In-vitro anti-inflammatory mechanism of *Averrhoa carambola* L. (Star Fruit) leaves on RAW 264.7 macrophage inflammation model induced by LPS.

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ABSTRACT:

The leaves of *Averrhoa carambola* L. (Oxalidaceae) have traditionally been used in folk medicine for alleviating pain and inflammation. This study explored the anti-inflammatory properties of *A. carambola* leaf extracts. The extraction was performed using the Soxhlet method, starting with petroleum ether and subsequently using 95% ethanol. The impact of the ethanol extract on the secretion of IL-1β, IL-6, and TNF-α by lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells was assessed and compared to indomethacin. The findings revealed that the extract inhibited IL-6, IL-1β, and TNF-α secretion at levels of 0.754, 3.981, and 2.136 µg/ml, respectively. At a final concentration of 50 µg/ml, *A. carambola* extract significantly reduced the production of all three pro-inflammatory cytokines compared to the LPS control. These results suggest that *A. carambola* leaves possess promising potential as a therapeutic agent for inflammatory conditions.

Keywords: *Averrhoa carambola* L., Inflammation, IL-1β, IL-6 and TNF-α.

INTRODUCTION:

Inflammation is the immune system's initial response to infection or irritation and plays a significant role in the development of diseases such as cancer, diabetes, atherosclerosis, and arthritis [1]. Macrophages, which are central to the inflammatory process, have three key functions: antigen presentation, phagocytosis, and immunomodulation through cytokine and growth factor production. They are instrumental in initiating, sustaining, and resolving

inflammation as they become activated and subsequently deactivated during the inflammatory response [2]. Activated macrophages release various pro-inflammatory cytokines and inflammatory mediators that are crucial to the inflammatory process. However, excessive production of these molecules can contribute to the onset of inflammation [3]. Upon stimulation with lipopolysaccharides (LPS), macrophages produce cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10), as well as inflammatory mediators like nitric oxide (NO) and prostaglandin E2 (PGE2) [4-6]. Therefore, in this study, LPS was used to activate macrophages to evaluate the anti-inflammatory effects of the leaf extract.

While steroidal and non-steroidal anti-inflammatory drugs are commonly used to treat inflammatory conditions, their severe side effects limit their widespread use. Consequently, traditional medicine continues to rely on medicinal plants as alternatives to allopathic treatments [7,8]. Plant-derived extracts have long been regarded as beneficial for maintaining overall health, preventing and managing various diseases, and enhancing immune function [9].

Averrhoa carambola, a member of the Oxalidaceae family, has historically been utilized as an appetite stimulant, diuretic, and for its anti-inflammatory, antidiarrheal, and febrifugal properties [10,11].

The objective of this study was to explore the therapeutic potential of an aqueous extract of *A. carambola* L. leaves in combating inflammation and to assess its potential as a commercial health-promoting product. Specifically, we examined the extract's effects on IL-1β, IL-6, and TNF-α

production in LPS-induced RAW 264.7 macrophage cells and compared its efficacy with that of indomethacin.

MATERIALS AND METHODS:

Collection and authentication of the plant:

The *Averrhoa carambola* leaves, from the Oxalidaceae family, were gathered from A Block in Baksa, Assam. The plant was identified and authenticated by Dr. Souravjyoti Borah, an Assistant Professor in the Department of Botany at Gauhati University, Assam.

Plant extraction:

The *Averrhoa carambola* leaves were air-dried in the laboratory and ground into a coarse powder. The plant material was then subjected to continuous extraction using the hot Soxhlet extraction method. The initial extraction was performed with 500 mL of petroleum ether as the solvent, lasting for 48 hours. The resulting extract was collected, and the solvent was evaporated using a rotary vacuum evaporator at 40°C. Following the petroleum ether extraction, the remaining plant residue (marc) was further extracted with 500 mL of 95% ethanol for another 48 hours. The ethanol extract obtained was then collected and concentrated by evaporation at 40°C using a rotary vacuum evaporator [12].

