidemic group with atherosclerosis): Group II was considered as disease group in which animals showing hyperlipidemia when fed with high fat diet were present.

Group III (Lakoocha extract): These hyperlipidemic rats were fed with extracts of A. lakoocha, extending from day 20up to day 60 of this experiment.

Induction of hyperlipidemia: All the experimental rats belonging to group-1 and group-2 were extensively fed on diet rich in (70% saturated fat, 12% protein, 18% carbohydrate, providing net energy content of 30.0 j/kg) at the recommended dosage of approximately100g/kg diet per day.

Sacrificing the animals and sample collection: At day 61, the animals were sacrificed by using the technique of cervical dislocation and using diethyl-ether anesthesia. After sacrifice, the livers of all animals were dissected out for further experimentation.

Determination of mRNA expression levels of L-5, IL-6, TNF-α and NF-κB

1. RNA extraction by using standard TRIzol technique

Following steps were followed under this method:

a) Homogenization of liver tissue

The extracted liver tissue was kept in each tube.300μl of TRIzol reagent was added in each tube. The combination was thoroughly mixed with the help of vortex mixer, solution homogenized after 12 minutes and was subsequently incubated at 25ºC room temperature for 5-6 minutes.

b) Phase separation

In each tube, 200μl of chloroform was added, and the solution was mixed briskly for 20-30 seconds in vortex mixer.The obtained solution was kept for 5 minutes at25ºC room temperature. Afterwards, the tubes were centrifuged at 6000 rpm, at 4ºC temperature for approximately 10 minutes.Discrete layers appeared in each tube. The upper clear supernatant layer comprised of RNA, and this layer was carefully separated from each individual tube by using a metallic spatula and a micropipette.

c) Method for RNA precipitation

Isopropanol was added in equal proportions in the tubes containing the aqueous phase, and the tubes were kept at 25ºC for 20 minutes, then tubes were centrifuged at the speed of 6000 rpm at 4ºC temperature. This resulted in formation of a small RNA pellet, which deposited at the bottom-most of each tube.

d) RNA washing

The RNA pellets obtained in the above step were washed with 1ml of 75% ethanol, and placed in the centrifuge machine at 8000 rpm at 4ºC. Then, ethanol was discarded from the tubes, and the RNA pellets were dried in air.

e) Method of Re-suspension

20μl of RNAse free water was added in the tubes containing RNA pellets, resulting in solubilization of RNA pellets in each tube, then these tubes were kept in dry heat oven for 20 minutes. After thorough drying, the resultant RNA was labeled, kept and stored at -80 ºC.

1. Quantification of obtained RNA with the help of spectrophotometer

Total RNA in each sample was measured by using the Nanodrop spectrophotometer device, by introducing 1 μl drop of each trial sample in the nanodrop spectrophotometer. The readings were measured and recorded for each sample introduced in the spectrophotometer.

2. Designing the primer

The primers used for this study were selected from data obtained from published researches. (Rana 2016 and Inam 2017).

Genes	Forward primer	Reverse primer	Product size
IL-5	5´-CCCTCGCCTGAAcCTCCTGG-3´	5´-CCCTGATGCAACGAAGACGA-3´	190
IL-6	5´-CGCAAAAAGAACGAAAGACA-3´	5´-CTCCGACTTGTGAAGTGGTA-3´	180
TNF-α	5 ' -TCGCCTCCGGCTCAACACTT-3 '	5 ' -ATCGGCTGGCACCACTAGTT-3 '	194
NF-κB	5-GACGTGTGTCTTAGGGCCTA-3′	5′-ACGCTCAGGTCCATCTCCTT-3′	158

3. Reverse transcription method

cDNA method was utilized by using First strand cDNA synthesis kit (ThermoFisher, Scientific America). The components of the kit were thawed, mixed and centrifuged, then placed on ice.

The components of the kit were added in the following order; as shown in the table below:

5X reaction Tris buffer	5 μl
RNase inhibitor Ribolock 2X	0.5 μl
dNTP combined (15 mM each)	1 μl
M-MuLV enzyme TDS (150 U/μl)	1 μl

A total of 20 μl mixture was formulated at the end of the procedure, the blend was mixed thoroughly and centrifuged for 10 minutes, and then the tubes were incubated at 50 ºC for approximately 45 minutes. The reaction ended at 70 ºC for 5 minutes in the auto-thermal cycler. The cDNA obtained at the end of the cycle was labeled and kept stored at -20 ºC.

4. Preparing the working solution of primers

First, the stock solution (200 μM) was prepared using all the primers, 250 μl RNase free water, which was added in each primer for dilution. Working solutions (20 μM) of each primers were formulated by using 10 μl from stock solution and adding 150 μl of RNase free water to adjust the volume up to 200 μl in each tube.

