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Research Article

Comparative Study of Anti-Inflammatory Activity of Two Formulations of Jatyadi thailam *in vitro* on Raw264.7 Macrophages and its Dual Inhibitor role of 5-LOX and COX

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ABSTRACT

Jatyadi thailam is a traditional formulation widely used to treat chronic wounds. Reverse pharmacology approach is employed to study the mode of action of two traditional formulations of thailam-AFI Jatyadi thailam (JT-AFI) and Jatyadi thailam-Yogagrantha (JT-YG) against radicals DPPH, ABTS, nitric oxide, hydroxyl, and superoxide radicals and inflammatory mediators *in-vitro*, namely nitric oxide, 5-Lipoxygenase and cyclooxygenase-1 (COX-1), and cyclooxygenase-2 (COX-2). Both formulations of Jatyadi thailam rendered dose-dependent inhibition of free radicals and demonstrated their reduction potential.The free radical formation is inextricably associated to the inflammatory process. The formulations significantly reduced nitrite at its highest concentration in lipopolysaccharide LPS-induced RAW264.7 macrophage cells. The formulations also unveiled subsequent dose-dependent inhibition of 5-Lipoxygenase, cyclooxygenase-1, and cyclooxygenase-2 enzymes. JT-AFI proved to be a selective inhibitor for COX-2 with a COX-1/COX-2 ratio of 1.924, while JT-YG was COX-1 selective (0.794). The study indicated that JT-AFI exhibited superior antioxidant and anti-inflammatory activities than JT-YG. The current investigation could shed insights on the scientific validation of using jatyadi thailam in chronic wound management.

Introduction

Oxidative stress is a global concept and has become a prominent focus of clinical and preclinical research. It occurs when pro-oxidant production surpasses the body's natural antioxidant defense mechanism. The production of reactive oxidants, including superoxide, hydroxyl radicals, and hydrogen peroxide, is an unavoidable byproduct of regular oxygen metabolism. One of the key factors implicated in persistent diabetic foot ulcers is oxidative stress. Excess and uncontrolled oxidative stress could lead to the deregulation of inflammation, which plays a vital role in the etiology of chronic non-healing wounds.[1] Excessive reactive oxygen species (ROS) formation damages redox equilibrium in chronic diabetic wounds, inducing antioxidant depletion and impairing wound healing.[2] Research that might throw light on the role of free radicals and their harmful effects in chronic wound healing will play a significant role.

Inflammation is a recurrent theme for many skin pathologies. The normal function of macrophages and neutrophils in wounds is affected by oxidative stress during the inflammation phase, resulting in prolonged inflammation. The inflammatory process is intrinsically tied to the creation of free radicals. Free radicals activate the immune system and attract inflammatory cells, which increases the risk of tissue injury. Pro-inflammatory mediators (Prostaglandins and nitric oxide) are the primary target mediators accountable for the induction, progression, tenacity, regulation, and eventual healing

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of the acute inflammatory state. Nitric oxide (NO) is a signaling molecule that serves a significant role in inflammatory aetiology.

On the other hand, NO is a pro-inflammatory mediator that causes inflammation when produced in excess under abnormal conditions.[3] Macrophages are extremely sensitive to lipopolysaccharide stimulation, which is primarily liable for several pathophysiological factors linked with gram-negative sepsis and other inflammatory conditions.[4] As a result, NO inhibitors significantly advance the treatment of inflammatory illnesses.

Inflammation of the wound is triggered by a number of mediators. The lipoxygenase (LOX) and cyclooxygenase (COX-1 and COX-2) pathways have been shown to play a key role in developing inflammatory disorders in recent years. COX-2 is activated in the early stages of inflammation in response to pro-inflammatory mediators and stimuli, including cytokines and endotoxins.[5] COX-1 has been connected to synthesizing physiologically significant prostaglandins that may help maintain homeostasis and also involved in inflammation, swelling, and pain.[6] There is a continuing attempt to find chemicals that suppress COX-2 rather than COX-1, as this would be a safer and more effective agent. COX appears to be linked to coordinated cell motility and new tissue formation in the skin and respiratory epithelium; therefore, it could be implicated in wound healing. Thus, dual COX-2/LOX inhibitor molecules could be devised into better and more efficient anti-inflammatory medications, as they could limit the synthesis of leukotrienes and prostaglandins without the side effects of nonsteroidal drugs[7] and also shown to improve tissue regeneration.

The rising prevalence of inflammatory illnesses necessitates newer alternatives for nonsteroidal anti-inflammatory drugs and a broader spectrum of inflammatory products. Blending medicinal plants and herbs with oils or clarified butter as oleaginous preparations is a prevalent practice in Traditional therapeutic systems, termed medicated oil (Thailam) in traditional texts.[8] Jatyadi thailam is an ayurveda-based polyherbal formulation extensively used in folk medicine to treat and manage chronic ulcers. There exist two kinds of formulation; according to ayurvedic formulary of India (AFI) guidelines and another based on the components in yogagrantha (YG) text. Preliminary clinical case studies[9,10] and *in-vivo* investigations on using Jatyadi thailam to heal chronic wounds have been reported in the excision wound model,[11] but the formulations have not been validated using scientific methods. A therapeutic drug should have anti-inflammatory and antioxidant effects for high healing efficacy. Therefore, this study focuses on evaluating the holistic potential of Jatyadi thailam in combating the reactive oxidants and pro-inflammatory mediators, which validates its use in treating non-healing wounds and thus, improving patient care.

Materials and Methods

Sample Preparation

Standardized AFI Jatyadi thailam (JT-AFI) and yogagrantha Jatyadi thailam (JT-YG) formulation was supplied by Arya Vaidhya Chikitsalayam, Coimbatore. To overcome the solubility problem, the samples were prepared according to w/v method using PEG-40 HCO and SPAN 80 in aqueous conditions.

Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMEM medium (Gibco), fetal bovine serum (FBS)(Gibco), penicillin/streptomycin (Penstrep) (Gibco) and Lipopolysaccharide (LPS) from *Escherichia coli*, Diclofenac, 5-lipoxygenase (5-LO), nordihydroguaiaretic acid (NDGA) were purchased from Sigma, USA. Cyclooxygenase 1 and Cyclooxygenase 2 (COX-1 and COX-2) were purchased from Cayman (USA). All other solvents and chemical reagents used were of analytical grade.