Cell culture:

The mouse macrophage cell line RAW 264.7 was obtained from the National Centre of Cell Science, Pune. The cells were cultured in phenol red-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% heat-

inactivated fetal bovine serum. The culture was maintained at 37 °C in an atmosphere of 5% CO_2. The cells were then washed with DMEM and detached using 0.25% trypsin-EDTA. Following detachment, they were resuspended in DMEM at a concentration of 5×10^4 cells per well. Cell viability was assessed using the trypan blue dye exclusion method.For the IL-1β, IL-6, and TNF-α assays, cells were seeded at a density of 5×10^4 cells per 100 µl in 96-well plates and incubated for 1 hour. Subsequently, the cells were stimulated with 2 µg/ml lipopolysaccharide (LPS), either alone or in combination with test compounds, including the plant extract and the anti-inflammatory drug indomethacin. After 48 hours of incubation, the supernatant was collected and analyzed using a commercial enzyme-linked immunosorbent assay (ELISA) kit [13-14].

LPS-induced cytokine production assay: [15-17]

Production of IL-1β, IL-6 and TNF-α were measured via ELISA according to the manufacturer's instructions. Briefly, a 96-well microtiter plate was pre-coated overnight with capture antibody. After blocking and several washings, 100 µl of working standards and samples were then added for incubation for 2 h. After washing, 100 µl of working detector solution containing biotinylated anti-mouse cytokine monoclonal antibody was added and incubated for 1 h. After further washing, 100 µl of avidin-horseradish peroxidase-conjugate was added to each well and incubated for 30 min. Then, 100 µl of substrate solution was added and the culture was incubated for 15 min, followed by the addition of 50 µl of stop solution, and the absorbance was read within 30 min using a microplate reader at 450 nm. The inhibition of cytokine production compared to the control (LPS) was calculated as: ([cytokine]c − [cytokine]t)/[cytokine]c, where [cytokine]c and [cytokine]t are the cytokine concentration in the control and test sample, respectively. A standard calibration graph was plotted (Abs Vs Conc.) and the concentrations of unknown samples have

been determined from the graph. Each group was added of RAW264.7 cells in triplicate (n=3) manner and has been treated with test chemical. The groups were as follows:

Group1: Normal control - Cells in growth medium, DMEM

Group 2: Lipopolysaccharide (LPS) control - 2µg/ml

Group 3: Treated group - LPS+ Averrhoa carambola, 50µg/ml

Group 4: Standard: LPS + Indomethacin, 2.5µg/ml

Statistical analysis:

The results are expressed as Mean ± SEM. Data were analyzed using statistical software Graph Pad Prism version 5. The significance of difference among the groups was assessed using one-way analysis of variance (ANOVA) followed by Tukey's test compared to Normal control (Untreated) Vs all groups $p<0.05$ were considered significant.

RESULTS:

Plant extraction:

The extract was obtained by freeze-drying, and the physical appearance of the extract was greenish brown. The percentage yields for the petroleum ether and ethanolic extracts of *Averrhoa carambola* leaves were at 12% and 20%, respectively.

***A. Carambola* extract inhibited IL-1β, IL-6 and TNF-α production from macrophage RAW 264.7 cells:**

The levels of IL-6, IL-1β, and TNF-α secreted by cells stimulated with bacterial LPS (2 µg/ml) and subsequently treated with *Averrhoa carambola* were measured at 0.754, 3.981, and 2.136