5. Polymerase chain reaction (PCR)

Polymerase chain reaction was utilized to amplify the cDNA. For template, of 2 μl cDNA was used. Following components were mixed in the PCR tubes in the given order:

cDNA	2 μl
Forward and reverse primers; each	1 μl
RNAse free water	3 μl
PCR master mix	5 μl

6. Gel electrophoresis

Preparation of 50X TAE buffer

Tris base buffer	234.0 g
0.5 M EDTA	150 ml
Glacial acetic acid (anhydrous acetic acid)	58.4 ml

Preparation of gel and conduction of gel electrophoresis

The obtained PCR product was visualized by using gel electrophoresis technique.1.1 g of agarose was added in 55 ml of 1X TAE buffer in conical flask, heated it in oven till all elements were solubilized completely, then cooled under running tap water. For visualization, 3 μl of ethidium bromide was added in the flask containing agarose and liquefied gel was poured in gel caster, then 1X TAE buffer in gel electrophoresis apparatus was added, and the gel was placed in it. Gel loading dye (1.5 μl) was added in all the samples tubes. The first well of the gel was loaded with 5 μl of 100 bp DNA ladder, while the continuing wells were filled with 5 μl of all the samples. Run the gel at 120 voltage for 45 minute. Gel documentation system was used for the visualization of gel and specific bands of varying density were observed and compared against standard ladder.

For Densitometry, ImageJ software was used for determining the results and semi-quantification of the obtained products in the procedure.

Results

The resultant data was analyzed with the help of Graph Pad prism version 5-software. One-way ANOVA was employed to observe the relative differences among the three experimental groups. Student t-test was then applied to the given data to analyze the comparison among the findings in different groups. Resultant data were represented as mean ± SD (standard deviation) and a P value of 0.05% was considered statistically significant.

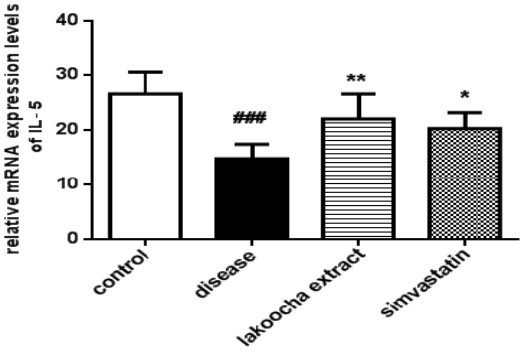
The results of the study obtained by Nano drop spectrophotometer are given below;

Results of quantification of RNA by Nano drop spectrophotometer		
Sample number	**Nucleic acid**	**Unit**
C-1	726.2	ng/μl
C-2	985.6	ng/μl
C-3	292.5	ng/μl
C-4	739.4	ng/μl
C-5	1304.6	ng/μl
C-6	246.3	ng/μl
D-1	976.8	ng/μl
D-2	3875.3	ng/μl
D-3	100.7	ng/μl
D-4	1022.7	ng/μl
D-5	897.3	ng/μl
D-6	545.5	ng/μl
LE-1	336.7	ng/μl
LE-2	428.7	ng/μl
LE-3	490.9	ng/μl
LE-4	612.4	ng/μl
LE-5	593.2	ng/μl
LE-6	622.5	ng/μl

(C stands for control group, D stands for disease group and LE stands for lakoocha extract)

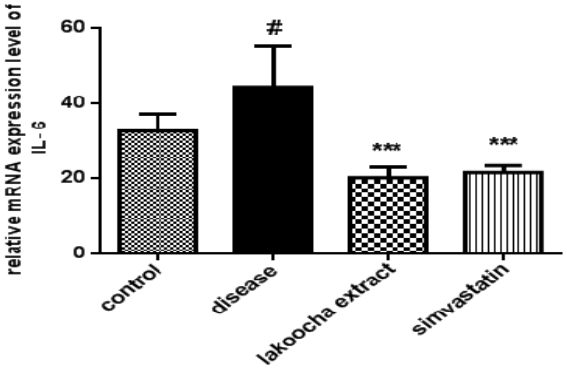
A. mRNA expressions of IL-5 were significantly enhanced in rats treated with A. lakoocha extracts

In the disease group, significantly decreased ($P < 0.001$) expression levels of IL-5 were found in (14.76 ± 2.65) compared with control group (26.54 ± 3.98). IN the group treated with A. lakoocha extracts (22.01 ± 4.53) significantly enhanced ($P < 0.01$) the expression levels of IL-5 were observed.



