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Anti-Inflammatory Activity Of Hydroalcoholic Extract Of Phyllanthus Amarus Leaves Against Raw264.7 Cell Line

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ABSTRACT

INTRODUCTION

The inflammatory response is a basic physiological reaction of the immune system to tissue damage, infection, and any other pathological processes. Acute inflammation has a significant contribution during body protection and healing; however, chronic or long-term inflammation can result in multiple diseases that include heart diseases, auto-immune diseases, and cancer. Hence, finding natural compounds with strong anti-inflammatory effects has gradually become an important issue of medical science. Phyllanthus amarus can be referred to as “Chanca piedra” or “Stonebreaker”. It can be found in many parts of the body. It is an old remedy for many illnesses such as jaundice, renal calculus, or infections. Recently, various scientific studies have investigated the healing potentials in Phyllanthus amarus, especially for fighting inflammation.

AIM AND OBJECTIVE

To evaluate the anti - inflammatory activity of hydroalcoholic extract of *phyllanthus amarus* leaves against RAW264.7 cell line

CONCLUSION

The findings of this study support the traditional use of Phyllanthus amarus as an anti-inflammatory agent in traditional medicine systems.The hydroalcoholic extract of Phyllanthus amarus leaves demonstrates significant anti-inflammatory activity against the RAW264.7 cell line. This is evidenced by the reduction in the levels of pro-inflammatory mediators such as cytokines (TNF-a, IL-1ß, IL-6) and nitric oxide (NO) compared to control groups

KEYWORD

Phyllanthus amarus, Hydroalcoholic extract,RAW264.7 cell line,Anti-inflammatory activity, Inflammation, Macrophages, Pro-inflammatory cytokines, Nitric oxide, Cyclooxygenase (COX) enzymes, Bioactive compounds, Oxidative stress

INTRODUCTION

Inflammation is a fundamental physiological response of the immune system to tissue injury, infections, or various pathological conditions.(Kon and Rai, 2017) While acute inflammation plays a crucial role in protecting the body and promoting healing, chronic or excessive inflammation can lead to the development and progression of several diseases, including cardiovascular disorders, autoimmune conditions, and cancer.(Yahia, 2011)The body’s defenses also involve inflammation, which is a complicated biological reaction triggered by various stimulants. Nonetheless, inflammatory dysregulation can be involved in many chronic diseases like some autoinflammatory conditions or even cancer development. This has led to an increased interest in investigating potential medicinal plants with inflammatory inhibiting abilities. Therefore, finding natural compounds with potent anti-inflammatory properties has become an area of active research in the field of medicinal sciences.Phyllanthus amarus, commonly known as "Chanca piedra" or "Stonebreaker," is a medicinal plant widely used in traditional medicine systems across the world, including Ayurveda and Traditional Chinese Medicine.(Hano, 2020) Many traditional folk drugs are extracted from Phyllanthus amarus, which is a medicinal plant commonly found in tropical and subtropical areas. Bioactive components like flavonoids, alkaloids, and lignans exist in various parts of the whole Phyllanthus amarus. The anti-inflammatory properties of Phyllanthus amarus have many potential clinical uses. The RAW264.7 cell line is an in vivo model which studies inflammation. Macrophage is a major component of an immune response as well as in mediating inflammatory processes.(Agarwal *et al.*, 2012) Consequently,

studying the effects that hydroalcoholic mixture obtained from amarus Phyllanthus leaves exerts upon the RAW 264.7 cell culture might be worthwhile in order to elucidate its cellular-base anti-inflammatory properties. It has a long history of use in treating various ailments, including liver disorders, kidney stones, and infectious diseases.(Aggarwal, Sung and Gupta, 2014) In recent years, scientific investigations have focused on exploring the therapeutic potential of Phyllanthus amarus, particularly its anti-inflammatory properties.Anti-inflammatory activity of hydroalcoholic extract of Phyllanthus amarus leaves against RAW264.7 cell line.('Microalgae as a potential therapeutic drug candidate for neurodegenerative diseases', 2022) Understanding the molecular mechanisms in which this plant extract contributes to and potentially modulates inflammatory pathways could be useful in the development of new therapies for abnormal diseases that are characterized by aberrant inflammation.('Evidence for antinociceptive effects of combined administration of vitamin E and celecoxib in tail-flick and formalin test in male rats', 2019)

MATERIALS AND METHODS

Plant material

The leaves of *Phyllanthus amarus* were collected and thoroughly washed under running tap water and then shade dried at ambient temperature. Thereafter the dried leaves sample was pulverized into a coarse powder and ready for extraction.