Free Radical Scavenging Activities of Jatyadi Thailam

DPPH Spectrophotometric Assay[12]

JT-AFI and JT-YG (20 µL) was combined with 0.5 mL DPPH solution and methanol to make the volume 1-mL. The reaction mixture was vigorously agitated and set aside for 30 minutes at room temperature. Purple color decolorization was measured at 518 nm. The positive control was DPPH in methanol, while the blank was methanol.

ABTS Radical Scavenging Assay[13]

ABTS 2 mM and potassium persulfate 70 mM were prepared in distilled water. A mixture of potassium persulphate (200 µL) and ABS (50 mL) was prepared and used after 2 hours. The assay was performed with this ABTS radical cation solution. 300 µL ABTS radical cation and 1.7 mL phosphate buffer (pH 7.4) were added to 500 µL of varied concentrations of samples. Instead of the test substance, methanol was utilized as a control. At 734 nm, the absorbance was measured and the experiment was done in triplicates.

Reducing Power Assay[14]

The samples at varying concentrations were mixed with an equal volume of 1% potassium ferricyanide and sodium phosphate buffer (0.2M, pH 6.6). At 50°C, the solution was left to react for 30 minutes. To the reactants, 2.5 mL of 10% trichloroacetic acid was added and the solution combination was centrifuged at 2000 g for 10 minutes. The outermost layer (2.5 mL) was mixed with 2.5 mL deionized water and 0.5 mL ferric chloride and the absorbance was measured spectrophotometrically at a wavelength of 700 nm.

Nitric Oxide Radical Inhibition Assay[15]

The reaction mixture consists of 0.5 mL of different concentrations of samples with 2.0 mL sodium nitroprusside and 0.5 mL PBS. The assay mixture was thoroughly mixed and incubated for 150 minutes at 25°C. The Griess reagent (0.5 mL) was added to the assay mixture and left for 30 minutes. The samples were not used in the control tube. The pink-tinted chromogen's absorbance was measured at 546 nm against a reagent blank.

H_2O_2 Scavenging Assay[16]

10 µL JT-AFI and JT-YG of different concentrations were mixed with 0.6 mL of H_2O_2 solution. Phosphate buffer was used to increase the total volume to 3.0 mL. At 230 nm, the absorbance of the reaction mixture was measured. Phosphate buffer without H_2O_2 was used in the blank solution.

Hydroxyl Radical Scavenging Assay[17]

The reaction mixture consists of each 0.1 mL of 2-deoxyribose, $FeCl_3$, EDTA, H_2O_2, ascorbate and buffer. By adding 20 µL of varying concentrations of samples, the final amount was increased to 1.0 mL. After incubating the reaction mixture for 1-hour at 37°C, 0.5 mL of it was added to 1.0 mL of TCA solution. The mixture was then incubated at 90°C for 15 minutes to allow for color development, after which 1-mL of 1% aqueous TBA was added. The absorbance was measured as 532 nm against a suitable blank solution after cooling.

Superoxide Radical Inhibition Assay[18]

0.02 mL of different concentrations of samples, 0.2 mL of EDTA, 0.1 mL of NBT, and 0.05 mL of riboflavin made up the test mixture. Phosphate buffer was used to increase the final volume to 3.0 mL. Instead of test sample, DMSO was used in the control tube. The tubes were vortexed, and the absorbance was measured at 560 nm after 30 minutes of uniform illumination and the percentage inhibition was estimated.

In vitro Anti-Inflammatory Activity

Nitric Oxide Inhibitory Assay[19]

- *Cell culture*

The RAW264.7 macrophage cell lines were obtained from NCCS, Pune. It was cultured in tissue culture flasks in DMEM supplemented with 10% FBS and 1% Penstrep solution at 37°C in a 5% CO_2 environment. Cells were seeded in 96 well-microtitre plates and were stimulated by incubation in medium containing LPS (1 µg/mL) and various concentrations of samples.

- *Cytotoxicity-tetrazolium-based Colorimetric Assay (MTT)*

Initially, the non-cytotoxic concentrations of samples in RAW264.7 cells were determined by standard MTT assay. RAW264.7 cells were seeded in 96-well plates at the density of 1.5×10^4 cells/well. After 24 hours of incubation, the adherent cells were treated with varying concentrations of the prepared samples in concentrations ranging from 500 to 2500 µg/mL. After changing the medium to 24 hours, 50 µL of MTT solution (1 mg/mL) was added, and the plates were incubated for another 4 hours. The formazan crystal was then dissolved with DMSO. An absorbance measurement at 570 nm on a microplate reader was used to determine cell survival (Biorad, USA).

Inhibition of Nitric Oxide (NO) Production

Macrophage cells RAW264.7 (4×10^5 cells/well) was seeded in 24-well plate overnight. Samples were added at varying concentrations and incubated for 1 hour. Then, they were activated with 1 µg/mL of LPS per ml of medium and further incubated for another 24 hours. In 96-well plate, culture supernatant was mixed with Griess Reagent (1:1 ratio), incubated in the dark for 10 minutes and spectrophotometrically read at 570 nm. The amount of nitrite was determined from a sodium nitrite standard curve.

5-LOX Inhibition Assay[20,21]

Different concentrations of 20 μL samples were dissolved in DMSO and plated in triplicate in a 96-well microtiter plate, followed by addition of 0.1M potassium phosphate buffer (pH 6.3) and kept at 25°C with 20 μL enzyme solution. After agitating the mixture, 10 μL of linoleic acid was added and incubated at 25°C for 10 minutes. At 234 nm, the absorbance was measured. The positive control was nordihydroguaiaretic acid (NDGA), and the percentage inhibition of enzyme was obtained by comparing the rates of reaction of the samples to the blank sample.

Peroxidase Endpoint Assay for COX-1 and COX-2[22]

20 μL of samples dissolved in DMSO were plated in triplicates at different concentrations in a 96-well microtiter plate followed by 20 μL of 10 U/mL of enzyme solution. Then, 160 μL of endpoint assay mix (100 μM bovine hemin chloride, 10 mM of AA, 17 mM of TMPD, and 1 M Tris HCl) was added and incubated at 25˚C for 10 mins. Absorbance was recorded at 611 nm using microtiter plate reader and Indomethacin (INDO) was utilized as a positive control and percentage inhibition of enzyme was determined.