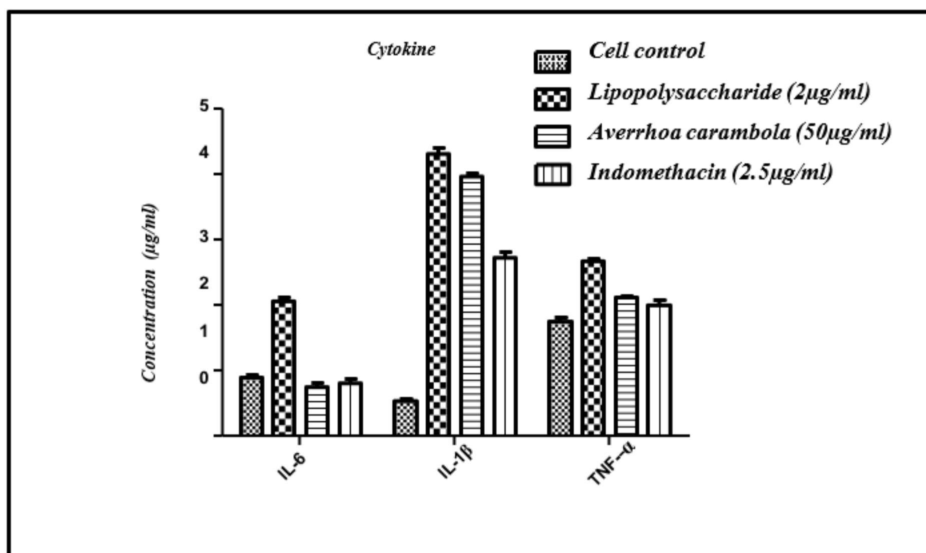
µg/ml, respectively. In the cell control group (without LPS or drug treatment), the concentrations of IL-6, IL-1β, and TNF-α were recorded as 0.898, 1.554, and 1.762 µg/ml, respectively. For the LPS control group (cells stimulated with LPS but without drug treatment), cytokine concentrations were found to be 2.060 µg/ml for IL-6, 4.316 µg/ml for IL-1β, and 2.276 µg/ml for TNF-α. Meanwhile, cells treated with Indomethacin (2.5 µg/ml) secreted IL-6, IL-1β, and TNF-α at concentrations of 0.809, 2.739, and 2.013 µg/ml, respectively. At a final concentration of 50 µg/ml, *Averrhoa carambola* significantly reduced the production of all three pro-inflammatory cytokines-IL-6, IL-1β, and TNF-α—compared to the LPS control group.

Table 1: Cytokine ELISA

Group	*Particulars*	Concentration (µg/ml)		
		IL-6	**IL-1β**	**TNF-α**
1	***Cell control***	0.898±0.04	1.554±0.03	1.762±0.06
2	***Lipopolysaccharide (LPS) control - (2 µg/ml)***	2.060±0.06	4.316±0.09	2.676±0.04
3	***LPS+ Averrhoa carambola (50µg/ml)***	0.754±0.06$^{\#\#}$	3.981±0.05$^{\#\#}$	2.136±0.02$^{\#\#\#}$
4	***LPS + Indomethacin (2.5µg/ml)***	0.809±0.07***	2.739±0.09***	2.013±0.08***

Values are expressed as Mean ± SEM; n=3 $^{***}p<0.001$ when compared with LPS, $^{\#\#\#}p<0.001$, $^{\#\#}p<0.01$ when compared with Indomethacin.

Figure 1: Cytokine ELISA



Values are expressed as Mean ± SEM; n=3

DISCUSSIONS:

Natural products serve as a rich source of novel bioactive secondary metabolites [18]. Various bioassays are available to assess the anti-inflammatory properties of these compounds, with macrophages playing a key role in many of these assays due to their direct involvement in the inflammatory response [19].Macrophages are responsible for producing a range of cytokines, reactive oxygen and nitrogen species, and growth factors in response to activation signals such as chemical mediators, cytokines, and bacterial lipopolysaccharides (LPS) [20-21].

Although the bioactive molecules generated by macrophages play a crucial role in inflammation, they can also have harmful and detrimental effects [22]. Therefore, modulating the production of these molecules presents a potential strategy for controlling inflammatory diseases. Of particular significance is the cytokine tumor necrosis factor-alpha (TNF-α), which regulates various biological processes, including the production of other cytokines [23]. By employing an *in vitro* bioassay to measure TNF-α production, natural product-derived compounds that inhibit this cytokine can be identified.

The ethanolic extract of *Averrhoa carambola* leaves demonstrated significant inhibition of TNF-α release. This effect is attributed to the presence of bioactive compounds such as quinones, total flavonoids, saponins, alkaloids, and phenolic acids, including sinapic acid, protocatechuic acid, and caffeic acid.

CONCLUSIONS:

At a final concentration of 50 µg/ml, Averrhoa carambola was found to significantly reduce the production of the three key pro-inflammatory cytokines—IL-6, IL-1β, and TNF-α—when compared to the LPS control. Its activity was found to be comparable to that of the standard anti-inflammatory drug, Indomethacin.

In conclusion, the A. carambola extract exhibited notable anti-inflammatory effects in vitro. The presence of phenolic and flavonoid compounds may partly explain this activity, while the synergistic effect of multiple bioactive compounds could enhance the pharmacological potential of the extract. Given its therapeutic properties, A. carambola leaves support their traditional medicinal use and hold promises for development as a commercial anti-inflammatory agent.

Further research is now required to elucidate the signaling mechanisms through which the extract modulates immune cell functions in vitro.

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