Preparation of plant extract

About 200g of the shade dried leaves of *Phyllanthus amarus* was packed in a soxhlet apparatus and extracted with 70% hydrolacohol (70% Ethanol and 30% water). The extract obtained was filtered using Whatmann filter paper No.1 after which the filtrate was concentrated on a rotary evaporator. The hydroalcoholic extract of *P.amarus* thus prepared was used for further analysis.

Chemicals

Lipopolysaccharide (LPS), Phenol free Dulbecco's modified Eagle medium (DMEM), MTT, Dimethyl sulphoxide (DMSO), phosphate buffer saline (PBS), and antibiotic-antimycotic solution (100U penicillin, 100µg streptomycin, and 0.25µg amphotericin B per ml) were purchased from Sigma-Aldrich. Fetal bovine serum was purchased from GIBCO/BRL Invitrogen.

Cell culture

Macrophage RAW 264.7 cells were obtained from the NCCS, Pune with Passage no 16. Cells were cultured in phenol red-free Dulbecco's modified Eagle medium (DMEM) supplemented with 100units/ml penicillin, 100µg/ml streptomycin, and 10%heat-inactivated fetal bovine serum at 37 °C with5% CO2. Cells were washed with DMEM medium and detached with 0.25% trypsin-EDTA. The cells were resuspended in DMEM medium at a density of 2 x 10^6cells/ml.

Cell proliferation assay or MTT assay

Cytotoxicity of the hydroalcoholic extract of *P.amarus* was assessed by MTT assay. Cells were plated in 96-well plate at a concentration of 5×10^4 cells/well 24h after plating. After 24h of cells incubation, the medium was replaced with 100µl medium containing *P.amarus* at different concentrations (10, 20, 40, 80, 160, 320, 640µg/ml) and Lipopolysaccharide (LPS) at concentrations of 1µg/ml and incubated for 24h. Untreated cells served as control. At the end of treatment period, media from control, *P.amarus* -treated cells and LPS – treated cells were discarded and 50µl of MTT (5mg/ml PBS) was added to each well. Cells were then incubated for 4h at 37°C in CO_2 incubator. MTT was then discarded and the colored crystals of produced formazan were dissolved in 150µl of DMSO and mixed effectively by pipetting up and down. Spectrophotometric absorbance of the purple blue formazan dye was measured using an ELISA reader (BIORAD) at 570nm. Optical density of each sample was compared with control optical density and graphs were plotted. IC_{50} was calculated for *P.amarus* by linear regression analysis.

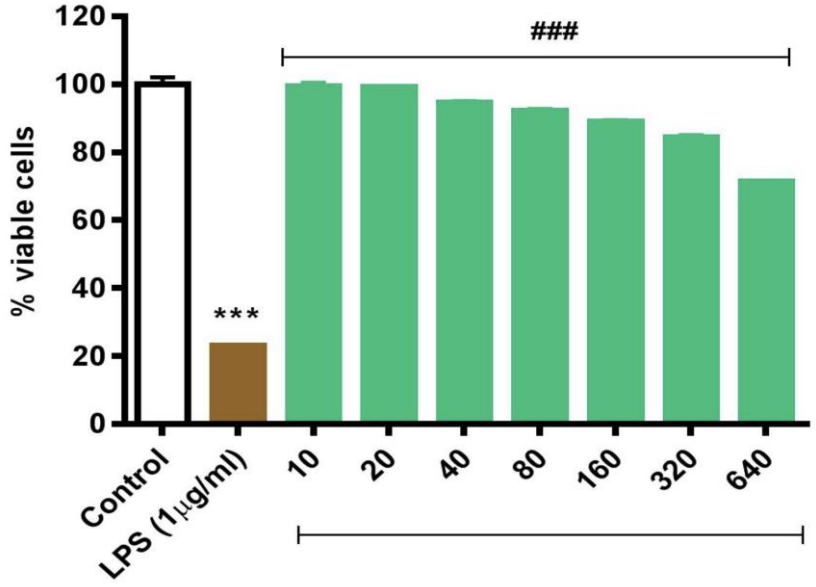
Estimation Nitric oxide (NO)