Statistical Analysis

Data were obtained from three independent experiments performed in triplicates and represented as mean ± SD. Students t-test was done and $p < 0.05$ was considered significant for all the assays.

Results and Discussion

Free Radical Scavenging Activity of Thailam

Free radicals are routinely produced in living systems and can cause serious harm to tissues and organelles, contributing to multiple ailments, particularly degenerative

disorders and extensive lysis.[1] Since there is a vast production of free radicals during systemic inflammation, antioxidants and radical scavengers are frequently utilized to alleviate specific inflammatory pathologies.

Jatyadi Thailam Scavenges DPPH and ABTS Radical

DPPH assay measures the reactivity of drugs in the presence of a stable free radical. When DPPH is combined with molecules that may donate hydrogen atoms, the absorption fades away, resulting in the reduced form (Diphenyl picryl hydrazine: non-radical), decolorizing violet color.[23] The experimental data showed that maximum scavenging activity was found to be 67.75 ± 0.06 by JT-AFI followed by JT-YG (50.65 ± 0.08) at a concentration of 2500 µg/mL, while the minimum scavenging activity was found at 500 µg/mL (Fig. 1). The IC_{50} value of JT-AFI was found to be 1069.84 ± 0.64 µg/mL and that of JT-YG to be 2440.60 ± 0.59 µg/mL. It was compared with the standard ascorbic acid, which showed 91.31 ± 0.97% radical scavenging at 500 µg/mL concentration. It may be inferred from the data that when the concentration increases, the free radical scavenging ability of Jatyadi thailam also increases. However, JT-AFI showed good radical scavenging ability than JT-YG.

The extent of discoloration of the ABTS mono cation was used to assess the efficiency and standard of the formulations. The suppression of a relatively stable ABTS radical by Jatyadi thailam is depicted in Fig. 1. Both JT-AFI and JT-YG exhibited significant reduction of the radical at all the concentrations tested with the highest scavenging activity was observed to be 89.31 ± 0.01 and 81.94 ± 0.39 at 2500 µg/mL and the minimum percentage inhibition was found at 500 µg/mL. Their IC_{50} was found to be 637.53 ± 0.06 and 932.69 ± 0.28 µg/mL. Thus, both the formulations hunt the free radicals efficiently, comparable to that of standard ascorbic acid in a concentration-dependent way; however, the JT-AFI had better scavenging activity.

When ABTS and potassium persulfate combine, a blue chromophore $ABTS^{\cdot+}$ is created. Upon addition of Jatyadi thailam to this already-formed radical cation, it was transformed into ABTS. DPPH is a recognized method for evaluating antioxidant activity due to its relatively quick turnaround time for analysis.[24] The findings of this investigation indicate that both formulations demonstrated radical scavenging activity through their capacity for electron exchange or hydrogen donation and by the reduction of the pre-formed $ABTS^{\cdot+}$ radical cation.

Jatyadi Thailam Reduces Ferric to Ferrous Ion

The reducing power of the JT-AFI was superior than JT-YG and its reducing capacity is an important predictor of its potential antioxidant activity. Antioxidant activity has been attributed to various mechanisms, including chain initiation prevention, peroxide breakdown, reducing power, and radical scavenging [25]. The reducing ability of the formulations could be assessed by their capability to transform Fe^{3+} to Fe^{2+} state and the results are illustrated in Fig. 2. Fe^{3+} transforming ability of both the formulations increased with a surge in dosage and the maximum reduction potential exemplified by JT-AFI was found to be statistically significant ($P < 0.05$) followed by JT-YG. This was correlated to the response by standard ascorbic acid. Reductants, which operate as antioxidants by donating a hydrogen atom to break the chains of free radicals, are typically linked to the existence of reducing power. In this experiment, the antioxidant content in Jatyadi thailam reduces the ferricyanide complex to the ferrous form. Both formulations showed good reducing power capability, which depended on concentration; however, the reducing ability was higher in JT-AFI.

Jatyadi Thailam Inhibits Nitric Oxide Radical Generation

Nitric oxide is a diffusible free radical that serves as an effector molecule in various biological systems-vasodilation, neural messenger, antibacterial and anticancer properties.[26] Both the formulations prevent the creation of nitrite by directly combating oxygen in the nitric oxide generation. Under aerobic conditions, nitric oxide is inherently unstable and the NO• scavenging ability of the thailam is shown in Fig. 3A. JT-AFI showed maximum inhibition of 81.69 ± 0.42 and its IC_{50} value was found to be 771.19 ± 0.31 µg/mL, whereas JT-YG exhibited

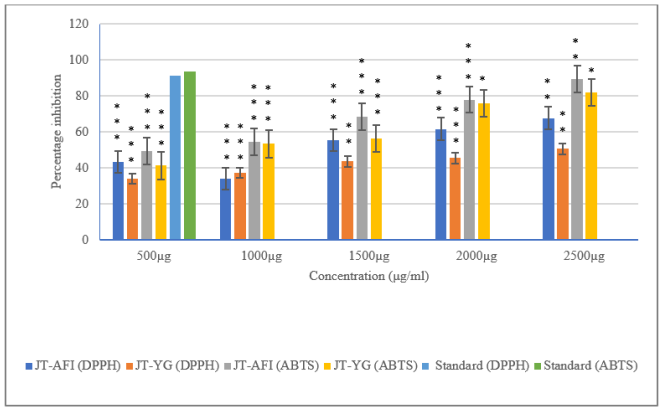

Fig. 1: DPPH and ABTS radical scavenging assay
(***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05; N= 3)

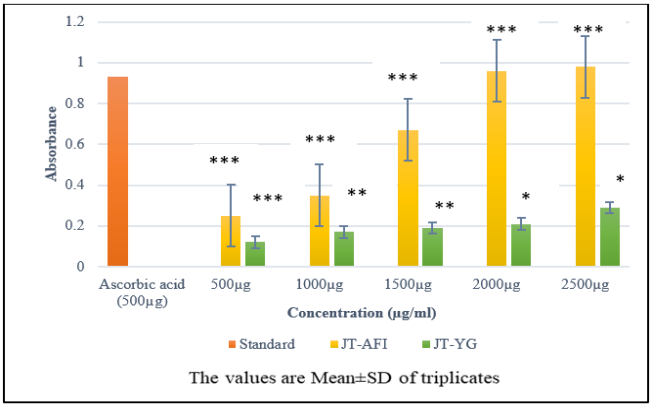

Fig. 2: Reducing power assay
(***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05; N= 3)

72.11 ± 0.33% as its highest scavenging ability with an IC_{50} value of 1391.29 ± 0.56 µg/mL. It was compared with the reference standard ascorbic acid at 500 µg/mL (89.76 ± 0.25%). The scavenging ability of both the formulations was dose-dependent. However, JT-AFI showed more efficient quenching of the radical.