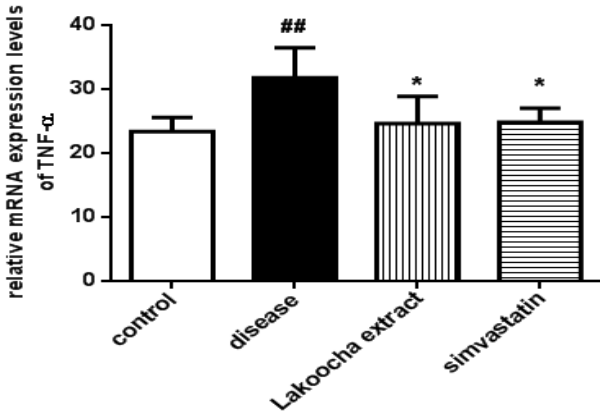
B. Significantly enhanced expression of IL-6 in rats treated with A. lakoochaextracts

The current study revealed that plasma IL-6 levels were significantly enhanced ($P < 0.05$) in the disease group (44.19 ± 10.90) compared with the control group (32.59 ± 4.51). Treatment of rats with A. lakoocha extract (20.22 ± 2.75) caused significant reduction ($P < 0.001$) in the expression levels of IL-6 as compared with the disease group.



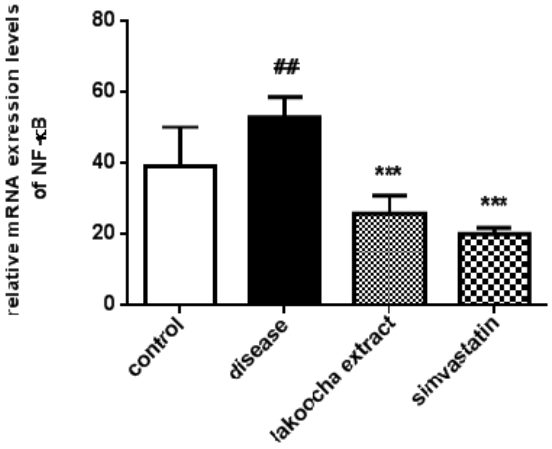
C. Significantly enhanced expressions of TNF- α levels in rats treated with A. lakoochaextracts

In the present study, there was significantly raised ($P < 0.01$) expression levels of TNF-α within the disease group (31.78 ± 4.71) compared with the control group (23.34 ± 2.25) group. Moreover, A. lakoocha extracts (24.60 ± 4.26) significantly reduced ($P < 0.05$) the expression of TNF-α levels, compared with the disease group.



D. Significantly reduced expression of NF-κBlevels in rats treated with A. lakoocha extracts

Significant up-regulation ($P < 0.01$) was observed in the levels of NF-κB in disease group (52.92 ± 5.58) compared with control group (39.01 ± 11.06). The expression of NF-κB was found considerably down-regulated in A. lakoocha extract treated group ($P < 0.001$; 25.59 ± 4.95), when compared with disease group.



Discussion

In the current study, significantly elevated expressions of mRNA levels of pro-inflammatory cytokines such as IL-6, TNFα and NF-κB were observed in the disease group, on the other hand, expression levels of atheroma-protective cytokine IL-5 were significantly reduced. Treatment with prepared extracts of A. lakoocha extract showed significantly reduced levels of pro-inflammatory cytokines in the disease group. IL-6 has been considered as an important pro-atherogenic cytokine, and is secreted from a variety of cells such as activated endothelial

cells, macrophages, fibroblasts and monocytes. IL-6 is primarily responsible for the progression of atherosclerosis by mediating endothelial and inflammatory dysfunction in the initial plaques, and simultaneously promotes pro-coagulant processes at the inflammatory site. In the present study it was observed that treatment with A. lakoocha extracts significantly decreased the mRNA expression of IL-6, while in the disease group, there was considerably high expression of mRNA of IL-6.

In the current study there was enhanced expression of mRNA of TNF-α in the disease group, on the contrary, treatment with A. lakoocha extracts significantly attenuated this mRNA expression of TNF-α. NF-κB pathway is predominantly activated at the inflammatory atherogenic site, stimulates enhanced transcription of pro-inflammatory cytokines, adhesion molecules and chemokines. In the present study, the expression of mRNA of NF-κB pathway was considerably increased within the disease group, however, treatment with A. lakoocha extracts exhibited decreased expression of mRNA of NF-κB.

Conclusion

Current study demonstrates that A. lakoocha extracts significant protective and anti-inflammatory properties in selected rat models, subsequently leading to atherosclerosis. The present study highlighted the down-regulation of inflammatory cytokines such as IL-6, TNF-α, IL-6 and NF-κB, while simultaneously up-regulating the plasma levels of IL-15, in rat models treated with extracts of A. lakoocha. Additional studies are, however, required to demonstrate the extent to which there is modification of intensities of inflammatory and immunomodulatory markers exerted by the extracts of A. lakoocha.

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