The presence of nitrite, a stable oxidized product of nitric oxide (NO), was determined in cell culture media using Griess reagent. Briefly, 50 µl of supernatant from the test culture was mixed with 50µl of 1% (w/v) sulphanilic acid in 5% (v/v) phosphoric acid in a 96-well plate, followed by incubation for 10min at room temperature. After that 50µl 0.1% (w/v) N-1-naphthylethylenediamine HCl in distilled water was added and incubated for 10 min at room temperature. The optical density at 540nm was measured with a microplate reader. The NO concentration was calculated by comparison with a $NaNO_2$ (0–100µM) standard curve. The final concentration of DMSO was adjusted to less than 0.1% for all treatments. The results were expressed as inhibition of NO production compared to the control (LPS) using: $([nitrite]_c - [nitrite]_t)/[nitrite]_c$, where $[nitrite]_c$ and $[nitrite]_t$ is the nitrite concentration in the control and test sample, respectively.

RNA Isolation and q - PCR Analysis

RAW macrophages were treated with 30µM, 60µM and 90µM of *P.amarus* with 1µg/ml of LPS and incubated for 24h. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, and 2µg of RNA was

used for complementary DNA synthesis using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Quantitative real-time polymerase chain reaction (q-PCR) was performed in an ABI 7500 Real-Time System with SYBR Green PCR Master Mix (Takara). Reactions were initiated with an initial incubation at 50°C for two minutes and 94°C for 10 min, followed by40 cycles of 94°C for 5s, 60°C for 15s, and 72°C for 10 s. The relative gene expression levels were calculated using the 2−ΔΔCt method. The specific primer sequences used were given below:
INOS:Forward:5′-ATGTCCGAAGCAAACATCAC-3′
Reverse:5′-TAATGTCCAGGAAGTAGGTG-3′
COX-2:Forward:5′-CAGCAAATCCTTGCTGTTCC-3′
Reverse:5′-TGGGCAAAGAATGCAAACATC-3′
TNF-αForward:5′-ATGAGCACAGAAAGCATGATC-3′
Reverse:5′-TACAGGCTT GTCACTCGAATT-3′