Nitric oxide produced by aqueous sodium nitroprusside (SNP) solution combines with oxygen at physiological pH to form nitrite ions, which can be measured by the Griess Illosvoy reaction. Certain carcinomas and inflammatory diseases, such as juvenile diabetes, multiple sclerosis, arthritis, and ulcerative colitis, are linked to persistent expression of the nitric oxide radical.[27] By immediately outcompeting oxygen in the reaction with nitric oxide, Jatyadi thailam prevents the creation of nitrite, exhibiting stronger nitric oxide scavenging action.

Jatyadi thailam Inhibits Hydroxyl Radical Formation

Hydroxyl radical (OH•) is a very labile oxygen-centered species and the accumulation of OH• leads to several health complications.[28] The findings of OH• forage ability by the formulations are shown in Fig. 3B. On increasing the concentration of the thailam from 500 to 2500 µg/mL, a subsequent increase is found in radical destruction by JT-AFI with percentage inhibition ranging between 51.72 ± 0.21 to 86.2 ± 0.86 (IC_{50}=353.49 ± 0.14) and in case of JT-YG, it ranged between 37.93 ± 0.62 to 79.31 ± 0.57% (IC_{50}=1049.71 ± 0.09). The results were comparable to the standard drug ascorbic acid, which possessed an inhibition of 78.26 ± 0.01% at 500 µg/mL. The results showed that both the formulations are effective against OH•; however, JT-AFI showed a significant decrease ($p < 0.05$) in the production of hydroxyl radical compared to JT-YG.

The hydroxyl radicals from the sugar were neutralized by Jatyadi thailam formulations when added to the reaction mixture, inhibiting the process. The IC_{50} value shows that the JT-AFI outperforms JT-YG as a hydroxyl radical scavenger.

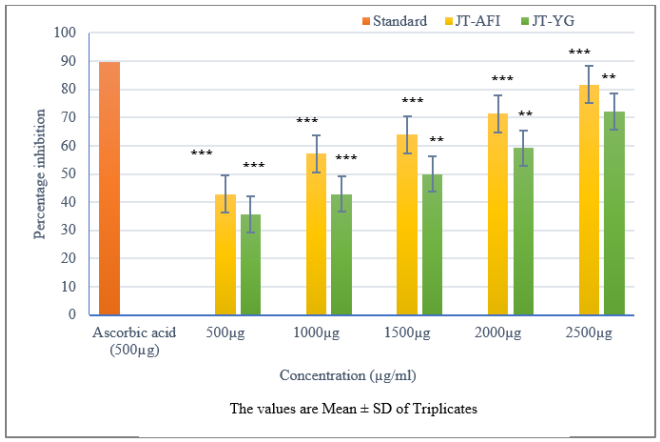

Fig. 3A: Nitric oxide scavenging assay
(***p ≤ 0.001, **p ≤ 0.01; N= 3)

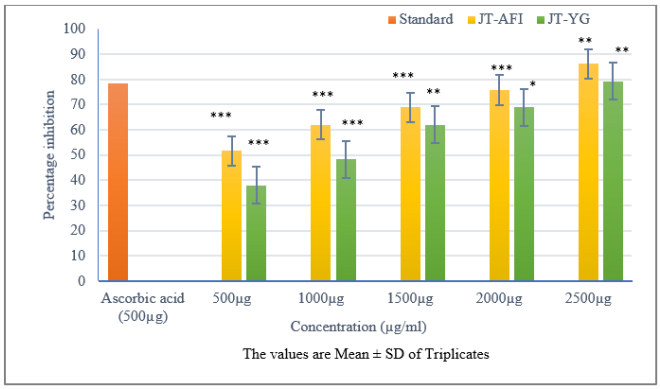

Fig. 3B: Hydroxyl radical scavenging assay
(***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05; N= 3)

Jatyadi thailam Quenches Superoxide Radical

The propensity of superoxide radicals released from ambient oxygen via PMS-NADH coupled reaction can be assessed by their capacity to diminish NBT. The efficacy of the formulations and the reference standard ascorbic acid to quell superoxide radicals can be known from the decline in absorbance at 560 nm. The data (Fig. 3C) revealed that the maximum reduction of the NBT was by JT-AFI followed by JT-YG at 2500 µg/mL and their percentage inhibition was found to be 87.18 ± 0.46 and 79.18 ± 0.24, respectively. IC_{50} of JT-AFI was 649.71 ± 0.48 µg/mL and for JT-YG. IC_{50} was 1026.277 ± 0.11 µg/mL. At 500 µg/mL, the percentage inhibition of the formulation was 48.12 ± 0.16% and 38.12 ± 0.37%, whereas that of ascorbic acid was 92.18 ± 0.58%. Thus, both the formulations responded in a dose-proportional basis and may act as a potential scavenger of superoxide generating system.