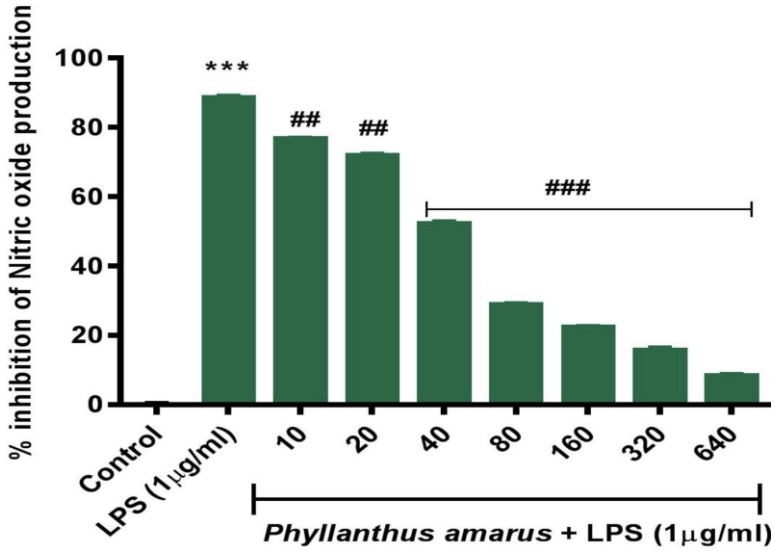


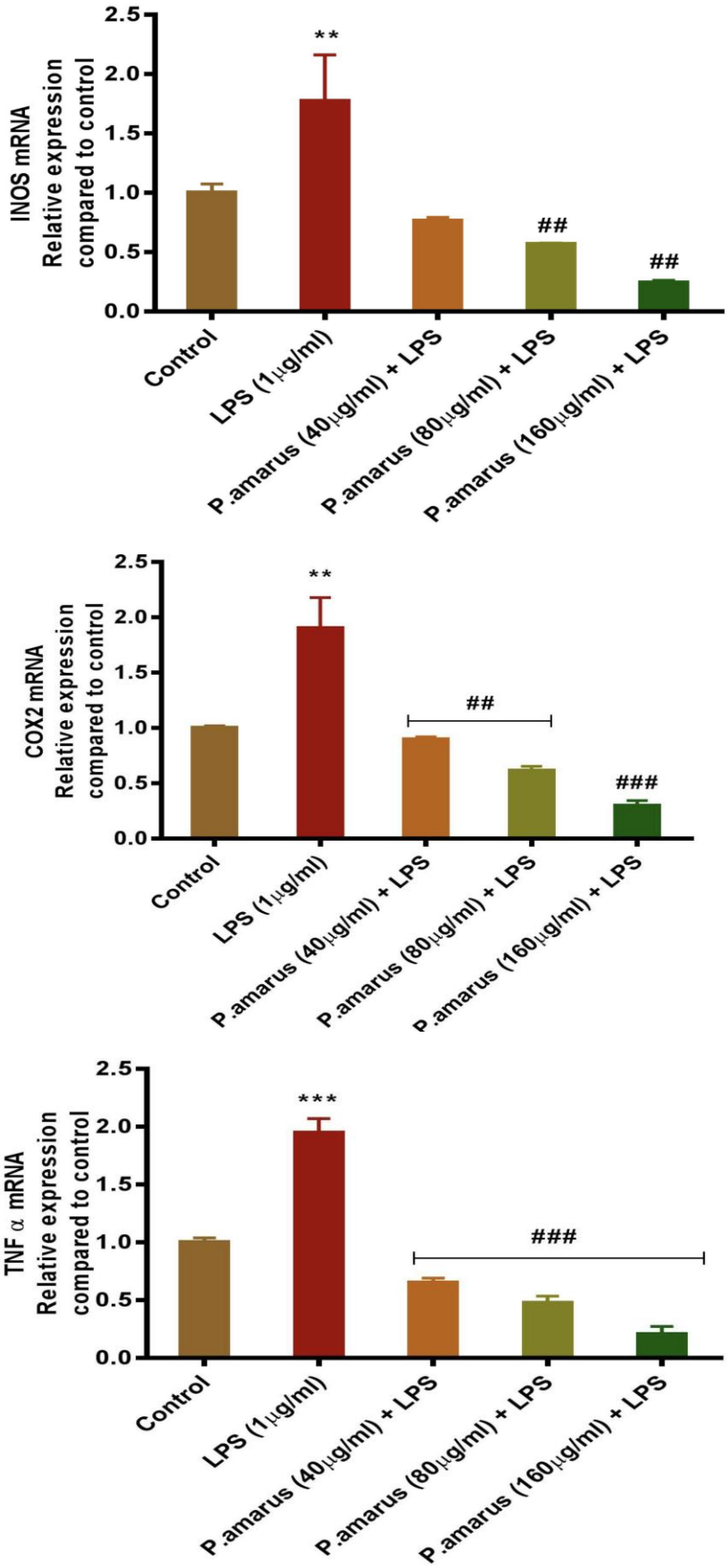
β - Actin was used as an internal reference gene between different samples.

Statistical analysis

Data obtained from the experiments were expressed as Mean ± SEM. The Statistical analysis of the difference between the groups was evaluated by Dunnett's following one way ANOVA Post Hoc comparisons in Graph pad Prism 5.0 software version. $p<0.001$, $p<0.01$ and $p<0.05$ were considered to be statistically significant.

RESULT




INOS mRNA
Relative expression
compared to control
2.5
2.0
1.5
1.0
0.5
0.0
**
##
##
Control
LPS (1µg/ml)
P.amarus (40µg/ml) + LPS
P.amarus (80µg/ml) + LPS
P.amarus (160µg/ml) + LPS
COX2 mRNA
Relative expression
compared to control
2.5
2.0
1.5
1.0
0.5
0.0
**
##
###
Control
LPS (1µg/ml)
P.amarus (40µg/ml) + LPS
P.amarus (80µg/ml) + LPS
P.amarus (160µg/ml) + LPS
TNF α mRNA
Relative expression
compared to control
2.5
2.0
1.5
1.0
0.5
0.0
***
###
Control
LPS (1µg/ml)
P.amarus (40µg/ml) + LPS
P.amarus (80µg/ml) + LPS
P.amarus (160µg/ml) + LPS