Thus, from the results of the free radical scavenging ability of JT-AFI and JT-YG, it could be inferred that both JT-AFI and JT-YG were effective against DPPH, ABTS, and Nitric oxide radicals. Hydroxyl radicals are short-lived yet highly reactive radicals produced by normal cellular metabolism, which can cause irreparable damage to DNA and proteins.[29] The inescapable byproduct of cellular respiration is the superoxide free radical. In the presence of Jatyadi thailam, there is a decreasing trend in absorbance with an increase in inhibition of these radicals exhibiting a potent

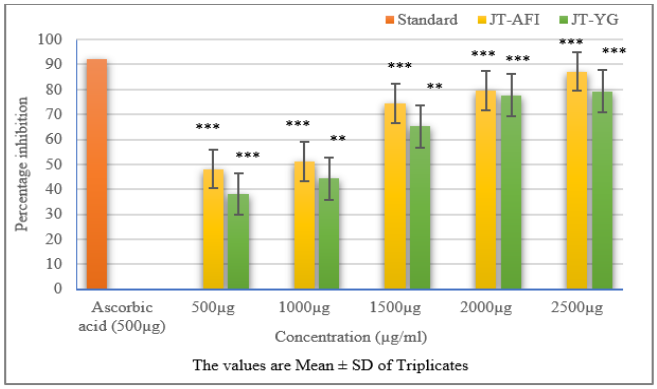

Fig 3c: Superoxide radical scavenging activity
(***p ≤ 0.001, **p ≤ 0.01; N= 3)

hydroxyl and superoxide radical scavenging capacity. The present findings showed that *in-vitro* free radical results give a perception of Jatyadi thailam in treating chronic wounds with JT-AFI outperforming JT-YG. Our results are in accordance with the study of Prabhu *et al.* (2020), who reported the potential antioxidant ability of Mahanarayana Thailam against free radicals.[30] Similar results were reported for Shivanar VembuKuzhi Thailam.[31] Thus, from the results, we could imply that the polyherbomineral formulation- JT-AFI and JT-YG could be an effective scavenger of free radicals and may help combat inflammation with much higher activity observed in JT-AFI.

Anti-inflammatory Activity of Jatyadi thailam

Innate immune cells are crucial for developing inflammatory responses and subsequent events in tissue damage. During this process, activated inflammatory cells release abnormal levels of NO, cytokines, and prostaglandin E2. These not only cause tissue injury and cell damage and stimulate macrophages in certain chronic inflammatory conditions. NO plays a crucial role in inflammatory pathogenesis and has been allegedly involved in endotoxin-induced tissue injury. Macrophages play a fundamental role in regulating various immunopathological phenomena during inflammation, including overproduction of NO [32].

Jatyadi thailam Inhibits NO in RAW264.7 Cells

Macrophages play an integral role in inflammation. The respective cellular reaction is stimulated by bacterial lipopolysaccharide (LPS) and can produce diverse inflammatory mediators, which include nitric oxide (NO), the most significant mediator in human health and pathology.[3] Nitric oxide (NO) is a pro-inflammatory messenger involved in various pathophysiological processes and also has a central role in host defense against pathogenic and stress environments. However, overproduction of NO may also lead to tissue injury related to acute and chronic inflammation [33]. Before analyzing the ability of the samples to suppress NO production in LPS-induced RAW264.7 macrophage cells, cytotoxicity was measured using MTT to evaluate the toxicity of the formulations to the cells while exercising their anti-inflammatory activity. Nevertheless, NO is very unstable in the biological matrix and easily oxidizes to nitrite (NO_2^-); thus the measurement of nitrite is habitually used as an index of NO output.

The results of the ability of different doses of JT-AFI and JT-YG on NO production in RAW264.7 cells are presented in Fig. 4B. RAW264.7 cells were stimulated with LPS and NO generation was assessed as nitrite concentration in the culture medium. Compared to the control, the pre-treated cells stimulated with LPS generated only a reduced amount of NO in the medium in the form of their stable non-volatile breakdown product nitrite. The JT-AFI and JT-YG showed a significant reduction ($p < 0.05$) of nitrite level from 79.47 ± 1.27 to 31.96 ± 0.50 µM and 82.6 ± 1.05 to 53.74 ± 1.00 µM at its highest tested concentration of 2500µg/mL. The result (Shown in Fig. 4A) of MTT cell viability assay confirmed that the repressive effect of these formulations was not caused by cell damage (viability>80%). The results are compared with the standard diclofenac, which showed a great drop in nitrite level upto 16.27 ± 0.98 µM. The data showed that the JT-AFI inhibited NO production more competently than JT-YG in LPS-stimulated RAW264.7 cells. This may be due to the viable efficiency of cells in the former than the latter. These observations imply that the JT-AFI may hold more pharmacological effectiveness in the treatment of wound healing.

The present study showed that JT-AFI significantly inhibited NO generation than JT-YG in LPS-stimulated RAW264.7 cells. No effective cytotoxicity of the formulations was detected in any tested doses, suggesting its protective role against NO generation. This may imply that Jatyadi

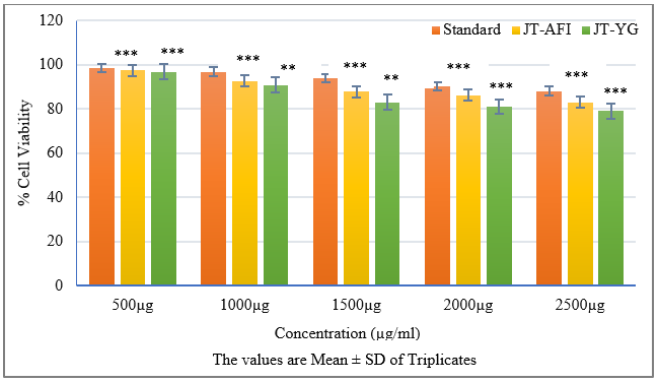

Fig. 4A: Effects of treatment on cell viability of RAW264.7 macrophages (Values shown are Mean ± SD; Significant difference ($p < 0.05$) was observed for all the tested concentrations (***p ≤ 0.001, **p ≤ 0.01; N= 3) when compared to control (100%) and Standard.

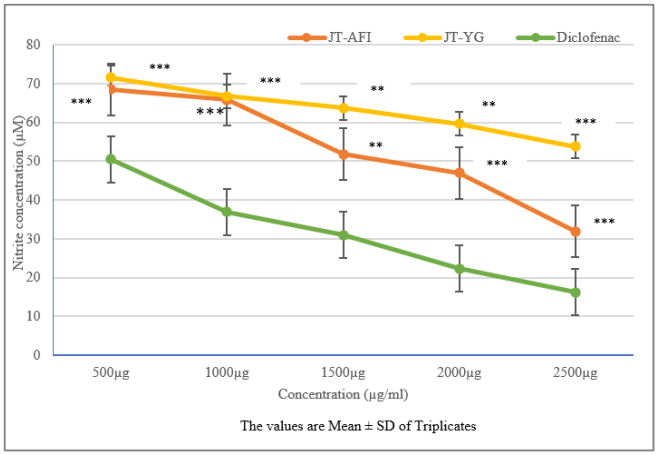

Fig. 4B: Effects of JT-AFI and JT-YG in comparison to standard drug on LPS-induced NO in RAW264.7 using the Griess assay. Macrophages were incubated in the presence of JT-AFI and JT-YG in combination with 1 µg/mL LPS for 24 h. Each value represents the Mean ± SD, N=3 and Significant difference (***p ≤ 0.001, **p ≤ 0.01) as observed for all the tested concentrations when compared to control (100%) and Standard.

thailam has many medicinally active compounds that might act as repressors of nitric oxide in LPS-activated macrophages. Our research work is in line with the study by Mandrika *et al.* (2021), who evaluated the anti-inflammatory potential of Jatyadi thailam extracts by inhibiting the mRNA expression and protein secretion of pro-inflammatory cytokines and chemokines in LPS-challenged macrophages[34] and Mazutti da Silva *et al.*, (2018) reported inhibition of NO production by essential oil extracted from *E. dysenterica* leaves.[35] Thus, the above reports hold up the promising therapeutic effectivity of thailam by inhibiting NO production.

5-Lox and COX Inhibitory Assay

Inhibitory activity of cyclooxygenases is a crucial tool for controlling inflammation in the human body. A comprehensive *in-vitro* anti-inflammatory enzymatic examination of the Jatyadi thailam suppresses COX-1 and 2 and 5-LOX enzymes significantly. An elevated number of leukotrienes and prostaglandins in the body prompts an inflammatory response, and chronic exposure of free radicals and pro-inflammatory mediators could disturb the normal inflammatory phases.[36]

The results of the ability of Jatyadi thailam to inhibit 5-LOX and COX enzymes are shown in Table 1. LOX converts Arachidonic acid to eicosanoids and leukotriene B4 (LTB4)[6], which is accompanied with the generation of thromboxanes and prostaglandins by cyclooxygenase at the start of the inflammatory phase (COX). These inflammatory mediators are accountable for strong chemo-attractive actions, as well as for enhanced pro-inflammatory cytokine release. The findings of LOX assay revealed that JT-AFI and JT-YG exhibited substantial dose-dependent enzyme inhibition of 5-lipoxygenase, notably JT-AFI possessing the most powerful activity with the highest percentage inhibition of 83.45 ± 0.01. Maximum percentage inhibition by JT-YG was observed to be 70.50 ± 0.02. From the result, the strength of 5-LOX activity was noted to be JT-AFI > JT-YG. JT-AFI has superior 5-LOX enzyme inhibition than JT-YG. The efficacy of formulations to inhibit COX, which is associated with inflammatory responses, was studied along with the standard drug Indomethacin. Both the formulations exhibited a dose responsible hike in inhibition of COX. JT-AFI inhibited COX-1 and COX-2 with the highest inhibition of 70.82 ± 0.01 and 82.04 ± 0.01 and possessed greater selectivity for COX- 2 (COX-1/COX-2 ratio=1.924), while inhibition by JT-YG was COX-1 selective (COX-1/COX-2 ratio=0.794) with a maximum percentage inhibition of 77.27 ± 0.01 (COX-1) and 65.45 ± 0.01 (COX-2). The aforesaid findings suggest that these formulations may have an effective anti-inflammatory effect on the 5-LOX and COX systems.

The present study's findings showed that JT-AFI and JT-YG exhibited a dose-dependent enzyme inhibition of 5-lipoxygenase and cyclooxygenase. Furthermore, our data revealed that both the formulations could effectively block the inducible COX-2 isoform, which plays a significant role in inflammatory events and is comparable to standard inhibitors used as a positive control. Because COX-2 is one of the most critical variables in regulating the inflammatory response and is a target for most antiphlogistic medicines, the ability of formulations to inhibit COX-2 *in vitro* could reveal one of the possible mechanisms of its action in-*vivo*.[37] The components in the thailam might decrease prostaglandin production, reducing inflammation and pain and possessing an efficient anti-inflammatory response. Thus, medicines that suppress both COX and 5-LOX (dual inhibitors) and diminish leukotriene and prostaglandin production while inhibiting inflammation totally[38] are a key criterion for a drug to be effective in inflammatory diseases. A COX-1 selective inhibitor will have a ratio of <1. while COX-2 will have >1.[39,40] The JT-AFI formulation was COX-2 selective, and JT-YG was COX-1 selective in this investigation, supporting the anti-inflammatory effects on the COX and 5-LOX systems. Our study could give insight into the efficacy of the formulations in fighting against radical-mediated damage and inflammatory mediators, where JT-AFI was more effective in rendering the anti-

Table 1: Percentage inhibition of JT-AFI And JT-YG against 5-Lipoxygenase and Cyclooxygenase enzymes

Samples		*Percentage inhibition at different concentration (%)*					
		500 µg/mL	*1000 µg/mL*	*1500 µg/mL*	*2000 µg/mL*	*2500 µg/mL*	*COX-1/COX-2 ratio*
JT-AFI	5-LOX	35.00 ± 0.01***	54.40 ± 0.01***	65.94 ± 0.02***	76.54 ± 0.01***	83.45 ± 0.03***	NA
	COX-1	36.31 ± 0.02***	41.02 ± 0.04**	57.14 ± 0.01***	65.92 ± 0.02***	70.82 ± 0.02***	1.92
	COX-2	47.35 ± 0.35***	54.89 ± 0.01***	65.92 ± 0.02***	74.69 ± 0.04***	82.04 ± 0.01***	
JT-YG	5-LOX	29.35 ± 0.03***	42.05 ± 0.01***	50.50 ± 0.02***	61.50 ± 0.14***	70.50 ± 0.07***	NA
	COX-1	31.81 ± 0.03***	45.00 ± 0.01***	59.09 ± 0.12***	67.27 ± 0.65**	77.27 ± 0.47***	0.794
	COX-2	27.27 ± 0.22***	38.18 ± 0.04***	50.45 ± 0.39**	63.18 ± 0.05**	65.45 ± 0.01***	
NDGA	5-LOX	53.12 ± 0.02	78.12 ± 0.46	84.37 ± 0.33	88.75 ± 0.12	96 ± 380.01	NA
INDO	COX-1	50.00 ± 0.14	72.60 ± 0.26	84.21 ± 0.15	92.18 ± 0.09	92.96 ± 0.06	1.84
	COX-2	52.63 ± 0.28	73.68 ± 0.02	89.47 ± 0.06	95.08 ± 0.04	98.06 ± 0.03	