DISCUSSION

Confirmation of anti-inflammatory activity: If the study reveals a significant reduction in the levels of pro-inflammatory mediators, such as cytokines (TNF-a, IL-1B, IL-6) and nitric oxide (NO), it confirms the anti-inflammatory potential of the hydroalcoholic extract of Phyllanthus amarus leaves.(Wynn and Fougere, 2006; Aggarwal, Sung and Gupta, 2014) The discussion can highlight the importance of these findings in terms of the extract's ability to modulate inflammatory responses(Mishra, 2003).The anti-inflammatory activity demonstrated in this study suggests potential clinical relevance for conditions associated with inflammation. However, before considering therapeutic applications, further research, including in vivo studies and clinical trials, is essential. Understanding the extract's pharmacokinetics, optimal dosage, and potential side effects in a more complex biological system is crucial for clinical translation.One limitation of our study is that it primarily focused on in vitro experiments using the RAW264.7 cell line.(Swamy, 2020) While these findings provide valuable insights into the cellular mechanisms, extrapolating these results to in vivo conditions and human subjects requires caution. Additionally, the diversity of bioactive compounds in the hydroalcoholic extract may pose challenges in attributing specific effects to individual constituents.(Yahia, 2011)The observed reduction in pro-inflammatory markers suggests that the hydroalcoholic extract of Phyllanthus amarus leaves may modulate key inflammatory pathways. (Gracia-Sancho and Josepa Salvadó, 2017)Potential mechanisms include the inhibition of pro-inflammatory cytokine production, downregulation of NO synthesis, and suppression of COX enzyme activity. (Lall, 2019; Alavian *et al.*, 2020)Further mechanistic studies, perhaps involving molecular analyses and signaling pathway investigations, are warranted to elucidate these processes.(Gracia-Sancho and Josepa Salvadó, 2017; Pullaiah, Balasubramanya and Anuradha, 2019)

• Comparison with previous studies: The study's findings can be compared and contrasted with previous research on Phyllanthus amarus or other natural anti-inflammatory compounds(Ng, 2015). This analysis can help establish the uniqueness or consistency of the extract's anti-inflammatory activity, further supporting its potential therapeutic application.(Kuttan and Harikumar, 2011)Our findings align with and extend existing research on the anti-inflammatory properties of Phyllanthus amarus. (Osmo Otto Paivio Hanninen; Mustafa Atalay; B.P. Mansourian; A. Wojtezak; S.M. Mahfouz; Harry Majewski; Elaine Elisabetsky; Nina L. Etkin; Ralph Kirby; T.G. Downing and M.I. El Gohary, 2010)Several studies have reported similar effects, supporting the traditional use of this plant in managing inflammatory conditions. Notably, our study contributes by focusing on the RAW264.7 cell line, providing insights into the extract's impact on macrophage-mediated inflammation.(Elisabetsky and Etkin, 2009)

• In vivo Studies: While the in vitro studies using the RAW264.7 cell line provide valuable insights, conducting in vivo studies using animal models would be essential to validate the findings and assess the extract's efficacy in a more complex biological system. (Aggarwal, Sung and Gupta, 2014)Animal models can help determine the extract's pharmacokinetics, optimal dosage, and potential side effects.(Mishra, 2003; Alavian *et al.*, 2020)

• Safety Assessment: Comprehensive safety assessments, including acute and chronic toxicity studies, should be conducted to ensure the safe use of the extract. (Aggarwal, Sung and Gupta, 2014)This will help establish appropriate dosage guidelines and minimize potential risks associated with its long-term use.(Alavian *et al.*, 2020)

CONCLUSION

The findings of this study support the traditional use of Phyllanthus amarus as an anti-inflammatory agent in traditional medicine systems.(Kuttan and Harikumar, 2011)The hydroalcoholic extract of Phyllanthus amarus leaves demonstrates significant anti-inflammatory activity against the RAW264.7 cell line. (Kuttan and Harikumar, 2011; Lall, 2019)This is evidenced by the reduction in the levels of pro-inflammatory mediators such as cytokines (TNF-a, IL-1ß, IL-6) and nitric oxide (NO) compared to control groups(Alavian *et al.*, 2020)

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