The experiments were done in triplicates (n= 3) and the values were represented as mean ± SD. ***p ≤ 0.001, **p ≤ 0.01 was observed for all the tested concentrations compared to standard and control (100%).

inflammatory property than JT-YG. The discrepancy in bioefficacy could be explained by differences in the content of exotic medicinal plants in the formulation, which could have different mechanisms of action to exert their anti-inflammatory activities. Our results are supported by Azad *et al.* (2018), who showed the dual inhibitor role of the *Premna integrifolia*.[41] Thus, Jatyadi thailam could act as a dual inhibitor of LOX and COX enzymes and serve as an effective formulation with anti-inflammatory activity.

To the best of knowledge, the current study is the first experimental work on the *in-vitro* free radical quenching ability of Jatyadi thailam and its inhibitory role against nitric oxide production in RAW264.7 macrophage cells and also is a dual inhibitor of 5-LOX and COX. Our study thereby provides scientific evidence and thus, justifies its use in the traditional practice of medicine. The JT-AFI possessed higher therapeutic efficacy than JT-YG. These observations could form a bridge towards the clinical validation of Jatyadi thailam to counteract inflammatory pathways in wound healing.

Abbreviations

JT-AFI : Jatyadi thailam Ayurvedic Formulary of India
JT-YG : Jatyadi thailam Yogagrantha
DPPH : 2,2-diphenyl-1-picrylhydrazyl
ABTS : (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid))
NO : Nitric Oxide
LPS : Lipopolysaccharide
5-LOX : 5-Lipoxygenase
COX-1 : Cyclooxygenase-1
COX-2 : Cyclooxygenase-2
NDGA : Nordihydroguaiaretic acid
INDO : Indomethacin
TMPD : N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride
AA : Arachidonic acid

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References

1. Deng L, Du C, Song P, Chen T, Rui S, David G, Amstrong DG, Deng W. The Role of Oxidative Stress and Antioxidants in Diabetic Wound Healing. Oxidative Medicine and Cellular Longevity. 2021; 11. https://doi.org/10.1155/2021/8852759
2. Sharifi-Rad M, Anil Kumar NV, Zucca P, Varoni EM, Dini L, Panzarini E, Rajkovic J, Tsouh Fokou PV, Azzini E, Peluso I, Prakash Mishra A, Nigam M, El Rayess Y, Beyrouthy ME, Polito L, Iriti M, Martins N, Martorell M, Docea AO, Setzer WN, Calina D, Cho WC and Sharifi-Rad. Lifestyle, Oxidative Stress, and Antioxidants: Back and Forth in the Pathophysiology of Chronic Diseases. Frontiers in Physiology. 2020; 11:694. doi: 10.3389/fphys.2020.00694
3. Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaeili SA, Mardani F, Seifi B, Mohammadi A, Afshari JT, Sahebkar A. Macrophage plasticity, polarization, and function in health and disease. Journal of Cellular Physiology 2018;233(9):6425-6440. doi: 10.1002/jcp.26429.
4. Lee MJ, Seo HJ, Hwang GS, Choi S, Park SJ, Hwang SJ, Kang KS. Molecular Mechanism of *Cinnamomum cassia* against Gastric Damage and Identification of Active Compounds. Biomolecules. 2022, 12, 525. https://doi.org/10.3390/ biom12040525
5. Qureshi AA, Tan X, Reis JC, Badr MZ, Papasian CJ, Morrison DC, Qureshi N. Inhibition of nitric oxide in LPS-stimulated macrophages of young and senescent mice by δ-tocotrienol and quercetin. Lipids in Health and Disease. 2011;10(1):1-22.
6. Wang B, Wu L, Chen J, Dong L, Chen C, Wen Z, Hu J, Fleming I, Wang DW. Metabolism pathways of arachidonic acids: Mechanisms and potential therapeutic targets. Signal Transduction and Targeted Therapy. 2021. 26;6(1):1-30.
7. Nguyen HT, Vu TY, Chandi V, Polimati H, Tatipamula VB. Dual COX and 5-LOX inhibition by clerodane diterpenes from seeds of Polyalthia longifolia (Sonn.) Thwaites. Scientific Reports. 2020. 29;10(1):1-0.
8. Ayurvedic Formulary of India (AFI), Part I, Government of India, Ministry of Health and Family Welfare, Department of Indian system of Medicines and Homeopathy, New Delhi, India, 2nd edition, 2003.
9. Joshi R, Mashetti NB, Gujar RK. Comparative clinical study of Jatyadi taila and Jatyadi ghrita in the management of Dushta Vrana. Journal of Ayurveda and Integrative Medicine. 2018; 3(03):47-53.
10. Kulkarni YS, Emmi SV, Dongargaon TN, Wali AA. Wound healing effect of Vimlāpanakarma with Jātyādi tailam in diabetic foot. Ancient Science of Life. 2015;34(3):171.
11. Shailajan S, Menon S, Pednekar S, Singh A. Wound healing efficacy of Jatyadi Taila: *in vivo* evaluation in rat using excision wound model. Journal of Ethnopharmacology. 2011; 138(1):99-104.
12. Mensor LL, Menezes FS, Leitão GG, Reis AS, dos Santos TC, Coube CS, Leitão SG. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytotherapy Research. 2001;15(2):127-30.
13. Shirwaikar A, Shirwaikar A Rajendran K, Punitha IS. *In vitro* antioxidant studies on the benzyl tetra isoquinoline alkaloid berberine. Biological and Pharmaceutical Bulletin. 2006;29(9):1906-10.
14. Oyaizu M. Studies on products of browning reactions: Antioxidant Activities of Products of browning reaction prepared from glucose amine. Japanese Journal of Nutrition and Dietetics. 1986; 44:307–15
15. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Analytical Biochemistry. 1982;126(1):131-8.
16. Ruch RJ, Cheng SJ, Klaunig JE. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis. 1989;10(6):1003-1008.
17. Kunchandy E, Rao MN. Oxygen radical scavenging activity of curcumin. International Journal of Pharmaceutics. 1990; 58(3): 237-40.
18. Winterbourn CC, Hawkins RE, Brian M, Carrell RW. The estimation of red cell superoxide dismutase activity. Journal of Laboratory and Clinical Medicine. 1975;85(2):337-41.
19. Tam JC, Lau KM, Liu CL, To MH, Kwok HF, Lai KK, Lau CP, Ko CH, Leung PC, Fung KP, San Lau CB. The in vivo and in vitro diabetic wound healing effects of a 2-herb formula and its mechanisms of action. Journal of Ethnopharmacology. 2011;134(3):831-8.

20. Baylac S, Racine P. Inhibition of 5-lipoxygenase by essential oils and other natural fragrant extracts. International Journal of Aromatherapy. 2003;13(2-3):138-42.
21. Kamatou GP, Viljoen AM, Steenkamp P. Antioxidant, anti-inflammatory activities and HPLC analysis of South African Salvia species. Food Chemistry. 2010;119(2):684-8.
22. Gierse JK, Kob'oldt CM. Cyclooxygenase assays, Current Protocols in Pharmacology. unit 3.1, 1998
23. Shekhar TC, Anju G. Antioxidant activity by DPPH radical scavenging method of Ageratum conyzoides Linn. leaves. American Journal of Ethnomedicine. 2014;1(4):244-9.
24. Rahman MM, Islam MB, Biswas M, Kurshid Alam AHM. *In vitro* antioxidant and free radical scavenging activity of different parts of *Tabebuia pallida* growing in Bangladesh. *BMC Research Notes*. 2015; 8, 621.
25. Gangwar M, Gautam MK, Sharma AK, Tripathi YB, Goel RK, Nath G. Antioxidant capacity and radical scavenging effect of polyphenol rich *Mallotus philippenensis* fruit extract on human erythrocytes: an *in vitro* study. Scientific World Journal. 2014;12.
26. Aara A, Chappidi V, Ramadas MN. Antioxidant activity of eugenol in *Piper betel* leaf extract. Journal of Family Medicine and Primary Care . 2020. 28;9(1):327-331. doi: 10.4103/jfmpc.jfmpc_809_19.
27. Charrier JG, Anastasio C. Rates of hydroxyl radical production from transition metals and quinones in a surrogate lung fluid. Environmental Science & Technology. 2015. 4;49(15):9317-25.
28. Hazra B, Biswas S, Mandal N. Antioxidant and free radical scavenging activity of Spondias pinnata. BMC Complementary and Alternative Medicine. 2008; 8(1):1-0.
29. Srikanth G, Babu SM, Kavitha CH, Rao MB, Vijaykumar N, Pradeep CH. Studies on *in vitro* antioxidant activities of *Carica papaya* aqueous leaf extract. Research Journal of Pharmaceutical, Biological and Chemical Sciences. 2010;1(2):59-65.
30. Prabhu K, Rao MR, Ravi A, Kalaivannan J, Shruti Dinakar VN. Antioxidant studies of one ayurvedic medicine, Mahanarayana thailam. Drug Invention Today: 2020;13(4):641-5.
31. Arun D, Saraswathi U. Antioxidant potential of Shivanarvembu Kuzhi Thailam. International Journal of Herbal Medicine. 2015;3:1-5.
32. Kany S, Vollrath JT, Relja B. Cytokines in Inflammatory Disease. International Journal of Molecular Sciences. 2019. 28;20(23):6008. doi: 10.3390/ijms20236008.
33. Xue Q, Yan Y, Zhang R, Xiong H. Regulation of iNOS on immune cells and its role in diseases. International Journal of Molecular Sciences. 2018;19(12):3805.
34. Mandrika I, Kumar S, Zandersone B, Eranezhath SS, Petrovska R, Liduma I, Jezupovs A, Pirags V, Tracevska T. Antibacterial and Anti-Inflammatory Potential of Polyherbal Formulation Used in Chronic Wound Healing. Evidence Based Complementary and Alternative Medicine. 2021.
35. Mazutti da Silva SM, Rezende Costa CR, Martins Gelfuso G, Silva Guerra EN, de Medeiros Nóbrega YK, Gomes SM, Pic-Taylor A, Fonseca-Bazzo YM, Silveira D, Magalhães PD. Wound healing effect of essential oil extracted from Eugenia dysenterica DC (Myrtaceae) leaves. Molecules. 2019;24(1):2.
36. Ketha A, Vedula GS, Sastry AV. In vitro antioxidant, anti-inflammatory, and anticancer activities of methanolic extract and its metabolites of whole plant *Cardiospermum canescens* Wall. Future Journal of Pharmaceutical Sciences. 2020;6(1):1-0.
37. Sosa S, Altinier G, Politi M, Braca A, Morelli I, Della Loggia R. Extracts and constituents of *Lavandula multifida* with topical anti-inflammatory activity. Phytomedicine. 2005;12(4):271-7.
38. das Chagas Pereira de Andrade, F., Mendes, A.N. Computational analysis of eugenol inhibitory activity in lipoxygenase and cyclooxygenase pathways. Scientific Reports 10, 16204 (2020). https://doi.org/10.1038/s41598-020-73203-z
39. Burnett BP, Jia Q, Zhao Y, Levy RM. A medicinal extract of *Scutellaria baicalensis* and *Acacia catechu* acts as a dual inhibitor of cyclooxygenase and 5-lipoxygenase to reduce inflammation. Journal of Medicinal Food. 2007;10(3):442-51.
40. Yang MH, Yoon KD, Chin YW, Park JH, Kim J. Phenolic compounds with radical scavenging and cyclooxygenase-2 (COX-2) inhibitory activities from *Dioscorea opposita.* Bioorganic & Medicinal Chemistry. 2009;17(7):2689-94.
41. Azad R, Babu NK, Gupta AD, Reddanna P. Evaluation of anti-inflammatory and immunomodulatory effects of *Premna integrifolia* extracts and assay-guided isolation of a COX-2/5-LOX dual inhibitor. Fitoterapia. 2018; 131:189-99

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